frontiers

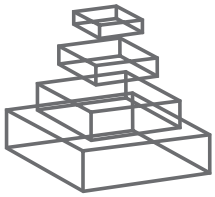
RESEARCH TOPICS

INDUCED PLANT RESPONSES TO MICROBES AND INSECTS



Topic Editors
Corné M. J. Pieterse, Marcel Dicke,
Saskia C. M. Van Wees and Erik H. Poelman

frontiers in
PLANT SCIENCE



frontiers

FRONTIERS COPYRIGHT STATEMENT

© Copyright 2007-2014
Frontiers Media SA.
All rights reserved.

All content included on this site, such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of or is licensed to Frontiers Media SA ("Frontiers") or its licensees and/or subcontractors. The copyright in the text of individual articles is the property of their respective authors, subject to a license granted to Frontiers.

The compilation of articles constituting this e-book, wherever published, as well as the compilation of all other content on this site, is the exclusive property of Frontiers. For the conditions for downloading and copying of e-books from Frontiers' website, please see the Terms for Website Use. If purchasing Frontiers e-books from other websites or sources, the conditions of the website concerned apply.

Images and graphics not forming part of user-contributed materials may not be downloaded or copied without permission.

Individual articles may be downloaded and reproduced in accordance with the principles of the CC-BY licence subject to any copyright or other notices. They may not be re-sold as an e-book.

As author or other contributor you grant a CC-BY licence to others to reproduce your articles, including any graphics and third-party materials supplied by you, in accordance with the Conditions for Website Use and subject to any copyright notices which you include in connection with your articles and materials.

All copyright, and all rights therein, are protected by national and international copyright laws.

The above represents a summary only. For the full conditions see the Conditions for Authors and the Conditions for Website Use.

ISSN 1664-8714
ISBN 978-2-88919-190-1
DOI 10.3389/978-2-88919-190-1

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

INDUCED PLANT RESPONSES TO MICROBES AND INSECTS

Topic Editors:
Corné M. J. Pieterse, Utrecht University, Netherlands
Marcel Dicke, Wageningen University, Netherlands
Saskia C. M. Van Wees, Utrecht University, Netherlands
Erik H. Poelman, Wageningen University, Netherlands



In this e-book, the model plant species *Arabidopsis thaliana* (thale cress) is often used to study mechanisms and function of induced plant responses to microbial pathogens, herbivorous insects, and beneficial microbes. The picture shows an *Arabidopsis* leaf under attack by a caterpillar of *Pieris rapae* (small cabbage white). Photo: Hans van Pelt, Utrecht University

Plants are members of complex communities and interact both with antagonists and beneficial organisms. An important question in plant defense-signaling research is how plants integrate signals induced by pathogens, insect herbivores and beneficial microbes into the most appropriate adaptive response. Molecular and genomic tools are now being used to uncover the complexity of the induced defense signaling networks that have evolved during the arms races between plants and the other organisms with which they intimately interact. To understand the functioning of the complex defense signaling network in nature, molecular biologists and ecologists have joined forces to place molecular mechanisms of induced plant defenses in an ecological perspective. In this Research Topic, we aim to provide an on-line, open-access snapshot of the current state of the art of the field of induced plant responses to microbes and insects, with a special focus on the translation of molecular mechanisms to ecology and vice versa.

Table of Contents

06 ***Induced Plant Responses to Microbes and Insects***
Corné M. J. Pieterse, Erik H. Poelman, Saskia C. M. Van Wees and Marcel Dicke

09 ***MAMP (Microbe-Associated Molecular Pattern) Triggered Immunity in Plants***
Mari-Anne Newman, Thomas Sundelin, Jon T. Nielsen and Gitte Erbs

23 ***Long-Distance Communication and Signal Amplification in Systemic Acquired Resistance***
Jyoti Shah and Jürgen Zeier

39 ***Reprogramming of Plants During Systemic Acquired Resistance***
Katrin Gruner, Thomas Griebel, Hana Návarová, Elham Attaran and Jürgen Zeier

67 ***The Arabidopsis NIMIN Proteins Affect NPR1 Differentially***
Meike Hermann, Felix Maier, Ashir Masroor, Sofia Hirth, Artur J. P. Pfitzner and Ursula M. Pfitzner

82 ***Induced Resistance in Tomato by SAR Activators During Predisposing Salinity Stress***
Matthew F. Pye, Fumiaki Hakuno, James D. MacDonald and Richard M. Bostock

91 ***Integrating Nitric Oxide into Salicylic Acid and Jasmonic Acid/Ethylene Plant Defense Pathways***
Luis A. J. Mur, Elena Prats, Sandra Pierre, Michael Anthony Hall and Kim Henrik Hebelstrup

98 ***A Component of the Sec61 ER Protein Transporting Pore is Required for Plant Susceptibility to Powdery Mildew***
Wen-Jing Zhang, Susanne Hanisch, Mark Kwaaitaal, Carsten Pedersen and Hans Thordal-Christensen

106 ***Lectin Receptor Kinases in Plant Innate Immunity***
Prashant Singh and Laurent Zimmerli

110 ***The Xylem as Battleground for Plant Hosts and Vascular Wilt Pathogens***
Koste A. Yadeta and Bart P. H. J. Thomma

122 ***Pathogenicity of and Plant Immunity to Soft Rot Pectobacteria***
Pär R. Davidsson, Tarja Kariola, Outi Niemi and E. Tapio Palva

135 ***Prospects for Advancing Defense to Cereal Rusts Through Genetical Genomics***
Elsa Ballini, Nick Lauter and Roger Wise

146 ***Metabolomics of Cereals Under Biotic Stress: Current knowledge and Techniques***
Dirk Balmer, Victor Flors, Gaetan Glauser and Brigitte Mauch-Mani

158 ***Using Fundamental Knowledge of Induced Resistance to Develop Control Strategies for Bacterial Canker of Kiwifruit Caused by Pseudomonas syringae pv. actinidiae***
Tony Reglinski, Joel L. Vanneste, Kirstin Wurms, Elaine Gould, Francesco Spinelli and Erik Rikkerink

162 ***Modeling Induced Resistance to Plant Disease Using a Dynamical Systems Approach***
Nurul S. Abdul Latif, Graeme C. Wake, Tony Reglinski, Philip A. G. Elmer and Joseph T. Taylor

165 ***Plant Systemic Induced Responses Mediate Interactions Between Root Parasitic Nematodes and Aboveground Herbivorous Insects***
Mesfin Wondafrash, Nicole M. Van Dam and Tom O. G. Tytgat

180 ***Differential Contribution of Transcription Factors to Arabidopsis thaliana Defense Against Spodoptera littoralis***
Fabian Schweizer, Natacha Bodenhausen, Steve Lassueur, Frédéric G. Masclaux and Philippe Reymond

192 ***Arabidopsis Redox Status in Response to Caterpillar Herbivory***
Jamuna Paudel, Tanya Copley, Alexandre Amirizian, Alberto Prado and Jacqueline C. Bede

205 ***Resistance to Sap-Sucking Insects in Modern-Day Agriculture***
Martin De Vos and Arjen VanDoorn

213 ***Where Do Herbivore-Induced Plant Volatiles Go?***
Jarmo K. Holopainen and James D. Blande

226 ***Herbivore-Induced Maize Leaf Volatiles Affect Attraction and Feeding Behavior of Spodoptera littoralis Caterpillars***
Georg E. von Mérey, Nathalie Veyrat, Marco D'Alessandro and Ted Turlings

235 ***E-2-Hexenal Promotes Susceptibility to Pseudomonas Syringae by Activating Jasmonic Acid Pathways in Arabidopsis***
Alessandra Scala, Rossana Mirabella, Cynthia Mugo, Kenji Matsui, Michel A. Haring and Robert C. Schuurink

246 ***Jasmonate-Mediated Induced Volatiles in the American Cranberry, Vaccinium Macrocarpon: From Gene Expression to Organismal Interactions***
Cesar R. Rodriguez-Saona, James Polashock and Edi A. Malo

263 ***Quantitative Patterns Between Plant Volatile Emissions Induced by Biotic Stresses and the Degree of Damage***
Ülo Niinemets, Astrid Kännaste and Lucian Copolovici

278 ***The Rhizosphere Revisited: Root Microbiomics***
Peter A. H. M. Bakker, Roeland L. Berendsen, Rogier F. Doornbos, Paul C. A. Wintermans and Corné M. J. Pieterse

285 ***ISR Meets SAR Outside: Additive Action of the Endophyte Bacillus Pumilus INR7 and the Chemical Inducer, Benzothiadiazole, on Induced Resistance Against Bacterial Spot in Field-Grown Pepper***
Hwe-Su Yi, Jung Wook Yang and Choong-Min Ryu

296 ***Getting the Ecology into Interactions Between Plants and the Plant Growth-Promoting Bacterium Pseudomonas fluorescens***
W. H. Gera Hol, T. Martijn Bezemer and Arjen Biere

305 ***Plant Growth in Arabidopsis is Assisted by Compost Soil-derived Microbial Communities***
Lilia C. Carvalhais, Frederico Muzzi, Chin-Hong Tan, Peer M. Schenk and Jin Hsien-Choo

320 ***Multiple Control Levels of Root System Remodeling in Arbuscular Mycorrhizal Symbiosis***
Uta Paszkowski and Caroline Gutjahr

328 ***Effector Candidates in the Secretome of Piriformospora indica, a Ubiquitous Plant-Associated Fungus***
Maryam Rafiqi, Lukas Jelonek, Ndifor Fidele Akum, Feng Zhang and Karl-Heinz Kogel

333 ***Deciphering the Hormonal Signalling Network Behind the Systemic Resistance Induced by Trichoderma harzianum in Tomato***
Ainhoa Martinez-Medina, Ivan Fernandez, Maria J. Sánchez-Guzmán, Sabine C. Jung, Jose A. Pascual and Maria J. Pozo

345 ***Investigating the Beneficial Traits of Trichoderma hamatum GD12 for Sustainable Agriculture—Insights from Genomics***
David J. Studholme, Beverley Harris, Kate Le Cocq, Rebecca Winsbury, Venura Perera, Lauren Ryder, Michael H. Beale, Jane L. Ward, Christopher R. Thornton and Murray Grant

frontiers in
PLANT SCIENCE

EDITORIAL
published: 21 November 2013
doi: 10.3389/fpls.2013.00475

Induced plant responses to microbes and insects

Corné M. J. Pieterse[1]*, Erik H. Poelman[2], Saskia C. M. Van Wees[1] and Marcel Dicke[2]

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands
[2] Laboratory of Entomology, Department of Plant Science, Wageningen University, Wageningen, Netherlands
*Correspondence: c.m.j.pieterse@uu.nl

Edited by:
Mary B. Mudgett, Stanford University, USA

Keywords: induced systemic resistance, systemic acquired resistance, insect-induced defense, priming, plant defense signaling, volatile organic compounds, microbiome, beneficial microbes

Plants play a key role in the sustainability of life on earth. They fix the solar energy that drives nearly all living processes. As a result, plants are members of complex communities and interact both with antagonists and beneficial organisms. To defend themselves against harmful pathogens and insects, plants have evolved a sophisticated immune system to perceive alien organisms, and to translate this perception into defense. The plant immune system is based on a surprisingly complex defense signaling network that is highly flexible in its capacity to recognize and respond to the invader encountered. Plant hormones and volatile organic compounds emerged as important signaling molecules in local and systemic induced defense responses to pathogen or insect attack.

Beneficial relationships between plants and microorganisms are frequent in nature as well, improving plant growth or helping the plant to overcome biotic or abiotic stress. Beneficial associations include root-colonizing microbes, such as plant growth-promoting rhizobacteria and fungi, and mycorrhizal fungi that form a symbiosis with ∼80% of all plant species. These ecologically and agriculturally important beneficial associations are based on the exchange of resources between the plant and the mutualist. Each gram of soil contains billions of microbes. However, the microbial community on plant roots is very different from that in bulk soil. Hence, plants are able to recruit specific microbes from the soil to their roots. The establishment of beneficial associations requires mutual recognition and a high degree of coordination of plant and microbial responses through a continuous molecular dialog between the plant and the beneficial. Because beneficial microbes are recognized as alien organisms, active interference with the plant immune system is fundamental for the establishment of intimate mutualistic relationships.

An important question in plant defense signaling research is: how do plants integrate signals induced by pathogens, insect herbivores and beneficial microbes into an adaptive response that maximizes both profitable and protective functions? Molecular and genomic tools are now being used to uncover the complexity of the induced signaling networks that have evolved during the arms races between plants and the organisms with which they intimately interact. To understand the functioning of this complex signaling network in nature, molecular biologists and ecologists have joined forces to place molecular mechanisms of induced plant defenses in an ecological perspective. Exciting new discoveries have greatly advanced our understanding of how the co-evolutionary arms race between plants and its social network has shaped the plant immune system into a sophisticated defensive shield capable of warding off the majority of harmful organisms in its environment. Tremendous progress has also been made in the understanding of how plants respond to and benefit from mutualistic soil-borne microbes to maximize growth and survival. With 31 original contributions, this Research Topic provides a snapshot of the current state of the art of the field of induced plant responses to microbial pathogens, insect herbivores, and beneficial root-associated microbes, with a special focus on the translation of molecular mechanisms to ecology and vice versa.

In this Research Topic, a nice mix of Mini Reviews, Reviews, Original Research Articles, and Opinion Articles provide up-to-date information on diverse aspects of the plant immune system and how it functions against microbial pathogens. Newman et al. (2013) provide an update on the wide variety of microbe-associated molecular patterns (MAMPS) that to date have been characterized in bacterial, fungal and oomycetal pathogens. Locally triggered immune responses are often associated with systemic acquired resistance (SAR). This requires long-distance communication and signal amplification in distal plant parts. In recent years, many candidate molecules that function as systemically transported signals have been characterized (reviewed by Shah and Zeier, 2013). Other aspects of SAR, such as detailed insight in transcriptional reprogramming during SAR (Gruner et al., 2013), the role of the NPR1 regulatory NIMIN proteins in SAR (Hermann et al., 2013), and the effect of abiotic stress on the expression of SAR (Pye et al., 2013) are discussed in this Research Topic and provide interesting new insights into the field of SAR research. Plant hormones emerged as important regulators of local and systemic induced defense responses. Besides the classic defense-related hormones salicylic acid, jasmonic acid and ethylene, nitric oxide emerged as an important regulatory signal as well. In their Mini Review, Mur et al. (2013) integrate nitric oxide into the network of hormone-regulated immune responses.

More specific topics on plant-pathogen interactions are addressed by Zhang et al. (2013), who report on the role of the Sec61 ER protein transporting pore in susceptibility to powdery mildew. Singh and Zimmerli (2013) provide a nice review on the role of lectin receptor kinases in plant immunity. Yadeta and Thomma (2013) zoom in on the xylem as site of action when it comes to plant defense against vascular pathogens. Davidsson et al. (2013) provide insight into the state-of-the-art of pathogenicity of soft rot pectobacteria and the defensive machinery of plants to protect themselves against this group of pathogens. Cereal pathogens have major impacts on future food security. Ballini et al. (2013) and Balmer et al. (2013) describe how modern technology such as genetical genomics and metabolomics

can help to investigate these scientifically and societally challenging host-pathogen interactions. On a more applied note, Abdul Latif et al. (2013) and Reglinski et al. (2013) describe how modeling approaches and fundamental knowledge on induced resistance can be used to develop control strategies in practice, such as to fight bacterial canker of kiwifruit.

A major topic in plant-insect interactions is the aspect of above-belowground interactions between plants, insects and other organisms. Wondafrash et al. (2013) review the field of systemic induced defense responses triggered by root parasitic nematodes and their effects against herbivorous insects on foliar tissues. In their original research articles, Paudel et al. (2013) and Schweizer et al. (2013) provide novel insights into the role of different transcription factors and the cellular redox status in the regulation of induced defense against caterpillar herbivory. Sap-sucking insects have a completely different mode of action. De Vos and Vandoorn (2013) review current knowledge on resistance to this group of insects in modern-day agriculture.

Plant volatiles emerged as important signals in the communication between plants, insect herbivores, and their enemies. In this Research Topic a number of contributions address the role of volatile organic compounds in the communication of the plant's social network. Von Mérey et al. (2013) describe the role of herbivore-induced plant volatiles in the attraction and feeding behavior of a caterpillar herbivore. Scala et al. (2013) investigated the effect of a common herbivore-induced plant volatile on a bacterial plant pathogen. Rodriguez-Saona et al. (2013) investigated the role of jasmonate-mediated induction of plant volatiles on multi-trophic level interactions in American cranberry, while Niinemets et al. (2013) addressed the question how volatile emission patterns induced by biotic stresses relate to the degree of damage. After release into the atmosphere, the plant no longer controls the action of its produced volatiles. In their review "Where do herbivore-induced plant volatiles go" Holopainen and Blande (2013) summarize the potential ecological and atmospheric processes that involve the reaction products of plant volatiles that are formed in the atmosphere upon release by the plant.

An emerging theme in the field of plant-microbe interactions is the importance of beneficial microbes in plant health. Bakker et al. (2013) reviewed the field of root microbiomics and highlights the role of root-associated beneficial microbes in plant growth and protection. Studies by Carvalhais et al. (2013), Hol et al. (2013) and Yi et al. (2013) provide additional insight into the roles of plant growth-promoting rhizobacteria in plant health, either as stimulants of the plant's immune system or through optimizing the root microbial ecology. Paszkowski and Gutjahr (2013) provide an excellent review on the mechanisms by which arbuscular mycorrhizal fungi shape the architecture of plants roots, which is essential for optimal acquisition of mineral nutrients and water from the soil. Furthermore, novel insights into mechanisms by which plant growth-promoting fungi, such as *Piriformospora indica* and *Trichoderma* spp., interact with the plant immune system or antagonize pathogens in the soil are provided by contributions of Martinez-Medina et al. (2013), Rafiqi et al. (2013) and Studholme et al. (2013).

With this Research Topic we aimed to provide a platform for scientists who liked to share their understanding of how induced plant responses shape the plant's social network. The excellent contributions are a demonstration of a highly active research community in plant science. Together they provide a detailed understanding of the intrinsic capacity of plants to simultaneously accommodate mutualists and ward off enemies to maximize both growth-stimulating and protective functions.

ACKNOWLEDGMENTS

Research in the laboratories of the Topic Editors is supported by ERC Advanced grant 269072 (Corné M. J. Pieterse), NWO-VIDI grant 11281 (Saskia C. M. Van Wees), NWO-VENI grant 863.10.012 (Erik H. Poelman), and the European Science Foundation EUROCORES Programme EuroVOL (Marcel Dicke, Erik H. Poelman, Corné M. J. Pieterse).

REFERENCES

Abdul Latif, N. S., Wake, G. C., Reglinski, T., Elmer, P.a.H., and Taylor, J. T. (2013). Modeling induced resistance to plant disease using a dynamical systems approach. *Front. Plant Sci.* 4:19. doi: 10.3389/fpls.2013.00019

Bakker, P. A. H. M., Berendsen, R. L., Doornbos, R. F., Wintermans, P. C. A., and Pieterse, C. M. J. (2013). The rhizosphere revisited: root microbiomics. *Front. Plant Sci.* 4:165. doi: 10.3389/fpls.2013.00165

Ballini, E., Lauter, N., and Wise, R. (2013). Prospects for advancing defense to cereal rusts through genetical genomics. *Front. Plant Sci.* 4:117. doi: 10.3389/fpls.2013.00117

Balmer, D., Flors, V., Glauser, G., and Mauch-Mani, B. (2013). Metabolomics of cereals under biotic stress: current knowledge and techniques. *Front. Plant Sci.* 4:82. doi: 10.3389/fpls.2013.00082

Carvalhais, L. C., Muzzi, F., Tan, C.-H., Schenk, P. M., and Choo, J. H. (2013). Plant growth in Arabidopsis is assisted by compost soil-derived microbial communities. *Front. Plant Sci.* 4:235. doi: 10.3389/fpls.2013.00235

Davidsson, P. R., Kariola, T., Niemi, O., and Palva, E. T. (2013). Pathogenicity of and plant immunity to soft rot pectobacteria. *Front. Plant Sci.* 4:191. doi: 10.3389/fpls.2013.00191

De Vos, M., and Vandoorn, A. (2013). Resistance to sap-sucking insects in modern-day agriculture. *Front. Plant Sci.* 4:222. doi: 10.3389/fpls.2013.00222

Gruner, K., Griebel, T., Návarová, H., Attaran, E., and Zeier, J. (2013). Reprogramming of plants during systemic acquired resistance. *Front. Plant Sci.* 4:252. doi: 10.3389/fpls.2013.00252

Hermann, M., Maier, F., Masroor, A., Hirth, S., Pfitzner, A. J. P., and Pfitzner, U. M. (2013). The Arabidopsis NIMIN proteins affect NPR1 differentially. *Front. Plant Sci.* 4:88. doi: 10.3389/fpls.2013.00088

Hol, W. H. G., Bezemer, T. M., and Biere, A. (2013). Getting the ecology into interactions between plants and the plant growth-promoting bacterium *Pseudomonas fluorescens*. *Front. Plant Sci.* 4:81. doi: 10.3389/fpls.2013.00081

Holopainen, J. K., and Blande, J. D. (2013). Where do herbivore-induced plant volatiles go? *Front. Plant Sci.* 4:185. doi: 10.3389/fpls.2013.00185

Martinez-Medina, A., Fernandez, I., Sánchez-Guzmán, M. J., Jung, S. C., Pascual, J. A., and Pozo, M. J. (2013). Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Front. Plant Sci.* 4:206. doi: 10.3389/fpls.2013.00206

Mur, L.a.J., Prats, E., Pierre, S., Hall, M. A., and Hebelstrup, K. H. (2013). Integrating nitric oxide into salicylic acid and jasmonic acid/ ethylene plant defense pathways. *Front. Plant Sci.* 4:215. doi: 10.3389/fpls.2013.00215

Newman, M.-A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Niinemets, U., Kännaste, A., and Copolovici, L. (2013). Quantitative patterns between plant volatile emissions induced by biotic stresses and the degree of damage *Front. Plant Sci.* 4:262. doi: 10.3389/fpls.2013.00262

Paszkowski, U., and Gutjahr, C. (2013). Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. *Front. Plant Sci.* 4:204. doi: 10.3389/fpls.2013.00204

Paudel, J., Copley, T., Amirizian, A., Prado, A., and Bede, J. C. (2013). Arabidopsis redox status in response to caterpillar herbivory. *Front. Plant Sci*. 4:113. doi: 10.3389/fpls.2013.00113

Pye, M. F., Hakuno, F., Macdonald, J. D., and Bostock, R. M. (2013). Induced resistance in tomato by SAR activators during predisposing salinity stress. *Front. Plant Sci*. 4:116. doi: 10.3389/fpls.2013.00116

Rafiqi, M., Jelonek, L., Akum, N. F., Zhang, F., and Kogel, K.-H. (2013). Effector candidates in the secretome of *Piriformospora indica*, a ubiquitous plant-associated fungus. *Front. Plant Sci*. 4:228. doi: 10.3389/fpls.2013.00228

Reglinski, R., Vanneste, J. L., Wurms, K., Gould, E., Spinelli, F., and Rikkerink, E. (2013). Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae*. *Front. Plant Sci*. 4:24. doi: 10.3389/fpls.2013.00024

Rodriguez-Saona, C. R., Polashock, J., and Malo, E. A. (2013). Jasmonate-mediated induced volatiles in the American cranberry, *Vaccinium macrocarpon*: from gene expression to organismal interactions. *Front. Plant Sci*. 4:115. doi: 10.3389/fpls.2013.00115

Scala, A., Mirabella, R., Mugo, C., Matsui, K., Haring, M. A., and Schuurink, R. C. (2013). E-2-hexenal promotes susceptibility to *Pseudomonas syringae* by activating jasmonic acid pathways in Arabidopsis. *Front. Plant Sci*. 4:74. doi: 10.3389/fpls.2013.00074

Schweizer, F., Bodenhausen, N., Lassueur, S., Masclaux, F. G., and Reymond, P. (2013). Differential contribution of transcription factors to *Arabidopsis thaliana* defense against *Spodoptera littoralis*. *Front. Plant Sci*. 4:13. doi: 10.3389/fpls.2013.00013

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci*. 4:30. doi: 10.3389/fpls.2013.00030

Singh, P., and Zimmerli, L. (2013). Lectin receptor kinases in plant innate immunity. *Front. Plant Sci*. 4:124. doi: 10.3389/fpls.2013.00124

Studholme, D. J., Harris, B., Le Cocq, K., Winsbury, R., Perera, V., Ryder, L., et al. (2013). Investigating the beneficial traits of *Trichoderma hamatum* GD12 for sustainable agriculture—insights from genomics. *Front. Plant Sci*. 4:258. doi: 10.3389/fpls.2013.00258

Von Mérey, G. E., Veyrat, N., D'alessandro, M., and Turlings, T. (2013). Herbivore-induced maize leaf volatiles affect attraction and feeding behavior of *Spodoptera littoralis* caterpillars. *Front. Plant Sci*. 4:209. doi: 10.3389/fpls.2013.00209

Wondafrash, M., Van Dam, N. M., and Tytgat, T. O. G. (2013). Plant systemic induced responses mediate interactions between root parasitic nematodes and aboveground herbivorous insects. *Front. Plant Sci*. 4:87. doi: 10.3389/fpls.2013.00087

Yadeta, K. A., and Thomma, B. P. H. J. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Front. Plant Sci*. 4:97. doi: 10.3389/fpls.2013.00097

Yi, H.-S., Yang, J. W., and Ryu, C.-M. (2013). ISR meets SAR outside: additive action of the endophyte *Bacillus pumilus* INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper. *Front. Plant Sci*. 4:122. doi: 10.3389/fpls.2013.00122

Zhang, W.-J., Hanisch, S., Kwaaitaal, M., Pedersen, C., and Thordal-Christensen, H. (2013). A component of the Sec61 ER protein transporting pore is required for plant susceptibility to powdery mildew. *Front. Plant Sci*. 4:127. doi: 10.3389/fpls.2013.00127

Received: 23 October 2013; accepted: 03 November 2013; published online: 21 November 2013.

*Citation: Pieterse CMJ, Poelman EH, Van Wees SCM and Dicke M (2013) Induced plant responses to microbes and insects. Front. Plant Sci. **4**:475. doi: 10.3389/fpls.2013.00475*

This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science.

Copyright © 2013 Pieterse, Poelman, Van Wees and Dicke. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 16 May 2013
doi: 10.3389/fpls.2013.00139

MAMP (microbe-associated molecular pattern) triggered immunity in plants

Mari-Anne Newman*, Thomas Sundelin, Jon T. Nielsen and Gitte Erbs

Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark

Edited by:
Saskia C. Van Wees, Utrecht University, Netherlands

Reviewed by:
Mahmut Tör, University of Worcester, UK
Jens Staal, Ghent University, Belgium

****Correspondence:***
Mari-Anne Newman, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark.
e-mail: mari@life.ku.dk

Plants are sessile organisms that are under constant attack from microbes. They rely on both preformed defenses, and their innate immune system to ward of the microbial pathogens. Preformed defences include for example the cell wall and cuticle, which act as physical barriers to microbial colonization. The plant immune system is composed of surveillance systems that perceive several general microbe elicitors, which allow plants to switch from growth and development into a defense mode, rejecting most potentially harmful microbes. The elicitors are essential structures for pathogen survival and are conserved among pathogens. The conserved microbe-specific molecules, referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), are recognized by the plant innate immune systems pattern recognition receptors (PRRs). General elicitors like flagellin (Flg), elongation factor Tu (EF-Tu), peptidoglycan (PGN), lipopolysaccharides (LPS), Ax21 (Activator of XA21-mediated immunity in rice), fungal chitin, and β-glucans from oomycetes are recognized by plant surface localized PRRs. Several of the MAMPs and their corresponding PRRs have, in recent years, been identified. This review focuses on the current knowledge regarding important MAMPs from bacteria, fungi, and oomycetes, their structure, the plant PRRs that recognizes them, and how they induce MAMP-triggered immunity (MTI) in plants.

Keywords: innate immunity, MAMPs, Flg22, EF-Tu, Ax21, PGN, LPS, Chitin

INTRODUCTION

Bacteria, fungi, oomycetes, and viruses attack plants in an attempt to gain nutrients from them. During the course of evolution both plants and pathogens have evolved features to combat each other; the plant is equipped with sophisticated and rapidly mounted defense mechanisms, while their cognate pathogens have developed counterstrategies to overcome those defenses, the so called "arms race" between plant and pathogens (Bent and MacKey, 2007). The interplay between the plant defense systems and its suppression by pathogens has been portrayed as a "zigzag model" by Jones and Dangl (2006). This model proposes that the plants' immune responses consist of two branches. The first line of defense in plants is the recognition of conserved molecules characteristic of many microbes. These elicitors are also known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (**Table 1**). MAMPs are essential structures for the microbes and are for that reason conserved both among pathogens, non-pathogenic and saprophytic microorganisms. MAMPs are recognized by pattern recognition receptors (PRRs), which are localized on the surface of plant cells; this first phase of defense induction is called MAMP-triggered immunity (MTI) (Ausubel, 2005; Jones and Dangl, 2006). All known plant PRRs are plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with modular functional domains. RLKs contain an extracellular domain (ECD), a single-pass transmembrane (TM) domain, and an intracellular kinase domain. RLPs contain an ECD and a TM but have only a short cytosolic domain without an obvious signaling domain (**Table 1**). Notably, in contrast to mammals, no intracellular nucleotide-binding-leucine-rich repeat (NB-LRR) protein recognizing a MAMP has yet been identified in plants (Maekawa et al., 2011). Bacterial effector proteins, injected directly into the host plants' cytoplasm via the pathogens type III secretion system (TTSS), have been demonstrated to suppress MTI (Jamir et al., 2004; He et al., 2006; Nomura et al., 2006), resulting in effector-triggered susceptibility (ETS). The second line of the plants' defense is direct or indirect recognition of a given effector through a set of plant resistance (R) gene products resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006); also named the gene-for-gene interaction as early as 1942 by Flor. ETI is generally an accelerated and amplified MTI response, and as such it is an effective defense response (resistance) that in most cases leads to a localized cell death, known as the hypersensitive response (HR). The majority of the R proteins are intracellular receptor proteins of the NB-LRR type. In most cases the interaction between NB-LRRs and the effectors are indirect (van der Biezen and Jones, 1998).

MAMP-induced defense responses include the production of reactive oxygen species (ROS, also called the oxidative burst), production of reactive nitrogen species such as nitric oxide (NO), alterations in the plant cell wall, induction of antimicrobial compounds and the synthesis of pathogenesis-related (PR) proteins. ROS and NO can act in signaling and have direct antimicrobial effects. ROS can also drive oxidative cross-linking of polymers in the plant cell wall to strengthen it against degradation, which may restrict pathogen spread. Other alterations in the plant

Table 1 | Microbe-associated molecular patterns (MAMPs) and Damage-associated molecular patterns (DAMPs).

Name	Corresponding plant receptor (PRR)	References
MAMPs		
Flagellin (Flg; flg22)	FLS2 (*Arabidopsis*)	Felix et al., 1999; Gómez-Gómez et al., 2001
Elongation factor TU (EF-Tu; elf18/26)	EFR (*Arabidopsis*; Brassicaceae)	Kunze et al., 2004
Peptidoglycan (PGN)	Lym1 and Lym3 (*Arabidopsis*)	Gust et al., 2007; Erbs et al., 2008a; Willmann et al., 2011
Lipopolysaccharide (LPS)	Not identified	Newman et al., 1995
Bacterial cold shock proteins (RNP1 motif)	Not identified	Felix and Boller, 2003b
Bacterial superoxide dismutase (Sod)	Not identified	Watt et al., 2006
Activator of XA21 (Ax21)	XA21 and XA21D (rice)	Song et al., 1995; Wang et al., 1998; Lee et al., 2009
Beta-Glycan (GE)	GEBP (putative receptor soyabean)	Darvill and Albersheim, 1984; Umemoto et al., 1997
Chitin	CeBip and CERK1 (rice); AtCERK1 (*Arabidopsis*)	Felix et al., 1993; Kaku et al., 2006; Miya et al., 2007; Shimizu et al., 2010
Avirulence on Ve1 tomato (Ave1)	Ve1 (putative tomato receptor)	Kawchuk et al., 2001; Thomma et al., 2011; de Jonge et al., 2012
Xylanase (EIX)	EIX (tomato)	Bailey et al., 1990; Ron and Avni, 2004
Pep-13 (An oligopeptide of 13 amino acids from *P. mega-sperma*)	Not identified	Nürnberger et al., 1994
Cellulose-binding elicitor lectin (CBEL) from *Phytophthora*	Not identified	Mateos et al., 1997; Séjalon-Delmas et al., 1997 Gaulin et al., 2006
DAMPs		
Systemin	Not identified	Narváez-Vásquez and Ryan, 2004
Pep1 (23 aa part of a cytosolic protein from *Arabidopsis*)	PEPR1 (*Arabidopsis*)	Huffaker et al., 2006; Yamaguchi et al., 2006
Oligogalacturonides (OGs)	WAK1 (*Arabidopsis*)	Nothnagel et al., 1983; Brutus et al., 2010
Cutin	Not identified	Schweizer et al., 1996; Kauss et al., 1999

wall include the deposition of the β-(1–3) linked glucan callose. PR proteins comprise a number of families that include enzymes, such as β-(1–3) glucanase and chitinase, which can directly attack pathogen structures, antimicrobial peptides and small proteins, and PR1, which is of unknown function [for reviews, see Hammond-Kosack and Jones (1996); Greenberg (1997); Lamb and Dixon (1997); Nürnberger and Kemmerling (2006)]. The induction of MTI in plants has been most extensively studied using the small peptides flg22 and elf18 derived from the bacterial MAMPs flagellin (Flg) and the translation elongation factor Tu (EF-Tu), respectively (Felix and Boller, 2003a; Zipfel et al., 2006). Bacterial glycoconjugates, such as the peptidoglycan (PGN), which provides rigidity and structure to the cell envelopes of both Gram-negative and Gram-positive bacteria (Erbs et al., 2008a; Willmann et al., 2011), and lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria have been found to act as elicitors of plant innate immunity (Silipo et al., 2005; Erbs and Newman, 2012). Oligosaccharides derived from cell wall polymers of fungi and oomycetes also act as MAMPs. Fungal chitin and its degradation products N-acetyl-chito-oligosaccharides, i.e., chitin oligomers induce various defense responses in both monocot and dicot plants (Kaku et al., 2006; Miya et al., 2007). In the oomycetes, the cell walls are composed of β-glucans and cellulose, rather than chitin, as in the fungi. Some of the earliest work on the role of glycosylated compounds in triggering plant defenses has examined the effects of β-(1→3/1→6)-linked glucans from the cell walls of *Phytophthora megasperma* f. sp. *glycinea* on the induction of phytoalexin accumulation in soybean [reviewed in Cheong and Hahn (1991)]. In the plant-virus interactions no conserved viral MAMP has been identified so far, and the primary plant defense is thought to be based mainly on RNA silencing (RNAi). By analogy with the zigzag model, viral-derived double-stranded RNA (dsRNA) is regarded as the MAMP inducing RNAi, a general plant defense mechanism or the MTI. To counteract this defense, plant viruses express RNA silencing suppressors (RSSs), many of which bind to dsRNA and attenuate RNAi (Csorba et al., 2009; Ruiz-Ferrer and Voinnet, 2009).

This review will focus on some of the important MAMPs from bacteria, fungi, and oomycetes and review the current knowledge of their structure, how they are recognized and how they induce MTI in plants. We include the slightly more unusual MAMP Ax21 from the rice pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). MAMPs in general activate MTI directly via their respective plant receptors, whereas Ax21 is secreted out of the bacterium via the type-I secretion system (TOSS), where it is then recognized by the rice receptor XA21, and induction of MTI follows. Finally, we will briefly describe damage-associated molecular patterns (DAMPs) of plant origin, which also induce MTI in the plant.

BACTERIAL MAMPs

FLAGELLIN (Flg)

Flagella are essential structures for the pathogenic bacteria as they provide motility and often increase adhesion of the bacteria to its host. Flg, the main building block of bacterial flagella, is well-established as a major activator of innate immunity in animals [reviewed by Ramos et al. (2004)]. Some of the first MAMP

recognition studies in plants were carried using Flg. Studies in mammals have shown that at least one of the conserved domains in the N-terminal and C-terminal part of the bacterial Flg, found to be involved in bacterial motility as well, is recognized by Toll-like receptor 5 (TLR5) (Hayashi et al., 2001; Smith et al., 2003). Studies in *Arabidopsis*, tomato, and other plants, revealed that plants respond to a highly conserved domain in the N-terminal part of the bacterial Flg, a 22 amino acid (aa) peptide, flg22 (Felix et al., 1999). In order to identify the gene involved in recognition and transduction of the flg22 elicitor signal, Gomez-Gomez and Boller (2000), used a genetic approach to screen *Arabidopsis* mutants after flg22 treatment and isolated several Flg sensing 2 (FLS2) mutants, which mapped to the FLS2 locus on chromosome 5. FLS2 belongs to the RLK family and has an ECD with 28 LRRs, a TM domain, and an intracellular serine/threonine kinase domain. No high-affinity binding site was found, after treatment with a radiolabeled derivative of flg22, in the Flg insensitive *Arabidopsis* ecotype Ws-0 and in plants carrying mutations in the LRR domain of the FLS2 gene, indicating a role for LRR in Flg binding (Gomez-Gomez and Boller, 2000; Bauer et al., 2001). Later work revealed that both an extracellular LRR domain and kinase activity of FLS2 were necessary for high affinity binding and binding specificity for Flg (Gómez-Gómez et al., 2001). Chinchilla et al. (2006) showed the specific interaction of flg22 with FLS2 in *Arabidopsis*. The recognized domain within Flg is not the same for all plant species. For example, flg15, an N-terminally shortened version of flg22, was shown to be highly active in tomato, while it only elicits immune responses at higher concentrations in *Arabidopsis*. Rice is able to recognize flg22, but its defense response is greater to the full length Flg (Takai et al., 2008). The functionality of the FLS2 receptor was tested by heterologous expression of the *Arabidopsis* FLS2 receptor in tomato cells. In these expression studies, tomato cells gained the Flg perception system characteristic for *Arabidopsis*, demonstrating that FLS2 represents the PRR that determines the specificity of Flg perception (Meindl et al., 2000; Bauer et al., 2001; Chinchilla et al., 2006). The difference in recognition of the Flg epitope is not restricted to different plant families; variations have also been found between species in the same family. A 15 aa peptide derived from *E. coli* Flg was shown only to be highly active in tomato (*Solanum lycopersicum* previously called *Lycopersicon esculentum*), but not in tobacco. Furthermore, the tomato Flg receptor, SlFLS2, an ortholog of the *Arabidopsis* FLS2 receptor, has now been identified and used in expression studies with *Nicotiana benthamiana*, where *N. benthamiana* expressing SlFLS2 gained the Flg perception system specific for tomato (Robatzek et al., 2007). In addition to this, studies focusing on host recognition of *Xanthomonas campestris* pv. *campestris* (*Xcc*) Flg have revealed within species and within pathovar variations for defense eliciting activity of Flgs among *Xcc* strains (Sun et al., 2006). Confirmation of FLS2 as a surface receptor came with studies using transgenic *Arabidopsis* Ws-0, expressing FLS2 fused to the green fluorescent protein (GFP), which revealed a cell membrane localization of FLS2. Additionally, FLS2 was found to undergo ligand-induced endocytosis; it is thought that this subcellular redistribution of FLS2, or any other surface receptor, from the plasma membrane to cytoplasmic vesicles may be a central point in signaling during immune responses (McCoy et al., 2004; Robatzek et al., 2006). Flg-induced activation of FLS2 in *Arabidopsis*, involves a complex formation with the Brassinosteroid-Insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1) (Chinchilla et al., 2007). BAK1 has also been reported to be involved in BRI1 endocytosis (Russinova et al., 2004). Furthermore, BAK1 is required for the immune responses triggered by multiple MAMPs other than Flg, including the bacterial elongation factor EF-Tu (see below) (Roux et al., 2011). The activities of MAP kinases (MAPK) were delayed and reduced or even absent in response to flg22 or elf18, a fully active EF-Tu derivative, in *bak1* mutants, compared to wild type plants. This indicates that BAK1 acts as a positive regulator of MAMP signaling in *Arabidopsis*. In addition, it was revealed that FLS2, after flg22 stimulation, interacts with BAK1 in a ligand-dependent manner (Chinchilla et al., 2007). This interaction allows phosphorylation and activation of the receptor complex (Schulze et al., 2010). Downstream of the FLS2-BAK1 receptor complex is a cytoplasmic receptor kinase *Botrytis*-induced kinase 1 (BIK1), which constitutively associate with FLS2. After FLS2-BAK1 dimerization, BIK1 dissociate from FLS2, possibly allowing BIK1 to phosphorylate downstream components, and thus linking the MAMP receptor complex to downstream intracellular signaling leading to MTI (Lu et al., 2010). However, the substrates of FLS2 and BAK1 kinases have yet to be identified, and how the MAMP signal is transmitted from the BAK1-associated receptor complexes at the plasma membrane to intracellular events is largely unknown.

ELONGATION FACTOR TU (EF-Tu)

In protein biosynthesis, the ribosomes translate the sequence of nucleotides in mRNA into the sequence of aa's in a protein. During the phase of elongation the ribosome is associated with elongation factors. One such elongation factor is EF-Tu, the most abundant protein in the bacterial cell (Jeppesen et al., 2005). The elicitor activity is attributed to a highly conserved part of the N-terminus of EF-Tu, either a 26 or 18 aa peptide named elf26 or elf18. The perception of EF-Tu by the EF-Tu Receptor (EFR) is independent of Flg perception, as EF-Tu is active in plants carrying mutations in FLS2 (Kunze et al., 2004). Although many of the signaling components downstream of EFR and FLS2 are shared between them (see above). EF-Tu recognition has only been found to elicit innate immunity in members of the family *Brassicaceae* (Zipfel et al., 2006). Studies using crosslinking assays in *Arabidopsis* cells, confirmed that elf18 and flg22 bind to different high-affinity binding receptors. Nevertheless, elf18 and flg22 were found by microarray analysis to induce the same pool of genes, and also a common set of responses in *Arabidopsis*. In addition to this, a combined treatment with both MAMPs, elf26/18 and flg22, was shown to induce the same kinases without an additive effect (Zipfel et al., 2006). An EF-Tu insensitive *efr-1* mutant did not respond with an oxidative burst, increased ethylene biosynthesis or induced resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 in response to EF-Tu-derived elicitors, whereas *Arabidopsis* Col-0 and the *fls2* mutant did respond to EF-Tu elicitors. Heterologous expression studies of EFR in *N. benthamiana*, a plant lacking a perception system for EF-Tu, resulted in *N. benthamiana* with a perception system for EF-Tu, confirming

the role for EFR as a functional receptor for EF-Tu (Zipfel et al., 2006). In addition to this, *efr* mutants were found to be more susceptible to *Agrobacterium tumefaciens (At)*-mediated transformation than wild type plants, indicating that EF-Tu recognition and the subsequent defense responses reduce *Agrobacterium*-mediated plant transformation (Zipfel et al., 2006).

Similar to FLS2, EFR belongs to the RLK family and has an ECD with 24 LRRs, a single TM domain and an intracellular serine/threonine kinase domain (Zipfel et al., 2006). Both FLS2 and EFR are members of the subfamily LRR-XII of RLKs (Shiu and Bleecker, 2003). Besides FLS2 and EFR from *Arabidopsis*, the rice pathogen recognition receptor, XA21 (see below), which confers resistance to *Xoo* strains is also a member of the LRR-XII subfamily (Song et al., 1995; Shiu et al., 2004; Lee et al., 2006). In contrast to FLS2, but like XA21, N-glycosylation is required for EFR functionality. Mutation of a single predicted glycosylation site compromised elf18 binding despite correct localization of the mutated protein to the plasma membrane (Häweker et al., 2010).

PEPTIDOGLYCAN (PGN)

PGN, a molecule never found in eukaryotes, is an essential and unique membrane envelope component of all bacteria, making it an excellent target for the eukaryotic innate immune system [reviewed by McDonald et al. (2005); Dziarski and Gupta (2006)]. PGN, which provides rigidity and structure to the cell envelope of both Gram-positive and Gram-negative bacteria, is a complex molecule consisting of numerous glycan chains that are cross-linked by oligo-peptides. These glycan chains are composed of altering N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), with short peptides attached by an amide linkage to the lactyl group of MurNAc. Several types of PGN, classified by the nature of the third residue of the stem peptide are commonly found. Typically, this is *m*-diaminopimelic acid (*m*DAP) PGN in Gram-negative bacteria and in some Gram-positive bacilli (genus *Bacillus* and *Clostridium*), whereas most other Gram-positive bacteria have L-lysine (LYS) PGN. In a recent study in tomato Nguyen et al. (2010) showed that pre-inoculation into tomato with *Staphylococcus aureus* PGN reduced the growth of a subsequent bacterial infection in PGN-treated tissue. This priming of defense with a MAMP is similar to that previously described for LPS (Newman et al., 2002). Early experiments with plant cells showed that the Gram-positive human pathogen *S. aureus* PGN was active as an elicitor in inducing extracellular alkalization of cultured tobacco cells, while no response was observed in cultured tomato cells, suggesting a different perception system for PGN within the *Solanaceae* (Felix and Boller, 2003a). PGN from both Gram-positive and Gram-negative bacteria was later found to act as elicitors of plant innate immunity in *Arabidopsis* (Gust et al., 2007; Erbs et al., 2008a). Gust et al. (2007) showed that it was the sugar backbone of the Gram-positive *S. aureus* PGN that was responsible for triggering immune responses and not the breakdown product of PGN, the muramyl dipeptide (MDP) or the muropeptide dimer, which is known to be the minimal chemical structure required for triggering the innate immune system in vertebrates and insects [reviewed by Traub et al. (2006)].

Erbs et al. (2008a), on the contrary, using PGN from two Gram-negative bacterial plant pathogens, *Xcc* and *At* found that both *Xcc* and *At* PGN and its constituents functioned as MAMPs in *Arabidopsis* and induced immune responses such as generation of ROS, extracellular pH increase, *PR1* gene expression, and callose deposition. Furthermore, they showed that the muropeptides were significantly more effective at inducing defense responses than the intact PGN molecule. These observations could be indicative of different perception systems for PGN from Gram-positive and Gram-negative bacteria or differences in structures, and therefore recognition sites, of the muropetides of human vs. plant pathogens. So far, the full structure of PGN from a Gram-positive plant pathogen has not been elucidated. In a study from 2009, Gimenez-Ibanez et al. showed that PGN from the bacterial pathogen *Pst* DC3000 induced the generation of ROS in *Arabidopsis cerk1* (chitin elicitor receptor kinase1) mutant plants, which indicated that *Pst* DC3000 PGN perception is independent of CERK1. In contrast, Willmann et al. (2011) reported that two of three *Arabidopsis* chitin oligosaccharide elicitor-binding proteins (AtCEBiP), LYM1, and LYM3, are involved in the perception/signaling of PGN (from various sources) together with AtCERK1, indicating the presence of a two-component receptor system similar to the rice chitin receptor OsCEBiP and OsCERK1 (Shimizu et al., 2010) (also see text below). All three proteins are required for PGN perception *in vivo* and for resistance to bacterial pathogens. Willmann et al.'s findings also showed that AtCERK1 is involved in the perception of at least two MAMP molecules, chitin and PGN in *Arabidopsis*. Interestingly, Shinya et al. (2012) showed that AtCERK1 serves both for chitin and PGN signaling, but AtCERK1 seems to contribute differently to the signaling. In the case of PGN signaling, the binding proteins LYM1 and LYM3 not only bind the ligand, but also contribute to the activation of AtCERK1 and downstream signaling, similarly to the function of OsCEBiP in rice. On the other hand, in the case of chitin signaling, AtCERK1 seems to function for both ligand perception and signaling (also see text below). Structurally, CEBiP is a receptor protein that contains extracellular LysM domains that are ∼40 aa in length lacking a recognizable intracellular signaling domain. The LysM domains are considered to generally mediate binding to GlcNAc-containing glycans, like chitin and the backbone of PGN [reviewed by Gust et al. (2012)]. Also in monocots two LysM-containing PRRs have been shown to recognize PGN. Liu et al. (2012) reported two homologous rice lysine-motif containing proteins, LYP4 and LYP6. Both proteins bound PGN and chitin, but not LPS *in vitro*. Silencing either of the two proteins impaired the PGN or chitin-induced defense responses, and compromised the resistance against *Xoo* or the fungal pathogen *Magnaporthe oryzae*. These results suggest that PGN and chitin have overlapping perception components in rice.

In mammals, the recognition of PGN is complex, *e.g.*, different receptors are found for PGN (extracellularly) and muropeptides (intracellularly). The cytosolic protein Nod2 can recognize MDP, from both Gram-positive and Gram-negative bacteria, and a lysine-containing muramyl tripeptide, but not a DAP-containing muramyl tripeptide (Girardin et al., 2003). In contrast, Nod1 only detects DAP-containing muropeptides. For instance, the human Nod1 recognizes the DAP-containing GlcNAc and MurNAc

tripeptide (Chamaillard et al., 2003). The structure of the mammalian NOD proteins is similar to that of the plant R proteins, which are intracellular receptor proteins of the NB-LRR type (Inohara et al., 2005). In plants these proteins are involved in the recognition of specialized pathogen effectors leading to ETI (see above). However, in animals they seem to be involved in MAMP recognition rather than recognition of pathogen effectors. The future will show if a system similar to the Nod system, detecting intracellular microbial molecules, could be a possibility in plants.

LIPOPOLYSACCHARIDES (LPS)

LPS, the major component of the outer membrane of Gram-negative bacteria, have been shown to have multiple roles in plant microbe interactions; it is thought to contribute to the restrictive Gram-negative membrane permeability, allowing bacterial growth in unfavorable environments. LPS and its derivatives act as MAMPs and induce innate immune responses in plants (Newman et al., 1995; Dow et al., 2000; Bedini et al., 2005; Silipo et al., 2005). Earlier studies in plants have shown that LPS can prevent the HR induced by bacteria. Pre-treatment of *Arabidopsis* leaves with LPS and its derivatives was found to prevent the HR caused by strains of *Pst* carrying the *avrRpm1* or the *avrRps4* genes, a phenomenon referred to as localized induced response (LIR; Newman et al., 2002; Silipo et al., 2005). The mechanisms behind HR prevention are still unknown, but the effects of LPS pre-treatment are considered to be associated with enhanced resistance of the plant tissue to pathogenic bacteria, which is thought to occur through an LPS-dependent potentiation of plant defense responses (Newman et al., 2002). LPS consists of a lipid, a core oligosaccharide, and an O-polysaccharide part. The lipid, referred to as lipid A, is embedded in the outer part of the phospholipid bilayer in the bacterial membrane. Lipid A and the core oligosaccharide are linked, usually by the sugar 3-deoxy-d-manno-2-octulosonate (KDO). The core oligosaccharide consists of a short series of sugars and ends in the O-antigen, which is composed of repeating oligosaccharide units (Raetz and Whitfield, 2002). The O-antigen of the LPS from many phytopathogenic bacteria has shown to consist of oligorhamnans (Bedini et al., 2002).

In order to know more about the structures within LPS that trigger immune responses in plants, synthetic O-antigen polysaccharides, oligorhamnans of increasing chain lengths, were tested in *Arabidopsis*. Tri-, hexa-, and nonasaccharides were synthesized and found to suppress the HR, as well as act as MAMPs and elicit the induction of the PR genes *PR1*and *PR2* in *Arabidopsis*. The efficiency of HR suppression and *PR* gene induction improved with increasing chain lengths of sugars in the synthetic O-antigen. In addition, a coiled structure was observed with the increasing chain length, indicating a role for this structure as a MAMP and by correlation a role for the O-antigen from phytopathogenic bacteria in plant innate immunity (Bedini et al., 2005). Studies in mammalian cells have shown that LPS is recognized through their lipid A moiety and this recognition was shown to govern the interactions with the innate immune system (Loppnow et al., 1989). In addition to this, the molecular shape of lipid A was found to directly correlate with its activity as a conical shape of lipid A was associated with endotoxicity and a cylindrical shape with antagonistic activity. A net negative charge of lipid A was found to influence its molecular conformation, and with that, its biological activity (Schromm et al., 1998, 2000). To study if the innate immune system from the mammalian system has parallels in the plant system, the role and mechanisms of action of LPS and its derivatives, the core oligosaccharide and the lipid A moiety, in plant-bacteria interactions were investigated in *Arabidopsis*. Initially, the complete structure of purified *Xcc* lipo-oligosaccharides (LOS), LPS without the O-chain, was determined. *Xcc* LOS was found to be a unique molecule with a high negative charge density and a phosphoramide group never found in such molecules before (Silipo et al., 2005). *Xcc* LOS and derivatives have been shown to elicit induction of the *PR* genes in *Arabidopsis*. LOS was found to induce the defense-related *PR1* and *PR2* genes in two temporal phases: the core oligosaccharide induced only the early phase and the lipid A moiety only the later phase, which suggests that both the core oligosaccharide and the lipid A are recognized by plant cells, e.g., both act as elicitors. These findings support the role of *Xcc* lipid A and the *Xcc* core oligosaccharide as MAMPs of innate immunity in plants. Silipo et al. (2005) speculated that the different LPS fragments are recognized by different plant receptors. This elicitor activity of *Xcc* lipid A correlates with earlier studies by Zeidler et al. (2004), who showed that lipid A preparations from various bacteria induced a rapid burst of NO production that was associated with the induction of defense-related genes in *Arabidopsis*. In a recent study by Madala et al. (2011) where the structure of *Burkholderia cepacia* strain ASP B 2D lipid A was determined, the role of lipid A as a MAMP in *Arabidopsis* was confirmed, and it was found to induce transcriptional changes associated with plant defense responses. Contrary to this, studies in tobacco cells, have shown that neither the lipid A nor the O-chain of the *Xcc* LPS molecule could induce the oxidative burst alone, but rather it was the inner core part of the LPS molecule that was responsible (Braun et al., 2005). The conflict in results could reflect the different defense responses measured after treatment with LPS and its derivatives in different plants.

In correlation to studies in the mammalian system, where it is well-established that the phosphorylation pattern of lipid A affects its biological activity [reviewed by Gutsmann et al. (2007)], it was tested whether de-phosphorylated *Xcc* LOS could be recognized in plants. After de-phosphorylation of *Xcc* LOS the molecule maintained only the negative charge of the KDO residue, and rendered the molecule unable to induce LIR, suggesting that the charged groups present in LOS play a key role in inducing defense responses in *Arabidopsis* (Silipo et al., 2005). Furthermore, from these experiments it could be concluded that the electrostatic interactions involving the phosphate groups seem to have a crucial function in binding not only lipid A, but also the core oligosaccharide, to putative receptors in plants (Silipo et al., 2005). LPS has been found, not only to induce defense responses, but also to prime expression of plant defense responses upon subsequent bacterial inoculation, e.g., promote an early triggering of the synthesis of the antimicrobial compounds feruloyl tyramine (FT) and p-coumaroyl tyramine (CT) (Newman et al., 2002, 2007; Prime-A-Plant Group, 2006). The O-antigen part of the LPS molecule is thought to be responsible for induced

systemic resistance (ISR) in *Arabidopsis*. Early studies showed that LPS from the rhizobacterium *Pseudomonas fluorescens,* as well as the live bacteria, induced ISR in carnation and radish, whereas mutant bacteria, lacking the O-antigen side chain could not induce ISR (Leeman et al., 1995; van Loon et al., 1998). In contrast to the rhizobacteria-mediated ISR, systemic activation of defense-related responses in plants upon local necrotizing pathogen infection is referred to as systemic acquired resistance (SAR). SAR is accompanied by a systemic increase in salicylic acid (SA), and SA is required for SAR signaling (Ryals et al., 1996; Schneider et al., 1996). However, recent studies suggest that recognition of the MAMPs, LPS, or Flg, and not necrotic lesion formations contribute to the bacterial induction of SAR in *Arabidopsis*. Treatment of *Arabidopsis* with *P. aeruginosa* LPS, Flg or non-host bacteria were shown to be associated with accumulation of SA, expression of the *PR* genes and expression of the SAR marker gene *Flavin-dependent monooxygenase 1* in treated as well as in distant leaves (Mishina and Zeier, 2006, 2007). The signaling cascades underlying SAR and NO production after perception of LPS by plant cells have not yet been resolved. Sun et al. (2012) investigated the biosynthetic origin of NO and the role of Non-expressor of Pathogenesis-Related Genes (NPR1) to gain insight into the mechanism involved in LPS-induced resistance of *Arabidopsis*. NPR1 is a key regulator of SAR, and is essential for SA signal transduction (Rockel et al., 2002). Analysis of inhibitors and mutants showed that LPS-induced NO synthesis was mainly mediated by an arginine-utilizing source of NO generation. LPS (Sigma)-activated defense responses, including callose deposition and defense-related gene expression, were found to be regulated through an NPR1-dependent pathway. In contrast, *Xcc* LPS can induce defense responses in pepper without triggering the oxidative burst or SA synthesis (Newman et al., 2002).

The activity of LPS in plants has mostly been described in dicots, but studies in rice cells have revealed that LPS, from various pathogenic and non-pathogenic bacteria, induce a generation of ROS and defense-related gene expression in monocots, indicating that the machinery recognizing LPS is evolutionary conserved in monocots and dicots (Desaki et al., 2006, 2012). Furthermore, the two MAMPs, LPS and chitin oligosaccharide, induced a close correlation of genes in rice cells, indicating a convergence in signaling cascades downstream of recognition. In addition, the effect of LPS from various bacteria was shown to be associated with a programmed cell death (PCD) in rice cells. In contrast, LPS has never been shown to elicit PCD in dicots (Desaki et al., 2006). The mechanism by which LPS is perceived by plants is still not understood. Recent studies with fluorescein-labeled *Xcc* LPS in cultured *N. tabacum* cells revealed that LPS was rapidly bound to the cell wall and then internalized into the cell, and eventually, LPS was found exclusively inside the vacuole. These findings suggest endocytosis, comparable to the mammalian system, of *Xcc* LPS in tobacco cells (Gross et al., 2005). However, no PRRs for LPS and its derivatives have been characterized in plants. In the mammalian immune system, LPS form complexes with LPS-binding proteins (LBP), and this LPS-LBP complex is recognized by the membrane-bound CD14 receptor, glycosylphosphatidyl inositol (GPI)-anchored glycoprotein (Wright et al., 1990), which again is thought to associate with TLR4-MD2 to participate in LPS-induced signaling (Jiang et al., 2000; Miyake, 2004). The concentrations of LPS required to elicit most of the effects described above are in the 5–100 μg per ml range, suggesting that plants do not have the sensitivity to LPS shown by mammalian cells, which can respond at concentrations in the pg to ng per ml range. These considerations have led to suggestions that plants possess only low affinity systems to detect LPS (Zeidler et al., 2004), although plants can detect other bacterial MAMPs such as the peptides derived from Flg and EF-Tu elongation factor at sub nM levels. One complicating factor is the aggregation of LPS molecules within the purified preparations, which may affect the ability of LPS to cross the matrix of the plant cell wall to reach presumed membrane-associated receptors (Aslam et al., 2009).

Many groups have attempted to identify plant components involved in LPS recognition and perception often with conflicting results. Livaja et al. (2008) found that in *Arabidopsis* cells, *B. cepacia* LPS induced a leucine-rich repeat RLK At5g45840 by nearly 17-fold after 30 min. Furthermore, in a proteomic analysis of the changes following perception of LPS from an endophytic strain of *B. cepacia* in *N. tabacum* BY-2 cells, 88 LPS induced/regulated proteins, and phosphoproteins were identified, many of which were found to be involved in metabolism and energy-related processes. Moreover, proteins were found that are known to be involved in protein synthesis, protein folding, vesicle trafficking, and secretion (Gerber et al., 2006, 2008). In a transcription profiling of *A. thaliana* cells treated with LPS from *B. cepacia*, Livaja et al. (2008) surprisingly did not find any genes involved in callose synthesis. Furthermore, genes involved in ROS production were found to be upregulated at a very low level by *B. cepacia* LPS. In addition, Livaja et al. (2008) found that *B. cepacia* LPS only induced the *PR* genes *PR3* and *PR4*, whereas studies in *B. cepacia* LPS treated *Arabidopsis* leaves revealed induction of several *PR* genes (Zeidler et al., 2004). Other LPS preparations, from *P. aeruginosa* and *Escherichia coli,* respectively, induce *PR1* and *PR5* in *Arabidopsis* leaves (Mishina and Zeier, 2007). The variation in results both reflects the different plant systems (*Arabidopsis* cell cultures contra the whole plant) and the origin of the LPS. All the above very specific effects show the ability of particular plants to recognize structural features within LPS that are not necessarily widely conserved.

Recognition of LPS/LOS in mammals is rather complex; how complex this recognition is in plants is still not known, and the mechanism of this recognition and consequent transduction steps in plants remains obscure. Gross et al. (2005) showed that, in tobacco cells, *Xcc* LPS was internalized 2 h after its introduction to a cell suspension, where it co-localized with Ara6, a plant homolog of Rab5 which is known to regulate early endosomal functions in mammals. It was speculated that this endocytosis in tobacco cells was, in correlation with the mammalian system, part of a down regulation of defense responses. In a recent study by Zeidler et al. (2010) localization and mobilization of fluorescein-labeled *Salmonella minnesota* LPS was studied in *Arabidopsis*. Leaves were pressure infiltrated with fluorescein-labeled *S. minnesota* LPS and the mobility of LPS was studied over time by fluorescence microscopy. After 1 h a fluorescent signal was observed in the intercellular space of the infiltrated leaf. The labeled LPS were visible in the midrib of the leaves after

4 h, whereas this fluorescence had spread to the smaller leaf veins near the midrib after 6 h. After 24 h it was detectable in the lateral veins. Moreover, cross-sections of the midrib 3 h after supplementation with fluorescein-labeled LPS revealed a fluorescent signal in the xylem. Using capillary zone electrophoresis a distribution of fluorescein-labeled *S. minnesota* LPS was found in the treated as well as in systemic leaves of the plant (Zeidler et al., 2010). In contrast to the results reported by Gross et al. (2005), no intracellular accumulation of the labeled LPS was observed in *Arabidopsis*. This disparity in outcomes might again be a reflection of the use of different plants, the difference in the age of the plants used (plant cell cultures vs. seedlings vs. fully developed plants) and the different defense responses measured after treatment with LPS and its derivatives.

Alterations in lipid A or other structures within LPS are known to occur during symbiotic interactions with plants (Kannenberg and Carlson, 2001) and in response to compounds in plant root exudates (Fischer et al., 2003) and may occur during plant pathogenesis. These alterations may serve both to increase the resistance of the bacteria against host defenses and to attenuate the activity of lipid A or LPS in triggering those defenses. Characterization of the structure and function of LOS from a non-pathogenic *Xcc* mutant strain 8530, which carries a Tn5 insertion in a gene of unknown function (Dow et al., 1995), revealed that this mutant had a truncated core region. The fact that *Xcc* strain 8530 was defective in core completion led to significant modifications in the acylation and phosphorylation patterns of its lipid A, and these changes had influence on its ability to trigger innate immune responses in *Arabidopsis* (Silipo et al., 2008). The core sugars provide protection against antimicrobial compounds and attenuate the endotoxic properties of lipid A, similar to lipid A modifications seen in mammalian pathogens (Raetz et al., 2007). These findings indicate that *Xcc* has the capacity to modify the structure of lipid A and thus reduce its activity as a MAMP in plants (Silipo et al., 2008). The acyl chains of lipid A can vary, as can the number and length of them depending on growth conditions and bacterial species. Studies in mammalian cells have shown that LPS from *Shigella flexneri* elicit a weaker TLR4-mediated response than *E. coli* LPS due to differences in the acylation status of their lipid A moieties (Rallabhandi et al., 2008).

Lipid A from *Halomonas magadiensis*, an extremophilic and alkaliphilic Gram-negative bacteria, isolated from a soda lake in an East African Rift Valley has been found to act as an LPS antagonist in human cells (Silipo et al., 2004). *H. magadiensis* lipid A, characterized by an unusual and very low degree of acylation, was verified to inhibit *E. coli* lipid A-induced immune responses in human cells (Ialenti et al., 2006). *E. coli* lipid A, which is an effective agonistic structure of immune responses in mammalian cells, is composed of a bis-phosphorylated hexa-acylated disaccharide backbone with an asymmetric distribution of the acyl residues. Studies have revealed that structural differences on the lipid A skeleton, for example, acylation can affect its agonist/antagonist activity (Munford and Varley, 2006). In consonant with the ability in blocking enteric LPS-induced human monocyte activation, our laboratory found that *H. magadiensis* lipid A was able to antagonize the action of *E. coli* lipid A when inducing *PR1* gene expression in *Arabidopsis*. Even though the mode of perception of LPS in plants is far less-understood than in mammals and insects, these results indicate that *Arabidopsis* is sensitive to the same structures of lipid A that determine biological activity in humans (Erbs et al., 2008b).

Thus far, LPS preparations used for the analysis of plant responses and for structural studies have been derived from bacteria grown in culture. We know nothing about the alterations in LPS that might occur when bacteria are within plants, although this may be highly relevant for recognition and signaling. Changes could occur in both the size distribution of LPS (alteration in the ratio of LOS to LPS) and/or in decoration of LPS with saccharide, fatty acid, phosphate, or other constituents. Increases in the sensitivity of mass spectrometric methodologies may allow development of micro-methods to analyse such changes in bacteria isolated from plants. Transcriptome or proteome profiling of bacteria isolated from plants may also give clues as to possible LPS modifications.

Intriguingly, although lipid A-like molecules have not been reported in plants, many plants, including *Arabidopsis*, encode full-length nuclear orthologs of six of the nine enzymes of the *E. coli* biosynthetic genes for lipid A. *Arabidopsis* mutants generated by knock-out of these genes are viable under laboratory conditions. However, they accumulate (wild type) or lose (mutant) the expected lipid A precursors (Li et al., 2011). The lipid A biosynthetic genes of higher plants may have been acquired from Gram-negative bacteria with the endosymbiosis of mitochondria. Plant lipid A may therefore play a structural role in mitochondrial or perhaps chloroplast membranes. Alternatively, lipid A-like molecules in *Arabidopsis* may be involved in signal transduction of plant defense responses. Although the mechanisms by which plants detect LPS remain unknown, lipid A-like molecules in plants might serve as signals to regulate cellular responses during plant pathogen invasion.

ACTIVATOR OF XA21-MEDIATED IMMUNITY (Ax21)

Even though the rice receptor XA21 has been known for a long time, the corresponding ligand Ax21 (previously known as avrXa21) was identified only recently (Lee et al., 2009). The conservation of Ax21 in all sequenced *Xanthomonas* spp., *Xylella fastidiosa,* and the human pathogen *Stenotrophomonas maltophila* suggests that it plays a key role in a biological function. *Ax21* encodes a 194 aa protein (Bogdanove et al., 2011). The minimal recognized epitope mimicking Ax21 activity is a 17 aa sulfated peptide, called axY^{s}22, which has been shown to be 100% identical among six different *Xanthomonas* spp. (Lee et al., 2009). XA21, is, together with FLS2 and EFR, among the best studied PRRs, they all belong to subfamily LRR XII of the non-RD class of receptor kinases (Shiu and Bleecker, 2003; Shiu et al., 2004; Dardick and Ronald, 2006). *Xa21* was originally identified as a dominant-resistant locus conferring resistance to multiple *Xoo* races in the wild rice species *O. longistaminata* (Khush et al., 1990). *Xa21* maps to chromosome 11, and already upon its discovery it was speculated to encode a gene product recognizing a determinant present in all *Xoo* races (Ronald et al., 1992). Later, the resistance of locus *Xa21* was linked to a single gene, also named *Xa21*, encoding a receptor kinase-like protein with predicted LRR, TM, juxtamembrane (JM), and intracellular kinase domains and that

this single gene was sufficient to confer resistance to a number of *Xoo* isolates (Song et al., 1995; Wang et al., 1996). *Xa21* is a member of a gene family with at least seven members in rice. The closest relative to *Xa21* is *Xa21D* and the spectrum of resistance is identical between the two genes, but the level of resistance differs as *XA21D* only confers partial resistance. The LRR domains of Xa21 and Xa21D are more than 99% identical, but Xa21D lacks the TM and kinase domains and it may therefore have an extracellular function, but the mode of action is unknown (Song et al., 1997; Wang et al., 1998).

Several proteins have been shown to interact with XA21. The ATPase XB24 (XA21-binding protein 24) promotes autophosphorylation of XA21, which is thereby kept in an inactive state. When Ax21 binds to XA21, the XB24/XA21 protein complex probably dissociates, and XA21 is activated (Chen et al., 2010). After activation, the phosphatase XB15 (XA21-binding protein 15) dephosphorylates XA21 in order to deactivate it again (Park et al., 2008). A recent study has shown that upon Ax21 recognitions by XA21, the intercellular kinase domain is released and translocated to the cell nucleus, a translocation that is necessary for the XA21-mediated immune response (Park and Ronald, 2012). The rice transcription factor OsWKKY62 (also called XB10), which has previously been shown to be a negative regulator of XA21 activity, is needed for this translocation (Peng et al., 2008; Park and Ronald, 2012). The E3 ubiquitin kinase XB3 (XA21-binding protein 3) is important for XA21-mediated resistance, as rice lines silenced in XB3 both has a decreased level of XA21 and display reduced resistance to *Xoo* (Wang et al., 2006). Less thoroughly studied are the genes *Rox1*, *2*, and *3* (Regulator of XA21-mediated immunity 1, 2, and 3 encoding a thiamine phosphokinase, a NOL1/NOL2/sun gene family member and a nuclear migration protein, respectively), which have also been shown to affect *Xoo* resistance in XA21-containing rice plants (Lee et al., 2011).

Interestingly, the *Arabidopsis* FLS2 and EFR receptors bind the artificial Ax21 derived peptide axY^{s}22-A1, this binding trigger responses similar to the ones triggered by Flg. axY^{s}22-A1 is identical to axY^{s}22, except that the first aa in the peptide has been changed from Ala to Glu (Danna et al., 2011). It was previously thought that FLS2 was specific to Flg. Even though the authors analyzed their axY^{s}22-A1 peptide stocks for the presence of flg22 by mass spectrometry, a question was later raised whether the observations were caused by flg22 contamination (Danna et al., 2011; Mueller et al., 2012). Mueller et al. (2012) had observed incidences of commercially produced peptides contaminated with flg22 and even minute amounts (in the range of 1 ppm) will activate FLS2 responses. Furthermore, *Arabidopsis* cell cultures did not respond to treatment with axY^{s}22 in their laboratory, therefore they concluded that the FLS2 binding observed by Danna et al. (2011) could be caused by contamination (Mueller et al., 2012). These concerns were dismissed by Danna et al. (2012), who believe that the difference in peptides used (axY^{s}22-A1 vs. axY^{s}22) and the differences in their experimental set-ups, could explain different results.

Ax21 is secreted from the bacterial cell through the TOSS—a fact that has been known longer than the identity of Ax21 (da Silva et al., 2004). TOSS is a relatively simple system consisting of only three protein subunits: a membrane fusion protein (MFP), an adenosine triphosphate-binding cassette (ABC) transporter, and an outer membrane protein (OMP). Three *Xoo* genes with homology to the TOSS components have been shown to be required for Ax21 activity (the so called *rax* genes). The genes *raxA*, *raxB*, and *raxC* are identified as coding for a MFP, an ABC transporter, and an OMP, respectively (da Silva et al., 2004). *raxA* and *raxB* are arranged in a putative operon (called *raxSTAB*) together with the gene *raxST*, which is not a part of the TOSS (da Silva et al., 2004). Instead *raxST* is a sulfotransferase (Shuguo et al., 2012), which catalyze the transfer of sulfate from PAPS (3′-phosphoadenosine 5′-phosphosulfate) to a tyrosine residue. Sulfation of secreted peptides is often important for their biological function. This is also the case for the Ax21-derived peptide axY^{s}22, as a non-sulfated version of the peptide, axY22 is not recognized by XA21 (Lee et al., 2009). Based on genetic similarity and complementation studies, the two genes *raxP* and *raxQ* have been suggested to be responsible for the PAPS synthesis as they encode proteins with ATP sulfohydrolase and APS kinase activities (Shen et al., 2002). Downstream of *raxSTAB* another putative operon, comprised of the two genes *raxR* and *raxH*, has been found. *Xoo* strains mutated in these two genes do not express full Ax21 activity. These two genes are probably encoding proteins involved in a bacterial two component regulatory system (a response regulator and a histidine protein kinase), and they could be involved in the regulation of a number of genes (Burdman et al., 2004). The two component system composed of RaxR and RaxH have also been found to regulate the expression of another two component system composed of PhoP and PhoQ. The PhoPQ system also seems to control the TTSS, important for delivering bacterial effector molecules to the host cell, through regulation of the *hrpG* gene (Lee et al., 2008).

As Ax21 was shown to be a secreted molecule (Lee et al., 2009) and the finding that the expression of *raxST*, *raxP*, *raxR,* and *raxC* are density-dependent it was suggested that Ax21 is a quorum sensing (QS) molecule (Lee et al., 2006). This was supported by a finding in *S. maltophila*, showing that mutants lacking Ax21 display reduced motility and biofilm formation. Also in this organism it appears that RaxH and RaxR are part of a two-component system (McCarthy et al., 2011). Han et al. (2011) published the evidence for this hypothesis, thereby making Ax21 the first QS factor also functioning as a MAMP. But unfortunately critical errors of a central *Xoo* strain used in the study has been found and the authors of the original paper are now in the process of repeating the experiments with a new validated strain in order to confirm the results (Comment on the PLoS homepage since January 2013). The outcome of these experiments should be followed with great interest. Knowledge of detection of small proteins, like QS in rice and other species, can be used to develop reagents to disrupt QS-mediated virulence activities.

FUNGAL AND OOMYCETE MAMPs

CHITIN AND β-GLUCAN

Examples of MAMPs from fungi and oomycetes include the fungal chitin and β-glucan from *P. megasperma*. However, data describing how these MAMPs are recognized and how the

following signal transduction is mediated has only in a few cases been accomplished.

In fungal cell walls branched β-glucan is cross-linked to chitin and in oomycetes to cellulose. In soybean the PRR recognizing *P. megasperma* β-glucan was identified as the β-glucan binding protein (GBP) (Umemoto et al., 1997). This MAMP and its corresponding PRR has not been studied further. On the other hand chitin and its fragments chitin oligosaccharides have been shown to trigger defense responses in both monocots and dicots. Together with CEBiP, CERK1 recognizes fungal chitin (Kaku et al., 2006; Shimizu et al., 2010). In rice, the RLP CEBiP binds chitin oligosaccharides at the cell surface and interacts with the LysM-RLK OsCERK1 for signaling. In *Arabidopsis*, only the LysM-containing RLK CERK1 was found to be essential for chitin elicitor signaling (Miya et al., 2007). Three CEBiP-like proteins, LYM1-3, have been identified in *Arabidopsis*. Using heterologous expression of these three *Arabidopsis* CEBiP homologous in tobacco BY-2 cells Shinya et al. (2012) tested for their ability to bind chitin oligosaccharides and found that only LYM2, also referred to as AtCEBiP, showed high affinity binding to chitin oligosaccharides. Even though affinity labeling with biotinylated $(GlcNAc)_8$ indicated that AtCEBiP represent a cell surface chitin-binding protein, knockout (KO) of AtCEBiP, LYM1, or LYM3, single or triple KO, together with AtCEBiP overexpression studies suggested that AtCEBiP does not contribute to chitin signaling in *Arabidopsis* (Shinya et al., 2012). These studies reveal that *Arabidopsis* and rice exploit different chitin receptor systems. Similar results were obtained by Wan et al. (2012) who showed that mutations in each of the three *Arabidopsis* CEBiP-like proteins 1, 2, or 3, or in a combination resulting in a triple mutant, had no effect on the plant response to chitin. *Arabidopsis* has five LysM RLKs1-5 (Lyk1, 2, 3, 4, and 5) one of them, Lyk1, is also known as CERK1. Wan et al. (2012) tested the *Arabidopsis lyk2*, *3*, *4,* and *5* KO mutants, respectively, to see if they were involved in chitin signaling. They found that the plant immune response to chitin was reduced only in the *lyk4* mutant suggesting Lyk4 to be involved in a chitin recognition receptor complex (Wan et al., 2012). Furthermore, the *lyk4* plants were more susceptible to the fungal pathogen *Alternaria brassicicola* and the bacterial pathogen *Pst* DC3000 than wild type plant (Wan et al., 2012). In addition to this it has been reported that two rice lysine-motif containing proteins, LYP4 and LYP6, could bind both PGN and chitin acting as dual functional PRRs in rice innate immunity (Liu et al., 2012). Their results further suggest that overlapping perception systems exist for bacterial PGN and fungal chitin in rice. In contrast, LYM1 and LYM3 the orthologs of LYP4 and LYP6 in *Arabidopsis* were only able to bind PGN and not chitin (Willmann et al., 2011). Further details on PGN recognition can be found in the text above.

AVE1 PEPTIDE AND ETHYLENE-INDUCING XYLANASE (EIX)

In tomato a *Verticillium* resistance locus *Ve* was identified that mediates resistance against race 1 strains of *Verticillium dahlia* and *V. albo-atrium*, respectively (Kawchuk et al., 2001). The characterization of the *Ve* locus revealed two genes *Ve1* and *Ve2* that encode cell-surface receptors belonging to the LRR class of RLP. Only *Ve1* was found to confer resistance in tomato. Moreover, tomato plants silenced in *BAK1*, showed higher susceptibility to infection with *Verticillium* indicating that BAK1 is involved in Ve1-induced defense responses in tomato (Fradin et al., 2009). A putative ligand for the LRR-RLP Ve1 is the Ave1 (avirulence on Ve1 tomato) peptide. Ave1, a conserved peptide identified in several fungi and in the plant pathogenic bacteria *Xanthomonas axonopodis* pv. *citri*, has been found to have homology to plant natriuretic peptides (PNPs). PNPs are extracellular signaling molecules that have been shown to have a role in regulation of homeostasis under several stress conditions (Wang et al., 2011). Ave1 acts as an elicitor of disease resistance mediated by the LRR-RLP Ve1 in tomato (de Jonge et al., 2012), but a direct binding between Ave1 and Ve1 remains to be shown. Ve1 has been referred to as a PRR or an R protein accompanied by speculations that the Ave1 peptide could be an effector acting as a MAMP (Thomma et al., 2011). Future results will reveal, if it is possible to differentiate as strictly, as we do today, between MAMPs and effectors, as between PRRs and R proteins.

Two other PRRs in tomato, the LRR-RLPs SlEix1 and SlEix2, which have been shown to have homology to the tomato Ve and Cf PRRs, recognize the fungal ethylene-inducing xylanase (EIX) (Ron and Avni, 2004). EIX is a 22-kD fungal protein (β-1-4-endoxylanase) from *Trichoderma viride* that independent of its endoxylanase activity can act as an elicitor of defense responses in tomato and tobacco plants (Furman-Matarasso et al., 1999; Ron and Avni, 2004). The aa sequence of SlEix1 and SlEix2 are 81.4% identical, SlEix1 and SlEix2 both bind EIX, but their functions differ. The SlEix2 receptor has been shown to be internalized upon EIX application (Bar and Avni, 2009) and only SlEix2 transmits the signal mediated by EIX leading to plant immune responses (Ron and Avni, 2004). SlEix1, on the other hand, block the EIX signaling and the authors suggested that SlEix1 functions in inhibition of plant defense signaling and plant cell death in response to EIX (Bar et al., 2010). Using BAK1-silenced tobacco plants Bar et al. (2010) further showed that BAK1 was required for this inhibitory activity of SlEix1 on SlEix2 signaling and endocytosis.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS (DAMPs)

The plant defense system is not only recognizing microbial elicitors, some plant-derived molecules also induce plant defense responses. This sensing of infectious-self or modified-self is mediated by DAMPs (Seong and Matzinger, 2004; Boller and Felix, 2009), also referred to as microbe-induced molecular patterns (MIMPs, Mackey and McFall, 2006). Similarly the mammalian immune system detects "danger" through a series of DAMPs, now also in in this system named damage-associated. The mammalian DAMPs are derived from other tissues activating intracellular cascades that lead to an inflammatory response (Lotze et al., 2007).

In Plants the 18 aa tomato peptide systemin is an endogenous elicitor of plant defense (Pearce et al., 1991; McGurl et al., 1992). The systemin precursor prosystemin is a cytoplasmic protein and upon cell damage the released systemin acts as a DAMP on surrounding cells (Narváez-Vásquez and Ryan, 2004). Early reports showed that the RLK SR160 (the tomato ortholog of BRI1) was the receptor for systemin (Scheer and Ryan, 1999, 2002), but later reports found that null mutants were sensitive to systemin

(Holton et al., 2007; Lanfermeijer et al., 2008). Also in *Arabidopsis* a system with a putative cytosolic peptide (Pep1) activates transcription of defense-related genes and induces alkalization in cell cultures. The 23 aa Pep1 and the seven homologous in *Arabidopsis* (Pep 1–7) are derived from the C-terminal part of their precursor proteins PROPEP1–7 (Huffaker et al., 2006). The Pep1 receptor, PEPR1, was found to be a LRR receptor belonging to the LRR XI subfamily (Yamaguchi et al., 2006). Based on sequence similarity a second receptor of Pep-peptides, PEPR2, has been identified. Transcription of both *PEPR1* and *2* is activated by wounding, Methyl-Jasmonate (MeJA), Pep-peptides, and specific MAMPs. A level of redundancy is found regarding ligand specificity of PEPR1 and 2 as they both bind Pep1 and 2, and in addition PEPR1 binds Pep3–6 (Yamaguchi et al., 2010).

Oligogalacturonides (OG) and cutin released from plant cell walls also function as DAMPs (Schweizer et al., 1996; Denoux et al., 2008). Using a domain swap approach Brutus et al. (2010) proved that WAK1 (Wall-Associated Kinase 1) function as an OG receptor whereas a receptor for cutin still remains to be found.

CONCLUDING REMARKS

Even though MAMPs are much conserved, they are under selective pressure in adapted pathogens to evade recognition. For example in the case of bacterial Flg, a potent inducer of MTI in most plants, mutations in key residues of the flg22 epitope that abolish recognition by the receptor FLS2 have been selected for in several plant pathogens and symbionts (Boller and Felix, 2009; Lopez-Gomez et al., 2012). Notably, this positive selection seems to be more rapid than previously thought, as modern natural isolates of *Pst* adapt to their tomato host through non-synonymous mutations in the Flg-encoding gene *fliC* (Cai et al., 2011). The best example so far of a glycosylated MAMP not being recognized in plants, is the LPS molecule from the nitrogen-fixing soil bacterium *Bradyrhizobium* sp. BTAi1, this LPS does not trigger the innate immune response in different plant families. *Aeschynomene indica* (the natural host of *Bradyrhizobium*), *Lotus japonicus,* and *Arabidopsis* were tested for perception of *Bradyrhizobium* LPS. Defense responses were not induced in any of the tested plants. The authors determined the structure of *Bradyrhizobium* LPS and found a unique LPS with an, in nature, unprecedented chemical structure of the monosaccharide forming the polymer, this "different" structure probably prevents recognition by the LPS receptor complex in plants (Silipo et al., 2011). MAMPs are necessary for microbial life and therefore under strong negative selection, but their immunogenic epitopes are under positive selection to evade host immune detection. These opposing evolutionary forces were recently used to identify novel candidate MAMPs from *Pseudomonas* and *Xanthomonas* species through an innovative bioinformatics approach. Identifying new MAMPs may prove to be a source of new antimicrobial agents (McCann et al., 2012).

Although plant receptors for bacterial PGN and the proteinaceous MAMPs Flg and EF-Tu elongation factor have been identified, those involved in perception of LPS remain obscure. In conclusion we expect that in the next few years we will see a substantial increase in our understanding of the processes of MAMPs perception and signal transduction in plants through the deployment of cross disciplinary approaches and ever expanding ranges of molecular experimental tools. Despite their critical role in immunity, we know remarkably little about the range and diversity of MAMPs. Most studies have focused on a limited number of MAMPs as described in this review. The identification of new MAMPs will give insight into the molecular and evolutionary mechanisms underlying host-pathogen interactions, and greater understanding of the mechanisms by which MAMPs elicits defense responses may have considerable impact on the improvement of plant health and disease resistance.

ACKNOWLEDGMENTS

Gitte Erbs and Mari-Anne Newman acknowledge funding by The Danish Council for Independent Research, Technology, and Production Sciences (FTP) and VILLUM FONDEN, Denmark.

REFERENCES

Aslam, S. N., Erbs, G., Morrissey, K. L., Newman, M.-A., Chinchilla, D., Boller, T., et al. (2009). MAMPs signatures, synergy, size and charge: influences on perception or mobility and host defense responses. *Mol. Plant Pathol.* 10, 375–387.

Ausubel, F. (2005). Are innate immune signalling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973–979.

Bailey, B. A., Dean, J. F. D., and Anderson, J. D. (1990). An ethylene biosynthesis inducing endoxylanase elicits electrolyte leakage and necrosis in *Nicotiana tabacum* cv *xanthi* Leaves. *Plant Physiol.* 94, 1849–1854.

Bar, M., and Avni, A. (2009). EHD2 inhibits ligand-induced endocytosis and signaling of the leucine-rich repeat receptor-like protein LeEix2. *Plant J.* 59, 600–611.

Bar, M., Sharfman, M., Ron, M., and Avni, A. (2010). BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1. *Plant J.* 63, 791–800.

Bauer, Z., Gomez-Gomez, L., Boller, T., and Felix, G. (2001). Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptorbinding sites. *J. Biol. Chem.* 276, 45669–45676.

Bedini, E., De Castro, C., Erbs, G., Mangoni, L., Dow, J. M., Newman, M.-A., et al. (2005). Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. *J. Am. Chem. Soc.* 127, 2414–2416.

Bedini, E., Parrilli, M., and Unverzagt, C. (2002). Oligomerization of a rhamnanic trisaccharide repeating unit of O-chain polysaccharides from phytopathogenic bacteria. *Tetrahedron Lett.* 43, 8879–8882.

Bent, A. F., and MacKey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45, 399–436.

Bogdanove, A. J., Koebnik, R., Lu, H., Furutani, A., Angiuoli, S. V., Patil, P. B., et al. (2011). Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic *Xanthomonas* spp. *J. Bacteriol.* 193, 5450–5464.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406.

Braun, S. G., Meyer, A., Holst, O., Pühler, A., and Niehaus, K. (2005). Characterization of the *Xanthomonas campestris* pv. campestris lipopolysaccharide substructures essential for elicitation of an oxidative burst in tobacco cells. *Mol. Plant Microbe Interact.* 18, 674–681.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457.

Burdman, S., Shen, Y., Lee, S. W., Xue, Q., and Ronald, P. C.

(2004). RaxH/RaxR: a two-component regulatory system in Xanthomonas oryzae pv. oryzae required for AvrXa21 activity. *Mol. Plant-Microbe Interact.* 17, 602–612.

Cai, R., Lewis, J., Yan, S., Liu, H., Clarke, C. R., Campanile, F., et al. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. *PLoS Pathog.* 7:e1002130. doi: 10.1371/journal.ppat.1002130

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., et al. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* 4, 702–707.

Chen, X., Chern, M., Canlas, P. E., Ruan, D., Jiang, C., and Ronald, P. C. (2010). An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8029–8034.

Cheong, J.-J., and Hahn, M. G. (1991). A specific, high-affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. *Plant Cell* 3, 137–147.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18, 465–476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, D. G. J., et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defense. *Nature* 448, 497–500.

Csorba, T., Pantaleo, V., and Burgyán, J. (2009). RNA silencing: an antiviral mechanism. *Adv. Virus Res.* 75, 35–71.

da Silva, F. G., Shen, Y., Dardick, C., Burdman, S., Yadav, R. C., de Leon, A. L., et al. (2004). Bacterial genes involved in type I secretion and sulfation are required to elicit the rice Xa21-mediated innate immune response. *Mol. Plant-Microbe Interact.* 17, 593–601.

Danna, C. H., Millet, Y. A., Koller, T., Han, S. W., Bent, A. F., Ronald, P. C., et al. (2011). The *Arabidopsis* flagellin receptor FLS2 mediates the perception of *Xanthomonas* Ax21 secreted peptides. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9286–9291.

Danna, C. H., Zhang, X. C., Khatri, A., Bent, A. F., Ronald, P. C., and Ausubel, F. M. (2012). FLS2-mediated responses to Ax21-derived peptides: response to the Mueller et al. commentary. *Plant Cell* 24, 3174–3176.

Dardick, C., and Ronald, P. (2006). Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathog.* 2:e2. doi: 10.1371/journal.ppat.0020002

Darvill, A. G., and Albersheim, P. (1984). Phytoalexins and their elicitors-a defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35, 243–275.

de Jonge, R., Peter van Esse, H., Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., et al. (2012). Tomato immune receptor *Ve1* recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5110–5115.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., et al. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Mol. Plant* 1, 423–445.

Desaki, Y., Miya, A., Venkatesh, B., Tsuyumu, S., Yamane, H., Kaku, H., et al. (2006). Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. *Plant Cell Physiol.* 47, 1530–1540.

Desaki, Y., Otomo, I., Kobayashi, D., Jikumaru, Y., Kamiya, Y., Venkatesh, B., et al. (2012). Positive crosstalk of MAMP signaling pathways in rice cells. *PLoS ONE* 7:e51953. doi: 10.1371/journal.pone.0051953

Dow, M., Newman, M.-A., and von Roepenack, E. (2000). The induction and modulation of plant defense responses by bacterial lipopolysaccharides. *Annu. Rev. Phytopathol.* 38, 241–261.

Dow, J. M., Osbourn, A. E., Wilson, T. J. G., and Daniels, M. J. (1995). A locus determining pathogenicity of *Xanthomonas-campestris* is involved in lipopolysaccharide biosynthesis. *Mol. Plant Microbe Interact.* 8, 768–777.

Dziarski, R., and Gupta, D. (2006). The peptidoglycan recognition proteins (PGRPs). *Genome Biol.* 7, 232–245.

Erbs, G., and Newman, M.-A. (2012). The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. *Mol. Plant Pathol.* 13, 95–104.

Erbs, G., Silipo, A., Aslam, S., De Castro, C., Liparoti, V., Flagiello, A., et al. (2008a). Peptidoglycan and muropeptides from pathogens *Agrobacterium* and *Xanthomanas* elicit innate immunity: structure and activity. *Chem. Biol.* 15, 438–448.

Erbs, G., Jensen, T. T., Silipo, A., Grant, W., Dow, J. M., Molinaro, A., et al. (2008b). An antagonist of lipid A action in mammals has complex effects on lipid A induction of defense responses in the model plant *Arabidopsis thaliana*. *Microbes Infect.* 10, 571–574.

Felix, G., and Boller, T. (2003a). Molecular sensing of bacteria in plants. *J. Biol. Chem.* 278, 6201–6208.

Felix, G., and Boller, T. (2003b). Molecular sensing of bacteria in plants - the highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. *J. Biol. Chem.* 278, 6201–6208.

Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18, 265–276.

Felix, G., Regenass, M., and Boller, T. (1993). Specific perception of subnanomolar concentrations of chitin fragments by tomato cells—induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. *Plant J.* 4, 307–316.

Fischer, S. E., Miguel, M. J., and Mori, G. B. (2003). Effect of root exudates on the exopolysaccharide composition and the lipopolysaccharide profile of *Azospirillum brasilense* Cd under saline stress. *FEMS Microbiol. Lett.* 219, 53–62.

Flor, H. H. (1942). Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 32, 653–669.

Fradin, E. F., Zhang, Z., Ayala, J. C. J., Castroverde, C. D. M., Nazar, R. N., Robb, J., et al. (2009). Genetic dissection of *Verticillium* wilt resistance mediated by tomato *Ve1*. *Plant Physiol.* 150, 320–332.

Furman-Matarasso, N., Cohen, E., Du, Q., Chejanovsky, N., Hanania, U., and Avni, A. (1999). A point mutation in the ethylene-inducing xylanase elicitor inhibits the β-1-4-endoxylanase activity but not the elicitation activity. *Plant Physiol.* 121, 345–351.

Gaulin, E., Dramé, N., Lafitte, C., Torto-Alalibo, T., Martinez, Y., Ameline-Torregrosa, C., et al. (2006). Cellulose binding domains of a *Phytophthora* cell wall protein are novel pathogen-associated molecular patterns. *Plant Cell* 18, 1766–1777.

Gerber, I. B., Laukens, K., De Vijlder, T., Witters, E., and Dubery, I. A. (2008). Proteomic profiling of cellular targets of lipopolysaccharide-induced signaling in *Nicotiana tabacum* BY-2 cells. *Biochim. Biophys. Acta* 1784, 1750–1762.

Gerber, I. B., Laukens, K., Witters, E., and Dubery, I. A. (2006). Lipopolysaccharide-responsive phosphoproteins in *Nicotiana tabacum* cells. *Plant Physiol. Biochem.* 44, 369–379.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J. P. (2009). The LysM receptor kinase CERK1 mediates bacterial perception in *Arabidopsis*. *Plant Signal. Behav.* 4, 539–541.

Girardin, S. E., Travassos, L. H., Herve, M., Blanot, D., Boneca, I. G., Philpott, D. J., et al. (2003). Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J. Biol. Chem.* 278, 41702–41708.

Gómez-Gómez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signalling in *Arabidopsis*. *Plant Cell* 13, 1155–1163.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011.

Greenberg, J. T. (1997). Programmed cell death in plant-pathogen interactions. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 525–545.

Gross, A., Kapp, D., Nielsen, T., and Niehaus, K. (2005). Endocytosis of *Xathomonas campestris* pathovar *campestris* lipopolysaccharides in non-host plant cells of *N. benthamiana*. *New Phytol.* 165, 215–226.

Gust, A., Biswas, R., Lenz, H. D., Rauhut, T., Ranf, S., Kemmerling, B., et al. (2007). Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. *J. Biol. Chem.* 282, 32338–32348.

Gust, A. A., Willmann, R., Desaki, Y., Grabherr, H. M., and Nürnberger, T. (2012). Plant LysM proteins: modules mediating symbiosis and immunity. *Trends Plant Sci.* 17, 495–502.

Gutsmann, T., Schromm, A. B., and Brandenburg, K. (2007). The physiochemistry of endotoxins in relation to bioactivity. *J. Med. Microbiol.* 297, 341–352.

Hammond-Kosack, K. E., and Jones, J. D. (1996). Resistance

gene-dependent plant defense responses. *Plant Cell* 8, 1773–1791.

Han, S. W., Sriariyanum, M., Lee, S. W., Sharma, M., Bahar, O., Bower, Z., et al. (2011). Small protein-mediated quorum sensing in a gram negative bacterium. *PLoS ONE* 6:e29192. doi: 10.1371/journal.pone.0029192

Häweker, H., Rips, S., Koiwa, H., Salomon, S., Saijo, Y., Chinchilla, D., et al. (2010). Pattern recognition receptors require N-glycosylation to mediate plant immunity. *J. Biol.Chem.* 285, 4629–4636.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., et al. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 410, 1099–1103.

He, P., Shan, L., Lin, N.-C., Martin, G. B., Kemmerling, B., Nürnberger, T., et al. (2006). Specific bacterial suppressors of MAMP signalling upstream of MAPKKK in *Arabidopsis* innate immunity. *Cell* 125, 563–575.

Holton, N., Caño-Delgado, A., Harrison, K., Montoya, T., Chory, J., and Bishop, G. J. (2007). Tomato BRASSINOSTEROID INTENSITIVE1 is required for systemin-induced root elongation in *Solanum pimpinellifolium* but is not essential for wound signaling. *Plant Cell* 19, 1709–1717.

Huffaker, A., Pearce, G., and Ryan, C. A. (2006). An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10098–10103.

Ialenti, A., Di Meglio, P., Grassia, G., Maffia, P., Di Rosa, M., Lanzetta, R., et al. (2006). A novel lipid A from *Halomonas magadiensis* inhibits enteric LPS-induced human monocyte activation. *Eur. J. Immunol.* 36, 354–360.

Inohara, N., Chamaillard, M., McDonald, C., and Nunez, G. (2005). NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 74, 355–383.

Jamir, Y., Guo, M., Oh, H.-S., Petnicki-Ocwieja, T., Chen, S., Tang, X., et al. (2004). Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. *Plant J.* 37, 554–565.

Jeppesen, M. G., Navratil, T., Spremulli, L. L., and Nyborg, J. (2005). Crystal structure of the bovine mitochondrial elongation factor Tu.Ts complex. *J. Biol. Chem.* 280, 5071–5081.

Jiang, S., Naito, M., Kaizu, C., Kuwata, K., Hasegawa, G., Mukaida, N., et al. (2000). Lipopolysaccharide-induced cytokine and receptor expression and neutrophil infiltration in the liver of osteopetrosis (op/op) mutant mice. *Liver* 20, 465–474.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Kaku, H., Nishizawa, Y., Ishll-Minami, N., Akimoto-Tomlyama, C., Dohmae, N., Taklo, K., et al. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091.

Kannenberg, E. L., and Carlson, R. W. (2001). Lipid A and O-chain modifications cause rhizobium lipopolysaccharides to become hydrophobic during bacteroid development. *Mol. Microbiol.* 39, 379–391.

Kauss, H., Fauth, M., Merten, A., and Jeblick, W. (1999). Cucumber hypocotyls respond to cutin monomers via both an inducible and a constitutive H2O2-generating system. *Plant Physiol.* 120, 1175–1182.

Kawchuk, L., Hachey, J., Lynch, D. R., Klcsar, F., van Rooijen, G., Waterer, D. R., et al. (2001). Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proc. Natl Acad. Sci. U.S.A.* 98, 6511–6515.

Khush, G. S., Bacalangco, E., and Ogawa, T. (1990). A new resistance gene to bacterial blight derived from *O.* longistaminata. *Rice Genet.* 7, 121–122.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16, 3496–3507.

Lamb, C., and Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275.

Lanfermeijer, F. C., Staal, M., Malinowski, R., Stratmann, J. W., and Elzenga, T. M. (2008). Micro-electrode flux estimation confirms that the *Solanum pimpinellifolium cu3* mutant still responds to systemin. *Plant Physiol.* 146, 129–139.

Lee, I., Seo, Y. S., Coltrane, D., Hwang, S., Oh, T., Marcotte, E. M., et al. (2011). Genetic dissection of the biotic stress response using a genome-scale gene network for rice. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18548–18553.

Lee, S. W., Han, S. W., Sririyanum, M., Park, C. J., Seo, Y. S., and Ronald, P. C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* 326, 850–853.

Lee, S.-W., Han, S.-W., Bartley, L. E., and Ronald, P. C. (2006). Unique characteristics of *Xanthomonas oryzae* pv. oryzae AvrXa21 and implications for plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 103, 18395–18400.

Lee, S. W., Jeong, K. S., Han, S. W., Lee, S. E., Phee, B. K., Hahn, T. R., et al. (2008). The *Xanthomonas oryzae* pv. oryzae PhoPQ two-component system is required for AvrXA21 activity, hrpG expression, and virulence. *J. Bacteriol.* 190, 2183–2196.

Leeman, M., Vanpelt, J. A., Denouden, F. M., Heinsbroek, M., Pahm, B., and Schippers, B. (1995). Induction of systemic resistance against fusarium-wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathol* 85, 1021–1027.

Li, C., Guan, Z., Liu, D., and Raetz, C. R. H. (2011). Pathway for lipid A biosynthesis in *Arabidopsis thaliana* resembling that of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A* 108, 11397–11392.

Liu, B., Li, J.-F., Ao, Y., Qu, J., Li, Z., Su, J., et al. (2012). Lysin motif-containing proteins LYP4 and LYP6 play dual roles in peptidoglycan and chitin perception in rice innate immunity. *Plant Cell* 24, 3406–3419.

Livaja, M., Zeidler, D., von Rad, U., and Durner, J. (2008). Transcriptional responses of *Arabidopsis thaliana* to the bacteria-derived PAMPs harpin and lipopolysaccharide. *Immunobiology* 213, 161–171.

Lopez-Gomez, M., Sandal, N., Stougaard, J., and Boller, T. (2012). Interplay of flg22-induced defense responses and nodulation in *Lotus japonicas*. *J. Exp. Bot.* 63, 393–401.

Loppnow, H., Brade, H., Durrbaum, I., Dinarello, C. A., Kusumoto, S., Rietschel, E. T., et al. (1989). IL-1 induction-capacity of defined lipopolysaccharide partial structures. *J. Immunol.* 142, 3229–3238.

Lotze, M. T., Zeh, H. J., Rubartelli, A., Sparvero, L. J., Amoscato, A. A., Washburn, N. R., et al. (2007). The grateful dead: damage associated molecular pattern molecules and reduction/oxidation regulates immunity. *Immunol. Rev.* 220, 60–81.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., and He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 496–501.

Mackey, D., and McFall, A. J. (2006). MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Mol. Microbiol.* 61, 1365–1371.

Madala, N. E., Leone, M. R., Molinaro, A., and Dubery, I. A. (2011). Deciphering the structural and biological properties of the lipid A moiety of lipopolysaccharides from *Burkholderia cepacia* strain ASP B 2D, in *Arabidopsis thaliana*. *Glycobiology* 21, 184–194.

Maekawa, T., Kufer, T. A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* 12, 817–826.

Mateos, F. V., Rickauer, M., and Esquerré-Tugayé, M. T. (1997). Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose-binding lectin-like activities. *Mol. Plant Microbe Interact.* 10, 1045–1053.

McCann, H. C., Nahal, H., Thakur, S., and Guttman, D. S. (2012). Identification of innate immunity elicitors using molecular signatures of natural selection. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4215–4220.

McCarthy, Y., Dow, J. M., and Ryan, R. P. (2011). The Ax21 protein is a cell-cell signal that regulates virulence in the nosocomial pathogen *Stenotrophomonas maltophilia*. *J. Bacteriol.* 193, 6375–6378.

McCoy, S. L., Kurtz, S. E., Hausman, F. A., Trune, D. R., Bennett, R. M., and Hefeneider, S. H. (2004). Activation of RAW264.7 macrophages by bacterial DNA and lipopolysaccharides increases cell surface DNA binding and internalization. *J. Biol. Chem.* 279, 17217–17223.

McDonald, C., Inohara, N., and Nuñez, G. (2005). Peptidoglycan signalling in innate immunity and inflammatory disease. *J. Biol. Chem.* 280, 20177–20180.

McGurl, B., Pearce, G., Orozco-Cardemas, M., and Ryan, C. A. (1992). Structure, expression, and antisese inhibition of the systemin precursor gene. *Science* 255, 1570–1573.

Meindl, T., Boller, T., and Felix, G. (2000). The bacterial elicitor flagellin activates its receptor in tomato cells according to the address-message concept. *Plant Cell* 12, 1783–1794.

Miyake, K. (2004). Innate recognition of lipopolysaccharide by Toll-like

receptor 4-MD-2. *Trends Microbiol.* 12, 186–192.

Mishina, T. E., and Zeier, J. (2006). The *Arabidopsis* flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* 141, 1666–1675.

Mishina, T. E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis*. *Plant J.* 50, 500–513.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618.

Mueller, K., Chinchilla, D., Albert, M., Jehle, A. K., Kalbacher, H., Boller, T., et al. (2012). Contamination risks in work with synthetic peptides: flg22 as an example of a pirate in commercial peptide preparations. *Plant Cell* 24, 3193–3197.

Munford, R. S., and Varley, A. W. (2006). Shield as signal: lipopolysaccharide and the evolution of immunity to Gram-negative bacteria. *PLoS Pathog.* 2:e67. doi: 10.1371/journal.ppat.0020067

Narváez-Vásquez, J., and Ryan, C. A. (2004). The cellular localization of prosystemin: a functional role for phloem parenchyma in systemic wound signaling. *Planta* 218, 360–369.

Newman, M.-A., Daniels, M. J., and Dow, J. M. (1995). Lipopolysaccharide from *Xanthomonas campestris* induces defense-related gene expression in *Brassica campestris*. *Mol. Plant Microbe Interact.* 8, 778–780.

Newman, M.-A., Dow, J. M., Molinaro, A., and Parrilli, M. (2007). Priming, induction and modulation of plant defense responses by bacterial lipopolysaccharides. *J. Endotoxin Res.* 13, 68–79.

Newman, M. A., von Roepenack-Lahaye, E., Parr, A., Daniels, M. J., and Dow, J. M. (2002). Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J.* 29, 487–495.

Nguyen, H. P., Chakravarthy, S., Velásquez, A. C., McLane, H. L., Zeng, L., Nakayashiki, H., et al. (2010). Methods to Study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant Microbe Interact* 23, 991–999.

Nomura, K., DebRoy, S., Lee, Y. H., Pumplin, N., Jones, J., and He, S. Y. (2006). A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science* 313, 220–223.

Nothnagel, E. A., McNeil, M., Albersheim, P., and Dell, A. (1983). Host-pathogen interactions: XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiol.* 71, 916–926.

Nürnberger, T., and Kemmerling, B. (2006). Receptor protein kinases – pattern recognition receptors in plant immunity. *Trends Plant Sci.* 11, 519–522.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Hahlbrock, K., and Scheel, D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* 78, 449–460.

Park, C. J., Peng, Y., Chen, X., Dardick, C., Ruan, D., Bart, R., et al. (2008). Rice XB15, a protein phosphatase 2C negatively regulates cell death and XA21-mediated innate immunity. *PLoS Biol.* 6:e231. doi: 10.1371/journal.pbio.0060231

Park, C. J., and Ronald, P. C. (2012). Cleavage and nuclear localization of the rice XA21 immune receptor. *Nat. Commun.* 3, 920.

Pearce, G., Strydom, D., Johnson, S., and Ryan, C. A. (1991). A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science* 253, 895–898.

Peng, Y., Bartley, L. E., Chen, X., Dardick, C., Chern, M., Ruan, R., et al. (2008). OsWRKY62 is a negative regulator of basal and *Xa21*-mediated defense against *Xanthomonas oryzae* pv. oryzae in rice. *Mol. Plant* 1, 446–458.

Prime-A-Plant Group: Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant-Microbe Interact.* 19, 1062–107.

Raetz, C. R. H., Reynolds, C. M., Trent, M. S., and Bishop, R. E. (2007). Lipid A modification systems in Gram-negative bacteria. *Annu. Rev. Biochem.* 76, 295–329.

Raetz, C. R. H., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700.

Rallabhandi, P., Awomoyi, A., Thomas, K. E., Phalipon, A., Fujimoto, Y., Fukase, K., et al. (2008). Differential activation of human TLR4 by *Escherichia coli* and *Shigella flexneri* 2a lipopolysaccharide: combined effects of lipid A acylation state and TLR4 polymorphisms on signaling. *J. Immunol.* 180, 1139–1147.

Ramos, H. C., Rumbo, M., and Sirard, J.-C. (2004). Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol.* 12, 509–517.

Robatzek, S., Bittel, P., Chinchilla, D., Kochner, P., Felix, G., Shiu, S. H., et al. (2007). Molecular identification and characterization of the tomato flagellin LeFLS2, an orthologue of *Arabidopsis* FLS2 exhibiting characteristically different perception specificities. *Plant Mol. Biol.* 64, 539–547.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20, 537–542.

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W. M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *J. Exp. Bot.* 53, 103–110.

Ron, M., and Avni, A. (2004). The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 16, 1604–1615.

Ronald, P. C., Albano, B., Tabien, R., Abenes, L., Wu, K. S., McCouch, S., et al. (1992). Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21*. *Mol. Gen. Genet.* 236, 113–120.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., et al. (2011). The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23, 2440–2455.

Ruiz-Ferrer, V., and Voinnet, O. (2009). Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* 60, 485–510.

Russinova, E., Borst, J.-W., Kwaaitaal, M., Cano-Delgado, A., Yin, Y., Chory, J., et al. (2004). Heterodimerization and endocytosis of *Arabidopsis* brassinosteroid receptors BRI1 and AtSERK3 (BAK1). *Plant Cell* 16, 3216–3229.

Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y., and Hunt, M. D. (1996). Systemic acquired resistance. *Plant Cell* 8, 1809–1819.

Scheer, J. M., and Ryan, C. A. (1999). A 160-kD systemin receptor on the Surface of *Lycopersicon peruvianum* suspension-cultured cells. *Plant Cell* 11, 1525–1535.

Scheer, J. M., and Ryan, C. A. (2002). The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9585–9590.

Schneider, M., Schweizer, P., Meuwly, P., and Metraux, J. P. (1996). Systemic acquired resistance in plants. *Int. Rev. Cytol.* 168, 303–340.

Schromm, A., Brandenburg, K., Loppnow, H., Moran, A., Koch, M., Rietschel, E., et al. (2000). Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion. *Eur. J. Biochem.* 267, 2008–2013.

Schromm, A., Brandenburg, K., Loppnow, H., Zahringer, U., Rietschel, E., Carroll, S., et al. (1998). The charge of endotoxin molecules influences their conformation and IL-6-inducing capacity. *J. Immunol.* 161, 5464–5471.

Schulze, B., Mentzel, T., Jehle, A. K., Mueller, K., Beeler, S., Boller, T., et al. (2010). Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J. Biol. Chem.* 285, 9444–9451.

Schweizer, P., Felix, G., Buchala, A., Müller, C., and Métraux, J. P. (1996). Perception of free cutin monomers by plant cells. *Plant J.* 10, 331–341.

Séjalon-Delmas, N., Mateos, F. V., Bottin, A., Rickauer, M., Dargent, R., and Esquerré-Tugayé, M. T. (1997). Purification, elicitor activity, and cell wall localization of a glycoprotein from phytophthora parasitica var. nicotianae, a fungal pathogen of tobacco. *Phytopathology* 9, 899–909.

Seong, S. Y., and Matzinger, P. (2004). Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat. Immunol.* 4, 469–478.

Shen, Y., Sharma, P., da Silva, F. G., and Ronald, P. (2002). The *Xanthomonas oryzae* pv. oryzae raxP and raxQ genes encode an ATP sulphurylase and adenosine-5'-phosphosulphate kinase that are required for AvrXa21 avirulence activity. *Mol. Microbiol.* 44, 37–48.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., et al. (2010). Two Lys M receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214.

Shinya, T., Motoyama, N., Ikeda, A., Wada, M., Kamiya, K., Hayafune, M., et al. (2012). Functional characterization of CEBiP and CERK1 homologs in *Arabidopsis* and rice reveals the presence of different chitin receptor systems in plants. *Plant Cell Physiol.* 53, 1696–1706.

Shiu, S. H., and Bleecker, A. B. (2003). Expansion of the receptor-like kinase/pelle gene family and receptor-like proteins in *Arabidopsis*. *Plant Physiol.* 132, 530–543.

Shiu, S.-H., Karlowski, W. M., Pan, R., Tzeng, Y.-H., Mayer, K. F. X., and Li, W.-H. (2004). Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. *Plant Cell* 16, 1220–1234.

Shuguo, H., Wei, Z., Chao, Z., and Daoji, W. (2012). One-step expression and tyrosine O-sulfonation of Ax21 in *Escherichia coli*. *Appl. Biochem. Biotechnol.* 166, 1368–1379.

Silipo, A., Leone, M. R., Erbs, G., Lanzetta, R., Parrilli, M., Chang, W.-S., et al. (2011). A unique bicyclic monosaccharide from the *Bradyrhizobium* lipopolysaccharide and its role in the molecular interaction with plants. *Angew. Chem.* 50, 12610–12612.

Silipo, A., Molinaro, A., Sturiale, L., Dow, J. M., Erbs, G., Lanzetta, R., et al. (2005). The elicitation of plant innate immunity by lipooligosaccharide of *Xanthomonas campestris*. *J. Biol. Chem.* 280, 33660–33668.

Silipo, A., Sturiale, L., Garozzo, D., de Castro, C., Lanzetta, R., Parrilli, M., et al. (2004). Structure elucidation of the highly heterogeneous lipid A from the lipopolysaccharide of the Gram-negative extremophile bacterium *Halomonas magadiensis* strain 21 M1. *Eur. J. Org. Chem.* 2004, 2263–2271.

Silipo, A., Sturiale, L., Garozzo, D., Erbs, G., Tandrup Jensen, T., Lanzetta, R., et al. (2008). The acylation and phosphorylation pattern of lipid A from *Xanthomonas campestris* strongly influence its ability to trigger the innate immune response in *Arabidopsis*. *Chembiochem* 9, 896–904.

Smith, K. D., Andersen-Nissen, E., Hayashi, F., Strobe, K., Bergman, M. A., Rassoulian Barrett, S. L., et al. (2003). Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial mobility. *Nat. Immunol.* 4, 1247–1253.

Song, W. Y., Pi, L. Y., Wang, G. L., Gardner, J., Holsten, T., and Ronald, P. (1997). Evolution of the rice *Xa21* disease resistance gene family. *Plant Cell* 9, 1279–1287.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., et al. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene Xa21. *Science* 270, 1804–1806.

Sun, A. Z., Nie, S. J., and Xing, D. (2012). Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by lipopolysaccharides. *Plant Physiol.* 160, 1081–1096.

Sun, W., Dunning, F. M., Pfund, C., Weingarten, R., and Bent, A. F. (2006). Within-species flagellin polymorphism in *Xanthomonas campestris* pv *campestris* and its impact on elicitation of *Arabidopsis* FLAGELLIN SENSING2-dependent defenses. *Plant Cell* 18, 764–779.

Takai, R., Isogai, A., Takayama, S., and Che, F. S. (2008). Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. *Mol. Plant Microbe Interact.* 21, 1635–1642.

Thomma, B. P. H. J., Nürnberger, T., and Joosten, M. H. A. J. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell* 23, 4–15.

Traub, S., von Aulock, S., Hartung, T., and Herman, C. (2006). MDP and other muropeptides -direct and synergistic effects on the immune system. *J. Endotoxin Res.* 12, 69–85.

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N., and Ishida, I. (1997). The structure and function of a soybean β-glucan-elicitor-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1029–1034.

van der Biezen, E. A., and Jones, J. D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23, 454–456.

van Loon, L. C., Bakker, P. A., and Pieterse, C. M. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483.

Wan, J., Tanaka, K., Zhang, X.-C., Son, G. H., Brechenmacher, L., Nguyen, T. H. N., et al. (2012). LYK4, a LysM receptor-like kinase, is important for chitin signaling and plant innate immunity in *Arabidopsis*. *Plant Physiol.* 160, 396–406.

Wang, Y. H., Gehring, C., and Irving, H. R. (2011). Plant natriuretic peptides are apoplastic and paracrine stress response molecules. *Plant Cell Physiol.* 52, 837–850.

Wang, Y. S., Pi, L. Y., Chen, X., Chakrabarty, P. K., Jiang, J., De Leon, A. L., et al. (2006). Rice XA21 binding protein 3 is a ubiquitin ligase required for full *Xa21*-mediated disease resistance. *Plant Cell* 18, 3635–3646.

Wang, G. L., Ruan, D. L., Song, W. Y., Sideris, S., Chen, L. L., Pi, L. Y., et al. (1998). *Xa21D* Encodes a receptor-like molecule with a leucine-rich repeat domain that determines race-specific recognition and is subject to adaptive evolution. *Plant Cell* 10, 765–779.

Wang, G. L., Song, W. Y., Ruan, D. L., Sideris, S., and Ronald, P. C. (1996). The cloned gene, *Xa21*, confers resistance to multiple *Xanthomonas oryzae* pv. oryzae isolates in transgenic plants. *Mol. Plant Microbe Interact.* 9, 850–855.

Watt, S. A., Tellstrom, V., Patschkowski, T., and Niehaus, K. (2006). Identification of the bacterial superoxide dismutase (SodM) as plant-inducible elicitor of an oxidative burst reaction in tobacco cell suspension cultures. *J. Biotechnol.* 126, 78–86.

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M.-A., Kolb, D., Tsuda, K., et al. (2011). *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19824–19829.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990). CD14 serves as the cellular receptor for complexes of lipopolysaccharides with lipopolysaccharide binding protein. *Science* 249, 1431–1433.

Yamaguchi, Y., Huffaker, A., Bryan, A. C., Tax, F. E., and Ryan, C. A. (2010). PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in *Arabidopsis*. *Plant Cell* 22, 508–522.

Yamaguchi, Y., Pearce, G., and Ryan, C. A. (2006). The cell surface leucine-rich repeat receptor for *At*Pep1, an endogenous peptide elicitor in *Arabidopsis*, is functional in transgenic tobacco cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10104–10109.

Zeidler, D., Dubery, I. A., Schmitt-Kopplin, P., Von Rad, U., and Durner, J. (2010). Lipopolysaccharide mobility in leaf tissue of *Arabidopsis thaliana*. *Mol. Plant Pathol.* 11, 747–755.

Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hertung, T., Bors, W., et al. (2004). Innate immunity in *Arabidopsis thaliana* lipopolysaccharides activate nitric oxide synthase NOS and induce defense genes. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15811–15816.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T., et al. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 March 2013; accepted: 23 April 2013; published online: 16 May 2013.

*Citation: Newman M-A, Sundelin T, Nielsen JT and Erbs G (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. Front. Plant Sci. **4**:139. doi: **10.3389/fpls.2013.00139***

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Newman, Sundelin, Nielsen and Erbs. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 22 February 2013
doi: 10.3389/fpls.2013.00030

Long-distance communication and signal amplification in systemic acquired resistance

Jyoti Shah*[1] ***and Jürgen Zeier*[2]***

[1] *Department of Biological Sciences, University of North Texas, Denton, TX, USA*
[2] *Department of Biology, Heinrich-Heine-University, Düsseldorf, Germany*

Edited by:
Saskia C. M. Van Wees, Utrecht University, Netherlands

Reviewed by:
Keiko Yoshioka, University of Toronto, Canada
Robin K. Cameron, McMaster University, Canada

****Correspondence:***
Jyoti Shah, Department of Biological Sciences, University of North Texas, Life Sciences Building-B, Room # 418, 1155 Union Circle #305220, Denton, TX 76203, USA.
e-mail: shah@unt.edu

Jürgen Zeier, Department of Biology, Heinrich-Heine-University, 40225 Düsseldorf, Germany.
e-mail: juergen.zeier@uni-duesseldorf.de

Systemic acquired resistance (SAR) is an inducible defense mechanism in plants that confers enhanced resistance against a variety of pathogens. SAR is activated in the uninfected systemic (distal) organs in response to a prior (primary) infection elsewhere in the plant. SAR is associated with the activation of salicylic acid (SA) signaling and the priming of defense responses for robust activation in response to subsequent infections. The activation of SAR requires communication by the primary infected tissues with the distal organs. The vasculature functions as a conduit for the translocation of factors that facilitate long-distance intra-plant communication. In recent years, several metabolites putatively involved in long-distance signaling have been identified. These include the methyl ester of SA (MeSA), the abietane diterpenoid dehydroabietinal (DA), the dicarboxylic acid azelaic acid (AzA), and a glycerol-3-phosphate (G3P)-dependent factor. Long-distance signaling by some of these metabolites also requires the lipid-transfer protein DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1). The relative contribution of these factors in long-distance signaling is likely influenced by environmental conditions, for example light. In the systemic leaves, the *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* (*ALD1*)-dependent production of the lysine catabolite pipecolic acid (Pip), *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) signaling, as well as SA synthesis and downstream signaling are required for the activation of SAR. This review summarizes the involvement and interaction between long-distance SAR signals and details the recently discovered role of Pip in defense amplification and priming that allows plants to acquire immunity at the systemic level. Recent advances in SA signaling and perception are also highlighted.

Keywords: azelaic acid, dehydroabietinal, glycerol-3-phosphate, methyl salicylate, pipecolic acid, DIR1

INTRODUCTION

Plants employ multiple layers of defense to combat pathogens. These defenses include a combination of preformed and inducible mechanisms (Jones and Dangl, 2006; Spoel and Dong, 2012). In the pathogen-inoculated tissues, recognition by the plant of molecular patterns that are conserved amongst groups of microbes results in the activation of PTI (PAMP-triggered immunity), which contributes to basal resistance that controls the extent of pathogen growth. By contrast to PTI, ETI (effector-triggered immunity), which is activated in response to plant recognition of race-specific effectors released by a pathogen, has a more pronounced impact on curtailing pathogen growth. Local infection by a pathogen can further result in immunization of the rest of the foliage against subsequent infections, a phenomenon that was reported as early as in the 1930s (Chester, 1933) and phrased "systemic acquired resistance (SAR)" by Ross (1966) (**Figure 1**). SAR confers enhanced resistance against a broad-spectrum of foliar pathogens. The beneficial effect of SAR has also been suggested to extend to the roots (Gessler and Kuc, 1982; Tahiri-Alaoui et al., 1993). The protective effect of SAR can be transferred to the progeny (Luna et al., 2012) and can confer a fitness advantage under conditions of high disease pressure (Traw et al., 2007).

Resistance in foliar tissues can also be enhanced by mycorrhizal associations and colonization of the rhizosphere by biocontrol fungi (Liu et al., 2007; Shoresh et al., 2010). Similarly, root colonization by plant growth-promoting rhizobacteria also enhances disease resistance in the foliage, a phenomenon that has been termed "induced systemic resistance (ISR)" (van Loon, 2007). SAR and ISR engage different mechanisms and as a result have an additive effect on foliar disease resistance (van Wees et al., 2000). SAR results in a heightened state of preparedness in the uninfected organs against subsequent infections. Furthermore, these tissues are primed to turn on defenses faster and stronger when challenged by pathogen (Conrath, 2011). Long-distance communication by the primary pathogen-infected organ with rest of the pathogen-free foliage is critical for the activation of SAR. Experiments by Joseph Kuc and colleagues led to the suggestion that this long-distance communication requires an intact phloem. In a series of grafting studies, they showed that the SAR signal can be transmitted from the pathogen-inoculated rootstock to the pathogen-free graft (scion) (Jenns and Kuc, 1979;

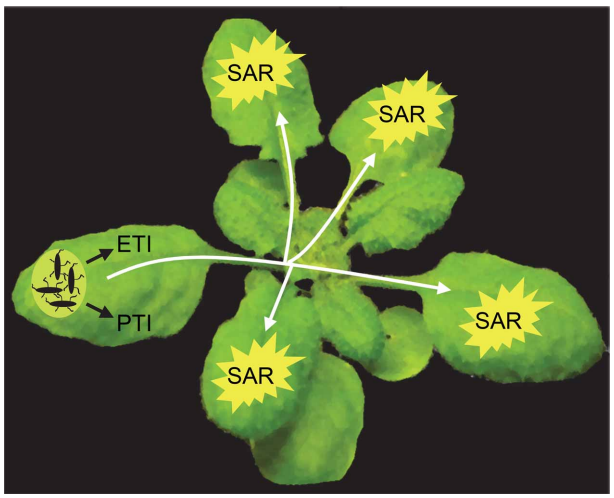


FIGURE 1 | Systemic acquired resistance. Pathogen infection results in the activation of defenses, for example PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), in the pathogen-infected organ. Simultaneously, the infected organ releases signals that are transported to rest of the foliage, where it induces systemic acquired resistance (SAR), which protects these organs against subsequent infections by a broad-spectrum of pathogens. The phloem is a likely conduit for the transport of these long-distance SAR signals. In the distal organs, effective signal amplification must take place to guarantee SAR establishment.

Tuzun and Kuc, 1985). Furthermore, long-distance transmission of the SAR signal in tobacco was disrupted when the phloem tissue in the stem above the pathogen-inoculated site was removed (Tuzun and Kuc, 1985). Similarly, girdling the petiole of the primary pathogen-inoculated leaf in cucumber (*Cucumis sativus*) prevented SAR from being activated in the distal leaves (Guedes et al., 1980). In *Arabidopsis thaliana*, the SAR-inducing activity can be recovered in the phloem sap-enriched petiole exudates (Pexs) obtained from leaves inoculated with a SAR-inducing pathogen (Maldonado et al., 2002; Chaturvedi et al., 2008; Jung et al., 2009), further suggesting that the phloem is a likely conduit for transmission of the long-distance SAR signal. It has been suggested, however, that the phloem may not be the exclusive conduit for transport of the long-distance SAR signal, since defenses were also induced in distal tissues that were not connected by the path of photoassimilate translocation from the primary-infected organ (Kiefer and Slusarenko, 2003). Pexs collected from pathogen-inoculated leaves of Arabidopsis are effective in inducing SAR in tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), and wheat (*Triticum aestivum*) (Chaturvedi et al., 2008, 2012). Similarly, the SAR signal generated in the pathogen-inoculated cucumber rootstocks was found to confer protection on watermelon (*Citrullus lanatus*), and muskmelon (*Cucumis melo*) grafts (Jenns and Kuc, 1979), thus suggesting that the SAR signal is not genus- or species-specific.

INVOLVEMENT OF SALICYLIC ACID SIGNALING IN SAR

SAR is accompanied by an increase in levels of salicylic acid (SA) and its derivative SA-glucoside (SAG), and elevated expression of SA-responsive genes in the pathogen-free organs. Elevated expression of the SA-responsive *PR1* (*PATHOGENESIS-RELATED 1*) gene has routinely been used as a molecular marker of SAR. SA accumulation and signaling in these organs are primed to further increase to higher levels upon challenge with a pathogen (Jung et al., 2009; Návarová et al., 2012). Genetic studies in Arabidopsis and tobacco have confirmed that SA accumulation and signaling are critical for the disease resistance conferred by SAR. The Arabidopsis *ics1* mutant, which is deficient in isochorismate synthase 1 activity that is required for SA synthesis, is SAR deficient (Wildermuth et al., 2001; Mishina and Zeier, 2007; Chaturvedi et al., 2008, 2012; Jung et al., 2009). Similarly, SAR is compromised in transgenic Arabidopsis and tobacco plants that express the SA degrading salicylate hydroxylase encoded by the *Pseudomonas putida nahG* gene (Vernooij et al., 1994; Lawton et al., 1995). In Arabidopsis, the *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE1*) gene is required for the systemic accumulation of SA that accompanies SAR (Mishina and Zeier, 2006; Chaturvedi et al., 2012). The role of *FMO1* in SAR is discussed later in this review. The activation of SAR requires the *NPR1* (*NON-EXPRESSER OF PR GENES1*) gene, which is an important regulator of SA signaling (Durrant and Dong, 2004; Chaturvedi and Shah, 2007). NPR1 is a transcription activator that is suggested to be one of the receptors for SA (Wu et al., 2012).

SA was found to accumulate at elevated levels in phloem sap collected from cucumber and tobacco leaves inoculated with SAR-inducing pathogens (Malamy et al., 1990; Métraux et al., 1990). Hence, till the early 1990s it was thought that SA is the likely long-distance signal in SAR. However, in 1994, Vernooij and coworkers provided genetic evidence arguing against a role for SA as the long-distance signal in SAR. They demonstrated that SAR was activated in wild-type tobacco scions that were grafted onto SA-deficient NahG rootstocks, which received the primary pathogen inoculation. In contrast, SAR was not activated in NahG scions grafted on wild-type rootstocks, thus confirming that although SA is required for the disease resistance conferred by SAR, SA *per se* is not the long-distance signal in SAR. These experiments also suggest that *de novo* synthesis of SA in the pathogen-free leaves is required for SAR. Studies with tobacco plants that were unable to accumulate SA due to epigenetic suppression of phenylalanine ammonia-lyase expression, also argued against a role for SA as the long-distance signal in SAR (Pallas et al., 1996).

FACTORS INVOLVED IN LONG-DISTANCE SAR SIGNALING

DIR1, A LIPID-TRANSFER PROTEIN, IS REQUIRED FOR LONG-DISTANCE SIGNALING IN SAR

As noted above, the SAR inducing activity can be recovered in Pex collected from leaves inoculated with a SAR-inducing pathogen. The SAR inducing activity in Pex was sensitive to Proteinase K and Trypsin treatment (Chanda et al., 2011; Chaturvedi et al., 2012), thus suggesting the involvement of a protein(s) in the accumulation and/or systemic translocation of the SAR signal. The DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1) protein, which exhibits structural similarities to the LTP2 family of lipid-transfer proteins, is a good candidate. *DIR1* is expressed in the phloem sieve elements and companion cells. Furthermore, DIR1 contains a signal peptide at its N-terminus that targets it

for secretion to the cell surface (Champigny et al., 2011). Earlier, Maldonado et al. (2002) had identified *dir1* in a genetic screen for Arabidopsis mutants that were defective in SAR. Unlike the wild-type plant, localized inoculation with pathogen was unable to confer enhanced resistance in the distal leaves of the *dir1* mutant in response to challenge inoculation with a virulent pathogen. Although the *dir1* mutant was responsive to the SAR signal present in Avr Pex collected from wild-type plants, similar exudates collected from *dir1* when applied to wild-type plants were unable to enhance *PR1* expression and disease resistance in the distal leaves (Maldonado et al., 2002; Chaturvedi et al., 2008). Thus, it was suggested that DIR1 is required for the accumulation and/or systemic movement of a SAR inducing factor. DIR1's function in defense seems to be specific to SAR since PTI was not compromised in the *dir1* mutant (Maldonado et al., 2002). DIR1 homologs also have an important function in systemic enhancement of disease resistance in tobacco (Liu et al., 2011b). DIR1 contains two SH3 domains (Lascombe et al., 2008). Since, SH3 domains are known to facilitate interaction between proteins, these domains in DIR1 might facilitate interaction with other proteins.

LONG-DISTANCE SIGNALING METABOLITES

The last 5 years have seen the identification of plant-produced metabolites (**Figure 2**) that are enriched in Pex after pathogen infection and/or can be systemically transported, and are thus possibly involved in long-distance signaling in SAR (Shah, 2009; Dempsey and Klessig, 2012). These metabolites can be divided into two broad groups. The first group includes methyl salicylate (MeSA) and dehydroabietinal (DA), which when locally applied promote SA accumulation in the distal leaves (Park et al., 2007; Chaturvedi et al., 2012). The second group includes azelaic acid (AzA) and pipecolic acid (Pip) that are implicated in priming the faster and stronger accumulation of SA in response to pathogen infection (Jung et al., 2009; Návarová et al., 2012). A glycerol-3-phosphate (G3P)-dependent factor has also been suggested to participate in SAR by facilitating the systemic translocation of DIR1 (Chanda et al., 2011). Evidence supporting the involvement of these molecules in long-distance communication and signal amplification in SAR is described below. **Table 1** lists Arabidopsis genes/proteins involved in the synthesis and/or signaling by these metabolites.

Methyl salicylate (MeSA)

The volatile SA derivative MeSA (**Figure 2**), also known as the oil of winter-green, has previously been associated with plant-insect interaction and inter-plant communication (Shulaev et al., 1997; Van Poecke and Dicke, 2002; Snoeren et al., 2010). More recently, MeSA has been suggested to be involved in long-distance signaling in SAR (Dempsey and Klessig, 2012). MeSA levels were reported to increase in the *Tobacco mosaic virus* (TMV)-infected and the distal virus-free leaves of tobacco, as well as in the Pex collected from TMV-infected leaves (Park et al., 2007). TMV infection-induced SAR was attenuated in tobacco plants in which expression of the *SAMT1* (*SA-METHYLTRANSFERASE1*) gene, which encodes a MeSA synthesizing S-adenosyl-L-methionine:

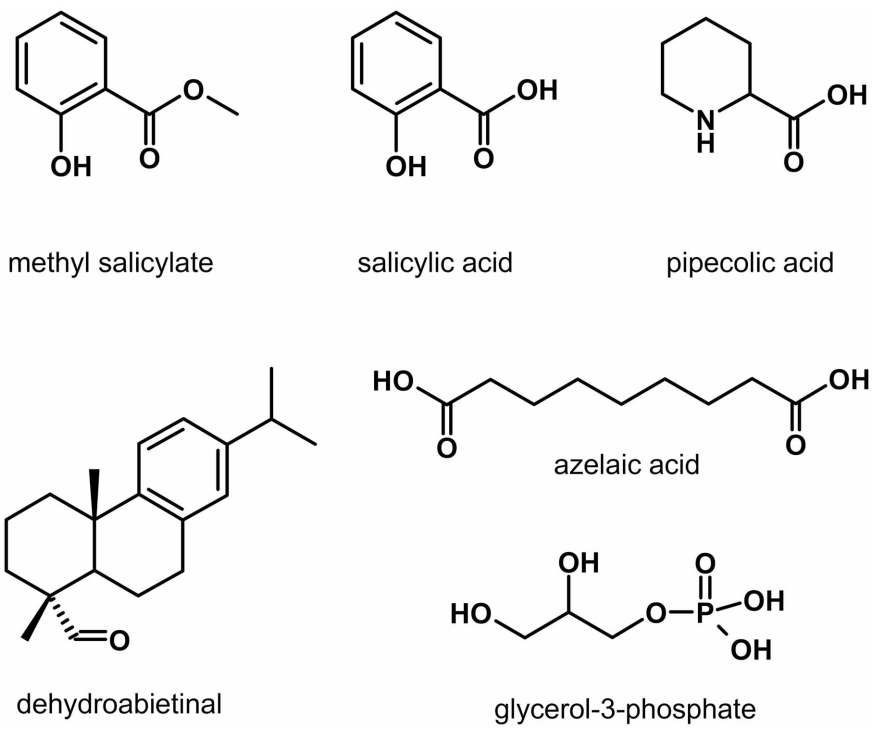


FIGURE 2 | Plant synthesized metabolites suggested to function in long-distance transport and/or signal amplification during systemic acquired resistance.

Table 1 | Arabidopsis genes involved in SAR.

Gene	AtG#	Function
ALD1	At2g13810	Aminotransferase required for pipecolic acid biosynthesis
AZI1	At4g12470	Putative lipid-transfer protein
BSMT1	At3g11480	Benzoic acid/salicylic acid methyl transferase; synthesizes MeSA
CBP60g	At5g26920	ACBP60 family transcription factor, involved in the control of *ICS1* expression
DIR1	At5g48485	Non-specific lipid-transfer protein
FMO1	At1g19250	Required for Pip-mediated resistance and systemic SA accumulation
ICS1 (*SID2*)	At1g74710	Isochorismate synthase required for stress-induced SA biosynthesis
MED15	At1g15780	Mediator subunit 15; transcriptional co-regulator
MED16	At4g04920	Mediator subunit 16; transcriptional co-regulator
MES9	At4g37150	MeSA esterase
MPK3	At3g45640	MAP-kinase
NPR1	At1g64280	SA receptor; transcriptional coactivator
NPR3	At5g45110	SA receptor involved in proteasomal turnover of NPR1
NPR4	At4g19660	SA receptor involved in proteasomal turnover of NPR1
PAD4	At3g52430	Lipase-like defense regulator controlling expression of several SAR regulatory genes
PHYA	At1g09570	Red/far-red light perception; required for light's influence on SAR
PHYB	At2g18790	Red/far-red light perception; required for light's influence on SAR
SARD1	At1g73805	ACBP60 family transcription factor, involved in the control of *ICS1* expression
SFD1 (*GLY1*)	At2g40690	Dihydroxyacetone phosphate reductase; synthesizes glycerol-3-phosphate in plastids

salicylic acid carboxyl methyl-transferase, was silenced by RNAi (Park et al., 2007). Reciprocal grafting between *SAMT1*-silenced and wild-type tobacco plants indicated that SAMT1 was required in the primary TMV-infected leaves for the induction of SAR. The MeSA esterase encoded by the tobacco *SABP2* (*SA-BINDING PROTEIN 2*) gene is also required for the activation of SAR in tobacco (Forouhar et al., 2005; Kumar et al., 2006; Park et al., 2007). A missense alteration ($Ser_{81} \rightarrow Ala_{81}$) in SABP2 that resulted in loss of its MeSA esterase activity, also resulted in the inability to restore SAR in tobacco plants lacking endogenous SABP2 activity (Park et al., 2007). Furthermore, competitive inhibition of SABP2's esterase activity by 2,2,2,2′-tetrafluoroacetophenone, prevented the induction of SAR (Park et al., 2009). It has been suggested, as shown in **Figure 3**, that during the activation of SAR, SAMT1-synthesized MeSA is transported out of the pathogen-inoculated leaf to the distal leaves. In the distal leaves, MeSA is hydrolyzed by the esterase activity of SABP2 to produce SA, which along with *de novo* synthesized SA contributes to the activation of downstream signaling in the pathogen-free organs (Dempsey and Klessig, 2012).

MeSA was also shown to be required for the induction of SAR in potato (*Solanum tuberosum*) by arachidonic acid (Manosalva et al., 2010). MeSA levels increased in the arachidonic acid-treated and the distal untreated leaves of potato. Blocking MeSA accumulation by RNAi-mediated silencing of the SABP2 homolog-encoding *METHYL ESTERASE 1* (*StMES1*) gene in potato compromised arachidonic acid-induced SAR. Furthermore, as in tobacco, 2,2,2,2′-tetrafluoroacetophenone prevented the induction of SAR in potato. 2,2,2,2′-tetrafluoroacetophenone also blocked SAR in Arabidopsis (Park et al., 2009). Knock-down of expression of multiple *AtMES* genes, which encode putative MeSA esterases in Arabidopsis, also attenuated SAR, however, only in 50% of experiments (Vlot et al., 2008; Chaturvedi et al., 2012). Similarly, while Liu et al. (2010) observed that SAR was weaker in the *Arabidopsis bsmt1* mutant, which lacks a MeSA synthesizing benzoic acid/salicylic acid methyl transferase 1, Attaran et al. (2009) noted that despite the MeSA deficiency, the *bsmt1* mutant plants were SAR competent. These studies suggest that the role of MeSA in SAR in Arabidopsis is likely impacted by additional factors. Light has been suggested to be a factor that likely influences the importance of MeSA in SAR in Arabidopsis (Liu et al., 2011a). Liu et al. (2011a) noted that when the primary inoculation with the SAR inducing bacteria was conducted early during the light period, MeSA was less important for SAR. However, when the primary inoculation occurred close to the onset of the dark period, MeSA was comparatively more important for SAR.

In comparison to the wild-type plant, expression of the *BSMT1* gene and MeSA content were higher in the pathogen-inoculated and the distal leaves of the *dir1* mutant (Liu et al., 2011b). In contrast, the content of free SA and SAG were lower in *dir1* tissues. Liu et al. (2011b) have suggested that DIR1 depresses the conversion of SA to MeSA, resulting in SA accumulation in the systemic organs expressing SAR. A similar correlation between *DIR1* and *SAMT1* expression was observed in tobacco as well (Liu et al., 2011b).

Dehydroabietinal (DA)

Terpenoids form one of the largest families of secondary metabolites in plants (Tholl, 2006). The abietane family of diterpenoids, which are components of oleoresin produced by conifers, have pharmacological and industrial applications (Trapp and Croteau, 2001; Bohlmann and Keeling, 2008). These compounds are also produced by angiosperms (Hanson, 2009), but their function in plants is unclear. Chaturvedi et al. (2012) purified DA, an abietane type diterpenoid, as a SAR-inducing factor from Avr Pex. Deuterated DA when applied to Arabidopsis leaves was rapidly

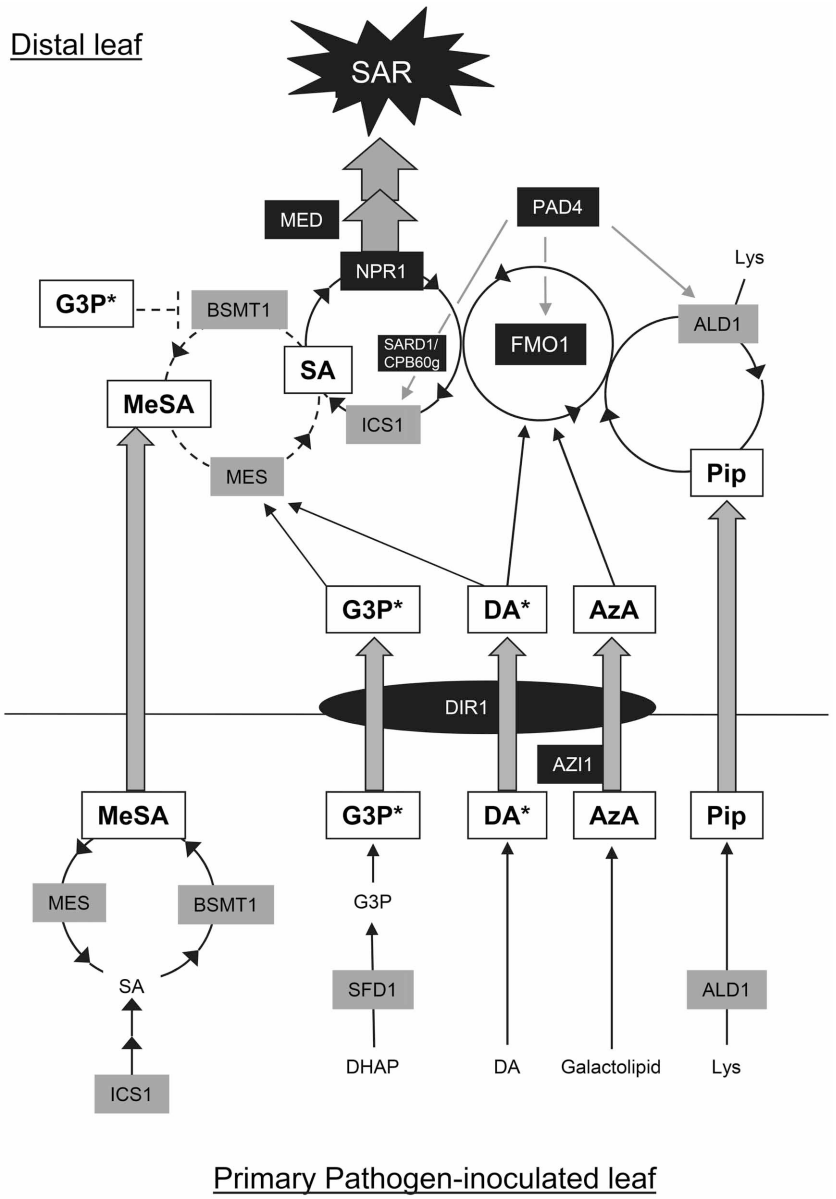


FIGURE 3 | SAR circuitry involving a network of signaling molecules. Studies in Arabidopsis and to a lesser extent in tobacco have indicated that multiple signaling molecules participate in SAR and that the role of some of these signals is influenced by the environment. The genes listed in this model are from Arabidopsis. *Events in the primary pathogen-infected leaf:* In Arabidopsis, increased activity of ICS1, resulting from pathogen-induced expression of the corresponding gene, provokes increased SA accumulation. A fraction of the accumulating SA is converted to MeSA by BSMT1. In tobacco, the high level of SA was simultaneously shown to inhibit the MeSA esterase (MES) activity of SABP2, thus ensuring increase in MeSA level. Glycerol-3-phosphate (G3P), azelaic acid (AzA), and pipecolic acid (Pip) levels also increase in response to pathogen inoculation. SFD1 (GLY1) catalyzes the synthesis of glycerol-3-phosphate from dihydroxyacetone phosphate (DHAP). AzA has been suggested to be synthesized from galactolipids by a non-enzymatic method. Pip is synthesized from lysine (Lys) via the ALD1 aminotransferase and heavily accumulates in infected leaves. Expression of the *ALD1* gene is induced in response to pathogen inoculation. Absolute levels of DA do not change. However, DA is mobilized from a non-signaling low-molecular weight to a high molecular weight signaling DA (DA*) complex in response to pathogen inoculation. Trypsin treatment destroys the high molecular weight DA* complex, suggesting the presence of proteins in this complex. The AzA-inducible *AZI1* gene is required for AzA-induced SAR and also promotes DA*-induced SAR. However, its involvement in SAR induced by the other factors is not known. DIR1, a putative non-specific lipid-transfer protein, is postulated to be involved in transport of a signal required for SAR. Genetic studies indicate that DIR1 is required for G3P, DA, and AzA-induced SAR. *Events in the distal (systemic) leaf:* Systemic transport of MeSA, a G3P-derived factor (G3P*), DA*, AzA, DIR1, and, possibly, Pip from the pathogen-inoculated leaf to the distal leaves occurs via the vasculature, most probably the phloem. G3P* and DIR1 have been suggested to facilitate long-distance transport of each other. DA* and G3P* promote accumulation of *MES* transcript (and likely the corresponding protein). Simultaneously, G3P* and DIR1 down-regulate expression of *BSMT1*, thus ensuring that the equilibrium is in favor of conversion of MeSA to SA. An amplification loop involving ALD1, Pip, FMO1, ICS1, SA, and the SA receptor NPR1, promotes Pip and SA accumulation. PAD4 regulates the expression of *ALD1, FMO1, SARD1, CPB60g,* and *ICS1.*

(Continued)

FIGURE 3 | Continued
NPR1 activation by SA leads to the expression of defense genes that contribute to SAR. MED transcriptional co-regulator subunits seem to act downstream of NPR1. Pip and FMO1 are required for the induction of *ICS1* expression and accumulation of SA in the pathogen-free distal leaves. *ICS1* expression is also controlled by SARD1 and CPB60g, a partly redundant pair of transcription factors. DA*, AzA and Pip signals converge at *FMO1*, which is required for activation of SAR by these signal molecules. It is likely that *FMO1* is also required for G3P* and MeSA-induced SAR. However, this needs to be tested. *ALD1* is a point of convergence of the AzA and Pip pathways. Pip acting through an amplification loop involving *FMO1*, promotes *ALD1* expression and thus its own synthesis. DIR1 is essential for SAR induced by MeSA, G3P*, DA*, and AzA. Whether it is required for Pip-induced SAR is not known. DA is shown to interact synergistically with AzA and the *SFD1*-dependent mechanism. White and gray boxes represent the signaling molecules and biosynthetic enzymes, respectively. Signaling/transport proteins are represented by black boxes/ovals. Gray-filled arrows represent possible long-distance transport. Black arrows indicate positive regulation (induction), while black lines ending with a bar indicate negative regulation. The solid line used for the Pip/SA amplification cycle symbolizes a robust requirement for this part of the circuit for SAR. The contributions of MeSA, DIR1, and G3P to SAR establishment seem less prominent when plants receive a prolonged period of light after pathogen contact.

transported out of the leaf and recovered from the untreated leaves. DA is one of the most potent inducer of SAR that is active when applied as picomolar solutions to leaves of Arabidopsis, tobacco, and tomato (Chaturvedi et al., 2012). Local application of DA systemically induced SA accumulation and *PR1* expression in the untreated leaves (Chaturvedi et al., 2012). DA induced SAR was attenuated in the SA deficient NahG transgenic and *ics1 ics2* double mutant plants and in the SA signaling-deficient *npr1* mutant, thus confirming that DA functions upstream of SA accumulation and signaling. The *FMO1* gene, although not required for SA accumulation in the DA-treated leaves, was required for systemic SA accumulation in DA-treated plants and DA-induced SAR.

Unlike the other SAR signal molecules described here (**Figure 2**), DA content did not increase in the pathogen-inoculated leaves and Pex during SAR. However, when Avr Pex collected from Avr pathogen-treated leaves was subjected to molecular sieve chromatography, DA was found to be enriched in the biologically active HMW fraction (>100 kD) (Chaturvedi et al., 2012). By comparison, in Pex derived from mock-inoculated leaves, DA was enriched in a LMW fraction (<30 kD) that was unable to induce SAR. Chaturvedi et al. (2012) have proposed that the rate limiting step in SAR is the mobilization of DA from the biologically inactive LMW pool into a biologically active signaling form (DA*) that is present in the HMW pool. Trypsin treatment, which destroys the SAR inducing activity of Avr Pex, also reduced DA content in HMW, suggesting that DA is associated with proteins in the HMW pool. What are the proteins in this HMW pool? Is DIR1 one of the proteins in this pool? Additional evidence with plants that are deficient in DA* are also needed to determine if DA* is essential for biologically-induced SAR.

Azelaic acid (AzA)

In tissues exhibiting SAR, SA accumulation is primed for faster and stronger induction in response to pathogen inoculation. Azelaic acid (AzA) (**Figure 2**), a nine carbon dicarboxylic acid has been suggested to be a factor involved in this priming response in Arabidopsis (Jung et al., 2009). AzA levels in Avr Pex collected from Arabidopsis leaves were found to be substantially higher than in Pex collected from mock-inoculated leaves. Local application of AzA systemically enhanced disease resistance. Deuterated AzA applied to Arabidopsis leaves was recovered in Pex and in the untreated leaves, suggesting that AzA is systemically translocated through the plant. AzA-mediated resistance required SA synthesis and signaling. However, unlike MeSA and DA, AzA application was not sufficient to promote SA accumulation and *PR1* expression in Arabidopsis leaves. Instead, pathogen-induced SA accumulation and *PR1* expression were faster and stronger in plants that were previously treated with AzA, suggesting that AzA is a priming factor. *FMO1* and *DIR1* were required for AzA-induced SAR. Also required for AzA induced SAR is ALD1, an aminotransferase that is involved in the synthesis of pipecolic acid (Pip), which as described below is involved in signal amplification during SAR (Návarová et al., 2012). The *AZI1* (*AZELAIC ACID-INDUCED 1*) gene, which encodes a putative lipid-transfer protein, was transiently expressed at elevated levels in AzA-treated plants. Experiments with the *azi1* mutant confirmed that *AZI1* is required for AzA- and biologically-induced SAR. The SAR associated priming of SA accumulation/signaling were attenuated in the *azi1* mutant. Unlike Avr Pex from wild-type plants, local application of Avr Pex collected from the *azi1* mutant was unable to systemically enhance disease resistance in wild-type plants. Furthermore, while locally applied Avr Pex and AzA were capable of enhancing disease resistance in the treated leaves of wild-type and *azi1* mutant, they were unable to promote disease resistance in the distal leaves of the *azi1* mutant compared to the wild-type plant. Thus, it has been suggested that *AZI1* is required for the accumulation and/or translocation of a SAR signal (Jung et al., 2009).

A potential mechanism for the synthesis of AzA is by oxidation of 9-oxononanoic acid synthesized from fatty acids by the action of 9-lipoxygenase and hydroperoxide lyase. Indeed, mutation in the *LOX1* gene, which encodes one of the two 9-lipoxygenase in Arabidopsis, disrupts SAR (Vicente et al., 2012). However, Avr pathogen inoculation-induced accumulation of AzA was retained in the *lox1 lox5* double mutant (Zoeller et al., 2012). Zoeller et al. (2012) suggested that AzA is a general marker of lipid peroxidation that is synthesized by a free-radical based mechanism from galactolipids, rather than a general immune signal. Moreover, Návarová et al. (2012) showed that SAR can occur without the concomitant accumulation of AzA in Pex collected from virulent pathogen-treated plants. Zoeller et al. (2012) reported that AzA content in virulent pathogen-inoculated leaves was only slightly higher than in mock-inoculated leaves. This could explain the lack of AzA increase in Pex collected from virulent pathogen-inoculated leaves (Návarová et al., 2012), compared to that observed in Avr Pex (Jung et al., 2009). None-the-less,

taken together these recent studies by Zoeller et al. (2012) and Návarová et al. (2012) suggest that systemic translocation of AzA is not essential for the establishment of SAR *per se*, but when it is translocated, AzA can add to the strength of systemic immunity observed during SAR.

SFD1-synthesized glycerol-3-phosphate-derived factor and its interplay with DIR1

sfd1 (*suppressor of fatty acid desaturase deficiency 1*) mutants were identified in a screen for suppressors of the constitutive SAR and dwarf phenotypes of the lipid metabolism *ssi2* (*suppressor of SA-insensitivity 2*) mutant (Nandi et al., 2003, 2004), which itself was identified as a suppressor of the *npr1* mutant (Shah et al., 2001). *sfd1* mutants had defects in lipid composition, in particular levels of the plastid-localized 34:6-MGDG (monogalactosyldiacylglycerol) were lower in the *sfd1* mutant, compared to the wild-type plant, while levels of 36:6-MGDG were higher in the *sfd1* mutant. Biologically-induced SAR was compromised in the *sfd1* mutant (Nandi et al., 2004; Chaturvedi et al., 2008, 2012). The SAR defect of the *sfd1* mutant was characterized by the lack of systemic increase in SA content and *PR1* transcript in response to localized pathogen inoculation. The *sfd1* mutant was responsive to SA (Nandi et al., 2004), and local application of Avr Pex from wild-type plants complemented the SAR defect of the *sfd1* mutant (Chaturvedi et al., 2008), suggesting that the *sfd1* mutant is sensitive to the long-distance SAR signal. In contrast, Avr Pexs collected from the *sfd1* mutant were unable to induce SAR when applied to wild-type plants, indicating that the *sfd1* mutant is defective in the accumulation and/or translocation of a long-distance translocated SAR signal (Chaturvedi et al., 2008). DA content was not adversely impacted in the *sfd1* mutant. However, in agreement with a role for *SFD1* in long-distance signaling leading to systemic SA accumulation, the *sfd1* mutant exhibited reduced sensitivity to the SAR-inducing activity of DA (Chaturvedi et al., 2012).

SFD1 encodes a plastid-localized dihydroxyacetone phosphate (DHAP) reductase that synthesizes glycerol-3-phosphate (G3P) (**Figure 2**) (Nandi et al., 2004), an important precursor in the synthesis of several biomolecules, including membrane and storage lipids. SFD1's DHAP reductase activity and its localization to the plastids were shown to be critical for its involvement in SAR, suggesting that SFD1 synthesized G3P, or a product thereof, is required for the accumulation and/or long-distance transport of a SAR signal (Lorenc-Kukula et al., 2012). More recently, Chanda et al. (2011) showed that SAR is also attenuated in the *gly1* mutant, which contains a mutation in the *SFD1* gene in Arabidopsis accession Columbia. However, unlike *sfd1*, which is in the accession Nössen, the *gly1*allele was not defective in the SAR associated systemic enhancement of SA accumulation and *PR1* expression. In Arabidopsis, G3P levels were reported to be elevated in the pathogen-inoculated and the distal pathogen-free leaves, as well as Avr Pex (Chanda et al., 2011). Chanda et al. (2011) further showed that SAR could be restored in the *gly1* mutant by co-applying G3P with Avr Pex, thus confirming an important role for G3P, or a G3P-derived factor in long-distance signaling associated with SAR. Since locally applied ^{14}C-labeled G3P could not be recovered in the systemic leaves, G3P *per se* is unlikely to be the systemically translocated SAR signal. Rather, a G3P-dependent factor is likely involved in long-distance signaling. These results also suggest that the systemic increase in G3P observed in SAR likely results from *de novo* synthesis.

Although G3P, when co-applied with Pex, was capable of enhancing disease resistance in the distal leaves, G3P by itself was not sufficient to induce systemic resistance (Chanda et al., 2011). These results suggest that additional factors that are present in Pex are required for G3P to induce SAR. An earlier study had shown that Avr Pex from *sfd1* to *dir1*, although ineffective in inducing SAR when applied individually, when co-applied were effective inducers of systemic disease resistance (Chaturvedi et al., 2008). This cross-complementation experiment suggested that the SFD1- and DIR1-dependent factors might function together in long-distance signaling. Indeed, G3P when co-applied with DIR1 protein was capable of enhancing systemic disease resistance (Chanda et al., 2011). G3P levels were also lower in Avr Pex from *dir1* mutant, leading to the suggestion that DIR1 and the G3P-dependent factor are required for systemic translocation of each other. Whether G3P or a G3P-dependent factor binds DIR1 is not known. G3P applied with Pex up-regulates *MES9* expression and simultaneously down-regulates *BSMT1* expression in the distal un-treated leaves (Chanda et al., 2011). As mentioned earlier, MES9 is a putative MeSA esterase, while BSMT1 is involved in MeSA synthesis. However, G3P application did not result in systemic increase in SA and SAG content (Chanda et al., 2011). Hence, the altered *MES9* and *BSMT1* expression may not be important for G3P-induced SAR, or alternatively their importance might be dictated by other factors. Liu et al. (2011b) showed that similar to its impact on the contribution of MeSA in SAR, light influenced the contribution of the G3P-dependent factor in SAR. The *gly1* mutant was SAR competent when the primary inoculation with the SAR-inducing microbe was conducted early during the light period. However, when the primary inoculation occurred close to the onset of the dark period, the *gly1* mutant was SAR-defective.

SAR SIGNALING AND SIGNAL AMPLIFICATION IN SYSTEMIC LEAVES

Long-distance signals generated and released from the primary pathogen-inoculated leaves are supposed to be perceived by the cells in the distal organs for SAR initiation at the whole plant level (**Figure 1**). The receptors of individual mobile signals which activate SAR signaling in the distal organs are yet to be identified. Early signaling events result in the systemic accumulation of SA, and subsequent increases in expression of a battery of defense-related genes (SAR genes) is thought to contribute to the enhanced state of broad-spectrum resistance (Sticher et al., 1997). Compared to PTI and ETI, local forms of induced resistance that are activated upon direct pathogen contact via recognition of microbial elicitors (Jones and Dangl, 2006), induction of systemic immunity is indirectly triggered by mobile, endogenous plant signals. The overall direct defense eliciting capacity of numerous PAMPs and/or pathogen released effectors at inoculation sites is probably higher than the elicitor strength of endogenous long-distance signals in distal leaves. It has been suggested that amplification of the stimulus delivered by the SAR signals

is important for SAR establishment (Mishina and Zeier, 2006). Recent findings provide evidence that pipecolic acid (Pip), a common lysine catabolite in plants and animals, acts as a central component of a feedback amplification mechanism that is critical for systemic SA accumulation and SAR (Návarová et al., 2012).

PIPECOLIC ACID—A CRITICAL SAR SIGNAL THAT ORCHESTRATES DEFENSE AMPLIFICATION

Pipecolic acid systemically accumulates in pathogen-inoculated plants

The cyclic non-protein amino acid L-Pip (homoproline; **Figure 2**) is present in plants throughout the plant kingdom (Morrison, 1953). L-Pip is a common catabolite of L-Lys in plants and animals (Broquist, 1991), and the pipecolate pathway represents the main degradation pathway of Lys in mammalian brains (Chang, 1976). In plants, Pip levels increase following chemical treatments that affect growth and upon osmotic stress (Yatsu and Boynton, 1959; Moulin et al., 2006). Pálfi and Dézsi (1968) reported that Pip accumulates both in virus-infected potato and tobacco and in fungus-infected rice leaves. They therefore described Pip as an indicator of abnormal protein metabolism in diseased plants. Since then, the physiological function of Pip in plants has remained elusive, albeit it was found to exert flower-inducing activity in the aquatic plant *Lemna gibba* (Fujioka et al., 1987).

Pip strongly accumulates, alongside with several other free amino acids, its precursor Lys, and another Lys catabolite, α-aminoadipic acid (Aad), in Arabidopsis leaves inoculated with SAR-inducing (virulent or Avr) *P. syringae* and in leaves treated with bacterial PAMPs (Návarová et al., 2012). Moreover, the only amino acid found to substantially increase in leaves distal from sites of pathogen inoculation in this study was Pip. Pip and SA therefore share the characteristic of systemically accumulating in plants upon localized pathogen inoculation. A time-resolved analysis in SAR-induced Arabidopsis indicates that systemic Pip levels start to significantly rise before marked elevations of SA are detectable in the systemic tissue (Návarová et al., 2012).

Pip biosynthesis and accumulation proceeds via ALD1, because the *ald1* mutant completely lacks local and systemic accumulation of Pip upon Avr or virulent *P. syringae*-inoculation (Návarová et al., 2012). *ALD1* transcript levels rise both locally and systemically in pathogen-inoculated Arabidopsis (Song et al., 2004a). *In vitro,* recombinant ALD1 has aminotransferase activity with strong substrate preference for Lys (Song et al., 2004b). It is conceivable that ε-amino-α-ketocaproic acid and Δ1-piperideine-2-carboxylic acid are direct reaction products of an ALD1-catalysed Lys aminotransferase reaction. However, the exact biochemistry of ALD1-mediated Pip production and the existence of a yet to postulate reductase that converts Lys transamination products to Pip remains to be clarified (Návarová et al., 2012).

The Pip resistance pathway is central for SAR

Pipecolate-deficient *ald1* plants fail to accumulate SA in distal leaf tissue following pathogen-inoculation and are fully compromised in SAR (Song et al., 2004a; Jing et al., 2011; Návarová et al., 2012). However, *ald1* plants regain the ability for systemic SA accumulation and SAR establishment when Pip is exogenously applied to the whole plant prior to pathogen treatment, demonstrating that Pip accumulation is critical for systemic SA production and SAR (Návarová et al., 2012). The *ald1* mutant also exhibits attenuated local resistance to compatible and incompatible *P. syringae*, and this is accompanied with reduced local defense responses such as SA biosynthesis, camalexin accumulation, and defense-related gene expression (Song et al., 2004a,b; Návarová et al., 2012). Exogenously applied Pip fully overrides the defects of *ald1* in PTI and ETI and increases the resistance of wild-type plants to bacterial infection. Moreover, Pip feeding of plants prior to inoculation boosts pathogen-triggered induction of SA biosynthesis, camalexin accumulation, and defense-related gene expression in wild-type and *ald1* plants, indicating that Pip strongly amplifies pathogen-triggered defense responses. The positive regulatory role of Pip on SA biosynthesis is particularly important for SA accumulation in distal leaves. It has been suggested that the early systemic increase of Pip at the onset of SAR functions as an initial trigger for signal amplification leading to the systemic increase in SA (Návarová et al., 2012).

Concomitant with SAR, localized *P. syringae* inoculation triggers enhanced expression of several hundred genes in the distal leaves of Arabidopsis wild-type plants. This massive switch in gene expression at the systemic plant level is totally lost in the *fmo1* mutant (Mishina and Zeier, 2006). The flavin-dependent monooxygenase FMO1 was previously identified as a critical regulator of SAR and found necessary for effective local resistance to several bacterial and oomycete pathogens (Bartsch et al., 2006; Koch et al., 2006; Mishina and Zeier, 2006; Jing et al., 2011). Like *ALD1*, *FMO1* is necessary for the systemic accumulation of SA upon SAR induction (Mishina and Zeier, 2006). In contrast to *ald1*, however, *fmo1* fails to establish Pip-induced resistance to bacterial infection. These data indicate that FMO1 functions downstream of Pip and upstream of SA in SAR (Návarová et al., 2012). Importantly, Pip enhances both its own biosynthesis and downstream signaling in SAR via amplification of pathogen-triggered *ALD1* and *FMO1* expression, indicating the existence of a positive feedback amplification loop with Pip as a central player (**Figure 3**; Návarová et al., 2012).

Biochemically characterized flavin-dependent monooxygenases from plants, animals, or fungi oxidize either N- or S-containing functional groups within small metabolic substrates. In Arabidopsis, FMOs of the YUCCA subgroup are capable of converting tryptamine to N-hydroxyl-tryptamine (Zhao et al., 2001), whereas members of the S-oxygenation subgroup ($FMO_{GS\text{-}OX}$) oxidize the sulfide group of Met-derived methylthioalkyl glucosinolates to sulfoxide moieties, thereby generating methylsulfinylalkyl glucosinolates (Li et al., 2008). A third subgroup consists of FMO1 and a pseudogene (Olszak et al., 2006; Schlaich, 2007). Interestingly, besides the inability of *fmo1* to mediate Pip-induced resistance, *fmo1* over-accumulates Pip in the pathogen-inoculated tissue during the later stages of infection. These observations are consistent with the hypothesis that FMO1 could be involved in the oxidation of Pip or a Pip derivative in the Pip signal amplification pathway (Návarová et al., 2012).

Besides FMO1, PHYTOALEXIN-DEFICIENT4 (PAD4) and NPR1 constitute two other necessary components of both SAR and Pip-mediated resistance (Mishina and Zeier, 2006; Jing et al., 2011; Návarová et al., 2012). The lipase-like protein PAD4 is a positive regulator of SA biosynthesis and downstream signaling in plant defense (Zhou et al., 1998; Jirage et al., 1999). A similar double regulatory role exists for PAD4 also in the Pip pathway, since PAD4 not only promotes pathogen-induced Pip production but is also required for resistance promoted by Pip application (Návarová et al., 2012). PAD4 seems to exert its central defense regulatory role via transcriptional control of Pip- and SA-pathway genes, including *ALD1*, *FMO1*, and *ICS1* (**Figure 3**; Song et al., 2004a; Bartsch et al., 2006; https://www.genevestigator.com).

How do the Pip and SA defense regulatory pathways relate to each other? The *ics1* mutant accumulates Pip in a wild-type-like manner in *P. syringae*-inoculated leaves, and exogenous Pip is able to significantly increase basal resistance to *P. syringae* in *ics1*, albeit not to the same extent as in the wild-type. These findings indicate that in the pathogen-inoculated leaves, Pip increases occur independently of *ICS1*-dependent SA biosynthesis, and suggest a partial competence for Pip to induce resistance in an SA-independent manner. By contrast, Pip-induced resistance is minimal in the *npr1* mutant. Thus, a function of NPR1 in Pip signal transduction that is unrelated to its well-described SA downstream regulatory function was proposed (Návarová et al., 2012).

These partly independent traits of the Pip and SA resistance pathways diminish when the distal rather than the locally infected tissue is considered. In the distal leaves of plants that were inoculated with pathogen on other leaves, SA content increase was fully dependent on *ALD1* and hence functional Pip biosynthesis, and downstream signaling involving *FMO1* (Song et al., 2004a; Mishina and Zeier, 2006; Návarová et al., 2012). Conversely, systemic Pip accumulation strongly relies on *FMO1* and *ICS1*-mediated SA biosynthesis (Návarová et al., 2012). This reflects the afore-mentioned strong subjection of SAR establishment on effective signal amplification involving feedback mechanisms that integrate both Pip and SA signaling (**Figure 3**).

Above-described findings implicate a central role for the Pip resistance pathway for SAR. This is corroborated by a recent high throughput forward genetic screen for SAR-deficient Arabidopsis mutants (Jing et al., 2011). Amongst the 16 independent SAR-defective mutants identified were six *fmo1*, four *ald1*, and one *pad4* alleles, as well as three *ics1* alleles. SAR is influenced by the availability of light and depends on intact phytochrome signaling (Zeier et al., 2004; Griebel and Zeier, 2008). A more recent study suggests that the duration of light exposure after bacterial infection influences the importance of individual signals for SAR. For instance, Arabidopsis *dir1*, *gly1*, and *bsmt1* mutants proved SAR-defective when the SAR-inducing inoculation occurred late during the daylight period but were SAR-competent when the primary inoculation was performed early during the daylight period (Liu et al., 2011a). This suggests that the contributions of DIR1, G3P, and MeSA to SAR establishment are less prominent when plants receive a prolonged period of light after pathogen contact. The same study indicates that *FMO1* is necessary for systemic resistance induction irrespective of the light regime applied (Liu et al., 2011a), suggesting that the FMO1 pathway is a point of convergence of various SAR signals, and a critical component for SAR under varying environmental conditions (**Figure 3**).

Is Pip a SAR long-distance signal?

In *P. syringae*-inoculated leaves, Pip production occurs along with the accumulation of several other pathogen-inducible metabolites (Griebel and Zeier, 2010; Ward et al., 2010; Chanda et al., 2011; Návarová et al., 2012). In distal leaves, a more specific response occurs and the increases in a relatively small number of metabolites, including SA, SA-glucoside (SAG), and Pip occurs (Návarová et al., 2012). Návarová et al. (2012) have performed a detailed comparative analysis of the composition of Pex collected from mock-treated and virulent *P. syringae* pv *maculicola* (*Psm*)-inoculated leaves between 6 and 48 h, a time window during which the SAR long-distance information is transduced from the pathogen-inoculated to the distal leaves in their experimental system (Mishina et al., 2008). The applied methods allowed the detection and quantification of 30 defense-related metabolites and amino acids in Pex, including free SA, SAG, MeSA, AzA, JA, camalexin, and Pip. Strikingly, the only substance that exhibited a substantial (7-fold) increase in Pex from *Psm*-inoculated compared to Pex from mock-treated leaves was Pip. SA, AzA, JA, and camalexin, were not enriched in Pex collected from *Psm*-inoculated leaves, and Phenylalanine, SAG and MeSA showed only a small, 1.5- to 2-fold increase. Notably, many substances that strongly accumulated in *Psm*-inoculated leaves during the sampling period were not enriched in the respective Pex.

This selective and marked enrichment of Pip in Pex collected from *Psm*-inoculated leaves during SAR induction is consistent with the hypothesis of a Pip-specific transport out of inoculated leaves and, possibly, translocation of Pip to systemic leaves Návarová et al. (2012). Thus, a scenario is feasible in which Pip, after massive local accumulation, is transported from inoculated to distal leaves, leading to initial, moderate rises in systemic Pip levels (**Figure 3**). Consistent with this hypothesis, Návarová et al. (2012) detected small but significant pathogen-induced rises in distal leaves of *fmo1* which are supposed to result from transport rather than *de novo* synthesis, because *fmo1* lacks systemic up-regulation of the Pip biosynthesis gene *ALD1*. These modest systemic rises in Pip originating from transport could then drive further Pip production in the wild-type via up-regulation of *ALD1* and subsequent FMO1-mediated activation of the Pip amplification cycle, and augmented Pip in systemic leaves would then potentiate the action of other SAR long-distance signals to fully realize SAR (**Figure 3**). However, further experimental evidence is needed to substantiate the hypothetical function of Pip as a long-distance signal. As a water-soluble amino acid, Pip would have ideal physicochemical properties to travel via the phloem.

REGULATORY ASPECTS OF THE SA PATHWAY

Regulation of ICS1 expression and SA accumulation during SAR

In Arabidopsis and *Nicotiana benthamiana*, stress- and pathogen-induced SA biosynthesis proceeds via isochorismate synthase (Nawrath and Métraux, 1999; Wildermuth et al., 2001; Catinot et al., 2008). Accumulation of SA in distal leaves of locally

inoculated Arabidopsis requires increased systemic expression of *ISOCHORISMATE SYNTHASE1* (*ICS1*; Attaran et al., 2009). Recent studies have provided new insight into the regulation of *ICS1* transcription. Zhang et al. (2010) identified two members of the plant-specific transcription factor family ACBP60, *SAR-DEFICIENT1* (*SARD1*) and *CALMODULIN-BINDING PROTEIN60G (CBP60g)* as SAR-relevant Arabidopsis genes. Both genes are locally and systemically up-regulated upon *P. syringae*-inoculation, and the single loss-of-function *sard1* and *cbpg60g* mutants exhibited attenuated SAR. SAR and SA accumulation in both local and systemic leaves are completely lost in a *sard1 cbpg60g* double mutant. Electrophoretic mobility shift analyses indicated that both SARD1 and CBPG60g bind to the *ICS1* promoter in a sequence-specific manner (Zhang et al., 2010). The function of CBP60g but not SARD1 is dependent on calmodulin binding, and the expression of both genes is regulated by PAD4. Moreover, expression profiling indicates that CBP60g and SARD1 affect defense responses other than SA biosynthesis, and suggests a more significant role for CBG60g and SARD1 during earlier and later stages of defense activation, respectively (Wang et al., 2011). Thus, pathogen-induced *ICS1* transcription is activated by a pair of partly redundant DNA binding proteins with different regulatory and temporal properties (Zhang et al., 2010; Wang et al., 2011).

Perception of SA and NPR1 regulation

Accumulating SA is sufficient to induce a subset of SA-responsive SAR genes such as the classical marker *PR1* (Sticher et al., 1997). The transcriptional co-activator NPR1 is essential for SAR and is required for the predominant part of SA downstream responses, including activation of defense gene expression (Durrant and Dong, 2004). NPR1 target genes include *PR1* and a number of genes involved in protein folding and secretion, implicating a critical role of the protein secretory pathway for SAR (Wang et al., 2005). T-DNA insertions in a subset of those genes, *LUMINAL BINDING PROTEIN (BIP2)*, *DEFENDER AGAINST APOPTOTIC DEATH1 (DAD1)*, and *SEC61α*, reduced secretion of the PR1 protein into the apoplast and the ability of the mutant plants to enhance disease resistance in response to S-methyl-1,2,3-benzothiadiazole-7-carbothioate (BTH), a chemical that triggers a SAR-like response (Wang et al., 2005). NPR1 can reside both in the nucleus and the cytosol, and nuclear localization is required to activate *PR1* transcription (Kinkema et al., 2000). In the cytosol, disulfide bridge-connected NPR1 oligomers are converted to monomers after treatment with chemical SAR inducers. SAR induction by chemical treatment or bacterial inoculation is thought to produce a reductive redox potential in the cytosol, and *in vitro* analyses indicate that similar redox changes are sufficient to trigger NPR1 oligomer to monomer transition, presumably by reduction of disulfide bonds. Moreover, NPR1 monomer transition is associated with its nuclear localization. Thus, a model was suggested in which SA accumulation during SAR provokes redox changes driving the transition from the inactive, cytosolic NPR1 oligomer to the active, nucleus-resident NPR1 monomer (Mou et al., 2003). In addition to NPR1 oligomer/monomer transitions, other mechanisms might control the subcellular localization of NPR1. Li et al. (2012) have suggested that in tobacco, the WD40 domain containing protein TRANPARENT TESTA GLABRA2 sequesters NPR1 from the nucleus and thus represses SA/NPR1-mediated defense responses.

Yeast-two-hybrid assays suggest that, in the nucleus, Arabidopsis NPR1 can interact with TGA2, TGA5, and TGA6, three closely related members of the TGA2 subclade of bZIP transcription factors that control *PR1* expression. The triple knockout mutant *tga2 tga5 tga6* is not able to establish SAR, but also exhibits about 50-fold higher basal *PR1* expression than the wild-type, suggesting that TGA factors suppress *PR1* transcription, in addition to promoting its induction in response to SA (Zhang et al., 2003). Indeed, the *PR1* promoter contains negative regulatory elements that can be bound by TGA2, in association with NPR1, thereby controlling the inappropriate activation of *PR1* in the absence of stress (Despres et al., 2000; Zhang et al., 2003; Kesarwani et al., 2007). Consistently, *in vivo* transcription assays by Rochon et al. (2006) demonstrated that TGA2 functions as a transcriptional repressor under basal conditions. In conditions of elevated SA, TGA2 is incorporated into a transactivating complex with NPR1 that stimulates *PR1* transcription. An N-terminal BTB/POZ domain of NPR1 interacts with and negates the function of the TGA repressor (Boyle et al., 2009). Moreover, a C-terminal transacting domain of NPR1 that contains two critical cysteines (Cys^{521} and Cys^{529}) in an oxidized form is necessary for the activation of *PR1* transcription (Rochon et al., 2006).

Since SA was attributed a key regulatory function in inducible plant immunity and SAR (Malamy et al., 1990; Métraux et al., 1990), a bona fide SA receptor required for SA-induced defense gene activation has remained elusive. Interestingly, when expressed in yeast, tobacco NPR1 is sensitive to SA and activates the expression of genes in a stimulus-dependent manner (Maier et al., 2011). Recently, Wu et al. (2012) have identified NPR1 as a direct SA receptor, unraveling that SA perception and subsequent transcriptional activation of defense genes are contiguous events. Using equilibrium dialysis, they determined that ^{14}C-labeled SA can bind to NPR1 protein with a dissociation constant comparable to those of other plant-hormone receptor-ligand interactions. Competitive binding experiments suggested that NPR1 interacts with the defense activators SA and BTH with higher affinities than with structurally related but inactive compounds such as MeSA, 4-hydroxybenzoic acid and catechol. Further, NPR1 can coordinately bind transition metals via Cys^{521} and Cys^{529}, and inductively coupled plasma-mass spectrometry analyses indicated that the protein is preferentially associated with copper. Wu et al. (2012) established that SA is bound to NPR1 via the NPR1-linked copper, presumably by the coordination of the oxygen atoms of the free carboxylate group and the phenolic hydroxyl group in *ortho* position of its aromatic ring. Further, SA binding to NPR1 causes a conformational change in the C-terminal transactivation domain that favors NPR1 oligomer disassembly and liberates the transactivation domain from an inhibitory interaction with the N-terminal BTB/POZ domain, thereby promoting nuclear localization and activation of transcription, respectively (Wu et al., 2012). According to Wu et al. (2012), SA binding, but not reducing conditions (Mou et al., 2003), induces NPR1 oligomer disassembly.

The BTB domain present in the N-terminus of NPR1 is generally found in proteins that interact with Cullin 3 (CUL3) ubiquitin E3 ligase which targets specific protein substrates for degradation by the proteasome. Cell-free degradation assays indicate that NPR1 is subject to protease-mediated degradation resulting in a continuous removal of NPR1 from the nucleus (Spoel et al., 2009). This abolishes the NPR1 coactivator activity and attenuates basal defense gene expression to prevent untimely activation of SAR. Moreover, SA treatment also promotes phosphorylation of NPR1, and thus facilitates ubiquitinylation by CUL3 ubiquitin E3 ligase and NPR1 degradation (Spoel et al., 2009). Spoel et al. (2009) further showed that this phosphorylation-mediated NPR1 turnover is necessary for SAR. Their model proposes that disposal of "exhausted" phosphorylated NPR1 from the target gene promoter allows "fresh" NPR1 to reinitiate the transcription cycle, thus allowing maximum *PR* gene transcription during SAR.

Like NPR1, its paralogues NPR3 and NPR4 contain a BTB and an ankyrin repeat protein-protein interaction domain, which are characteristic for CUL3 substrate adaptors. Fu et al. (2012) observed that *npr3 npr4* mutant plants, unlike the wild-type, lacked SA-induced NPR1 degradation, and *in vitro* pull down and co-immunoprecipitation assays indicated that both NPR3 and NPR4 interact with CUL3 ubiquitin ligase. Moreover, a yeast-two-hybrid assay established that NPR1 can interact with both NPR3 and NPR4, whereby SA promotes the NPR1-NPR3 and disrupts the NPR1-NPR4 interaction. Fu et al. (2012) also demonstrated direct binding of [^{3}H]-labeled SA to NPR3 and NPR4, identifying NPR3 as a low affinity and NPR4 as a high affinity receptor for SA. In contrast to the findings of Wu et al. (2012), binding assays employed by Fu et al. (2012) did not detect a considerable binding affinity of SA to NPR1. In summary, the results of Fu et al. (2012) suggest that NPR3 and NPR4 function as adaptors of CUL3 ubiquitin E3 ligase and control NPR1 stability in an SA-dependent manner. This control mechanism seems to be required for ETI and SAR, because the *npr3 npr4* double mutant exhibited attenuated ETI and reduced HR. Fu et al. (2012) also observed that systemic resistance could not be enhanced further by prior exposure to an Avr strain of *P. syringae* in the *npr3 npr4* mutant. Hence, they concluded that the *npr3 npr4* double mutant is SAR-defective. However, results presented in Fu et al. (2012) also show that PTI associated basal resistance was significantly higher in the *npr3 npr4* double mutant than in wild-type plants. In fact, basal resistance in the *npr3 npr4* double mutant was higher than the heightened resistance observed in SAR expressing wild-type plants (Fu et al., 2012). Thus, any interpretations on SAR in the *npr3 npr4* double should take into consideration the hyper-resistant state of the *npr3 npr4* double mutant plant. Fu et al. (2012) present a model in which NPR4 binds to and promotes NPR1 degradation in the presence of low SA levels to attenuate defense gene expression under basal conditions. The model also proposes that elevated SA following SAR establishment promotes the disruption of the NPR1-NPR4 complex but is not sufficient for promoting association of the low affinity SA receptor NPR3 with NPR1, thereby liberating NPR1 to activate defense gene expression.

In addition to *NPR1*, a genetic screen has identified *Non-Recognition-of-BTH4 (NRB4)* as a mediator of SA responses in Arabidopsis (Canet et al., 2012). Plants carrying weak *nrb4* alleles exhibit strong SA insensitivity and show, to a varying degree, attenuated SAR and compromised basal resistance to *P. syringae*. Like *npr1*, *nrb4* mutants fail to develop SA- or BTH-induced resistance and over-accumulate SA in the course of *P. syringae*-infection. *nrb4* null alleles also express severe growth defects, indicating a role of *NRB4* in plant development. *NRB4* is allelic to Mediator subunit 15 (*MED15*). Mediator represents a multiprotein complex that functions as a transcriptional co-activator or co-repressor in eukaryotes, depending on the nature of associated protein components. Individual Mediator subunits transduce diverse signals to the general transcriptional machinery and can thereby convey plant transcriptional responses to specific stimuli (Kidd et al., 2011). An Arabidopsis screen for reduced *PR1* activation upon exogenous NAD^+ application, a treatment that induces *PR* gene expression and disease resistance in Arabidopsis (Zhang and Mou, 2009), identified Mediator subunit 16 (MED16) as an essential SAR component (Zhang et al., 2012). M*ed16* knockout lines exhibit increased susceptibility to Avr and virulent *P. syringae* and are unable to establish SAR. Following bacterial inoculation, *med16* plants locally and systemically accumulate SA to similar levels than the wild-type but are impaired in *PR* gene expression. Zhang et al. (2012) demonstrated that *MED16* functions downstream of SA and positively regulates NPR1 protein accumulation. Beyond its function in the SA pathway, *MED15* is also required for plant defense toward necrotrophic pathogens and activation of jasmonic acid (JA)/ethylene (ET) pathway genes. Thus, MED16 seems to relay signals from the SA pathway and the JA/ET pathway to the general transcription machinery. MED16 might regulate SA responsiveness via the modulation of NPR1 protein accumulation, but it is not clear yet whether NPR1 or TGA factors are physically associated with the Mediator subunit (Zhang et al., 2012).

SAR—AN ALARMED STATE OF PLANTS THAT CONFERS DEFENSE PRIMING VIA PIP ACCUMULATION

Several PR proteins exhibit antimicrobial activities *in vitro* and overexpression studies indicate that increased expression of single *PR* genes can render plants more resistant to particular pathogen types (Sticher et al., 1997). This suggests that PR proteins that accumulate during SAR contribute to increased pathogen resistance by directly exerting harmful effects to microbial invaders. A second phenomenon supposed to confer resistance during SAR is defense priming or conditioning (Conrath, 2011). Defense priming can be interpreted as an alarmed or sensitized state of plants during which they are able to react more quickly and effectively to pathogen attack.

Although plant conditioning has been associated for a long time with biologically induced SAR (reviewed in Sticher et al., 1997), the phenomenon has been most convincingly described for experimental setups in which plants or plant cell cultures were exogenously treated with chemical enhancers of resistance. These compounds include plant-derived substances such as SA, thiamine and riboflavin (Thulke and Conrath, 1998; Ahn et al., 2007; Zhang et al., 2009), but often also synthetic or unnatural substances like BTH or β-amino butyric acid (BABA; Katz et al.,

1998; Zimmerli et al., 2000). Recently, a high-throughput chemical screen identified a series of novel synthetic compounds that confer defense priming by targeting SA glycosyltransferases and thus increasing endogenous SA accumulation (Noutoshi et al., 2012).

Recent studies indicate that a primary inoculation with a SAR-inducing pathogen leads to defense priming in distal leaves, enabling the whole plant to more effectively mobilize defenses in the course of a subsequent challenge infection (Jung et al., 2009; Návarová et al., 2012). Jung et al. (2009) demonstrated that biological SAR induction, similar to exogenous AzA treatment [see section "Azelaic Acid (AzA)"], enables plants to accumulate higher levels of SA and *PR1* transcripts. This effect was not observed in plants disrupted for the *AZI1* gene, which is transiently expressed at elevated levels in response to AzA treatment (Jung et al., 2009). However, genetic evidence that AzA is responsible for priming of SA production and responsiveness during biological SAR is lacking. Beckers et al. (2009) reported enhanced activation of mitogen-activated protein kinases (MPK) MPK3 and MPK6 upon mechanical stress (pressure infiltration of water) or *P. syringae*-exposure of leaves when Arabidopsis plants were previously treated with BTH. They found that full BTH-mediated priming of *PAL1*- and *PR1*- expression in response to mechanical stress was dependent on both *MPK3* and *MPK6.* MPK3 but not MPK6 was also required for *P. syringae*-induced SAR. However, the role of the MPKs in priming of SAR-related defense responses to pathogen challenge following biological SAR induction was not investigated (Beckers et al., 2009). Another study established that BTH application and localized *P. syringae*-treatment systemically primed Arabidopsis for enhanced expression of the WRKY transcription factor genes *WRK6*, *WRKY29*, and *WRKY53* in response to the stress associated with pressure-infiltration of water into leaves (Jaskiewicz et al., 2011). This priming of *WRKY* genes by BTH was dependent on *NPR1*. Concomitantly, BTH-treatment and *P. syringae*–inoculation also induced histone modifications in the chromatin at the promoters of these *WRKY* genes, suggesting that these histone modifications provide a form of memory of a previous stress. However, whether this histone modification-associated memory has a role in SAR-mediated priming and establishment of systemic immunity remains to be determined.

The recent study of Návarová et al. (2012) demonstrated that biologically-induced SAR in Arabidopsis plants promotes an alarmed state that accelerates the responses to subsequent pathogen attack on several levels. On the metabolite level, SAR priming is characterized by a strongly potentiated induction of both Pip biosynthesis and accumulation of the phytoalexin camalexin after *P. syringae* inoculation, and by a more moderate stimulation of SA accumulation. Moreover, biological SAR prepares plants for a stronger induction of defense genes after a challenge infection, including the two essential SAR regulatory genes *ALD1* and *FMO1*, and the SA-inducible *PR1*. The Pip-deficient *ald1* plants are defective in these SAR-associated conditioning events, suggesting that Pip accumulation is critical for SAR priming. This is corroborated by the findings that exogenous Pip promotes a sensitized state highly similar to that occurring after biological induction of SAR and compensates priming defects in *ald1*. Therefore, genetic and physiological evidence indicates that Pip accumulation is necessary and sufficient to promote a primed state after biological SAR induction (Návarová et al., 2012). Interestingly, the biosynthesis of the endogenous priming regulator Pip is also potentiated during biological SAR, indicating that feedback amplification mechanisms similar to those described in section "The Pip Resistance Pathway Is Central for SAR" for SAR establishment contribute to defense priming in the course of the challenge infection. Moreover, the observations that Pip also accumulates in BABA-treated plants to physiological levels, and that Pip-deficient *ald1* plants are defective in BABA-induced resistance to *P. syringae* suggest that BABA-induced resistance to hemibiotrophic bacteria is regulated via Pip-mediated priming events (Návarová et al., 2012).

THE MEMORY OF SAR IS PASSED ON TO THE PROGENY

SAR confers a fitness advantage under conditions of disease stress (Traw et al., 2007). A recent study indicated that the memory of SAR in Arabidopsis is passed on to the next generation, thus benefiting the progeny plants as well (Luna et al., 2012). The progeny of plants in which SAR had been activated by inoculation with a virulent strain of *P. syringae* pv *tomato* exhibited heightened resistance to *P. syringae* pv *tomato* as well as the unrelated oomycete *H. parasitica* than the progeny of plants that received a control mock-treatment. Although the basal content of defense hormones SA, JA, and JA-Ile were not altered in these next generation SAR plants, SAR associated defenses were more responsive to SA, as indicated by the more robust expression of *PR1* and the *WRKY* genes, *WRKY6*, *WRKY53* and *WRKY70* in these progeny when treated with SA, than in progeny of plants in which SAR was not induced (Luna et al., 2012). NPR1 was required for the next generation SAR. By contrast, the sensitivity of these next generation SAR progeny to JA was reduced, resulting in the weaker induction of JA-inducible genes (*PDF1.2* and *VSP2*) in response to exogenously applied JA and a concomitant increase in susceptibility to the necrotrophic pathogen *Botrytis cinerea*. Similarly, enhanced protection in progeny plants has also been reported for plants treated with an Avr strain of *P. syringae* or BABA (Slaughter et al., 2012). Progeny of the BABA-treated plants were primed for SA-dependent resistance against *P. syringae* and *H. arabidopsidis*.

Luna et al. (2012) showed that next generation SAR was accompanied by changes in the methylation and acetylation status of histones at the promoters of various *NPR1* regulated or SAR associated genes, including *PR1*, *WRKY6,* and *WRKY53*. Promoters of these genes in plants exhibiting next generation SAR contained elevated levels of histone 3 with acetylated Lys9 (H3K9ac), which is considered a transcription activation mark. By contrast, the *PDF1.2* promoter contained elevated levels of H3K27me3, which is normally associated with transcriptional silencing. These results suggest that plants exhibiting next generation SAR have chromatin marks that likely are involved in retaining memory of an infection in the parental generation. In the absence of any evidence that histone modifications *per se* can be transmitted via the gametes, Luna et al. (2012) suggested that DNA methylation patterns, which can be transferred from one generation to another, are likely connected with transmission of memory associated with SAR from the parental generation to

the progeny. Bacterial infection is known to cause hypomethylation (Pavet et al., 2006). Similarly, JA and SA treatment also have been reported to impact the DNA methylation status (Verhoeven et al., 2010). Luna et al. (2012) noted that basal resistance was higher in the *drm1 drm2 cmt3* triple mutant in which non-CpG DNA methylations are reduced. In addition, the *drm1 drm2 cmt3* plants also responded more robustly to SA thus mimicking the priming effect associated with next generation SAR. However, Slaughter et al. (2012) did not see any relationship between next generation protection conferred by BABA or bacterial inoculation and the methylation status at the *PR1* promoter, thus suggesting that if DNA methylation changes are associated with transmission of the priming memory from the parent to the progeny, it is exerted not directly at the *PR1* promoter, but rather at the level of upstream regulatory genes. Next generation stress protection is not limited to defense against pathogens. It has also been reported in Arabidopsis and tomato (*Solanum lycopersicum*) subjected to mechanical damage or herbivory (Rasmann et al., 2012). In this case, the next generation protection was accompanied by priming of JA-dependent defenses. Epigenetic changes associated with next generation protection offer the advantage that they are not permanent and hence offer plasticity, which allows plants to better adapt to a changing environment.

CONCLUDING REMARKS

Although SAR confers a fitness advantage that can benefit multiple generations of plants (Traw et al., 2007; Luna et al., 2012), it needs to be tightly regulated since it is an energy-driven process that diverts resources from growth and development (Heidel et al., 2004; Pajerowska-Mukhtar et al., 2012). Hence, uncontrolled and untimely activation of SAR is detrimental for plant growth and development. Pathogens are also known to target plant defenses to facilitate infection. A circuitry involving networking between multiple signals (**Figure 3**) offers plants the advantage of having sufficient flexibility to better control SAR under different environmental conditions. The coming years will be important for understanding the molecular components of this circuitry, its regulation, conservation amongst plants and the application of this knowledge to sustainable agriculture.

ACKNOWLEDGMENTS

This work was supported by grants to Jyoti Shah from the National Science Foundation (IOS-1121570 and MCB-0920600) and the U.S. Department of Agriculture as a cooperative project with the U.S. Wheat & Barley Scab Initiative (Agreement No. 59-0790-8-060), and to Jürgen Zeier from the Swiss National Science Foundation (SNF grant No. 3100A-125374) and the German Research Foundation (DFG Graduate program IRTG 1525).

REFERENCES

Ahn, I. P., Kim, S., Lee, Y. H., and Suh, S. C. (2007). Vitamin B1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in Arabidopsis. *Plant Physiol.* 143, 838–848.

Attaran, E., Zeier, T. E., Griebel, T., and Zeier, J. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. *Plant Cell* 21, 954–971.

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J. L., Bautor, J., et al. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monoxygenase FMO1 and the nudix hydrolase NUDT7. *Plant Cell* 18, 1038–1051.

Beckers, G. J., Jaskiewicz, M., Liu, Y., Underwood, W. R., He, S. Y., Zhang, S., et al. (2009). Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. *Plant Cell* 21, 944–953.

Bohlmann, J., and Keeling, C. I. (2008). Terpenoid biomaterials. *Plant J.* 54, 656–669.

Boyle, P., Le Su, E., Rochon, A., Shearer, H. L., Murmu, J., Chu, J. Y., et al. (2009). The BTB/POZ domain of the Arabidopsis disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* 21, 3700–3713.

Broquist, H. P. (1991). Lysine-pipecolic acid metabolic relationships in microbes and mammals. *Annu. Rev. Nutr.* 11, 435–448.

Canet, J. V., Dobón, A., and Tornero, P. (2012). Non-recognition-of-BTH4, an Arabidopsis mediator subunit homolog, is necessary for development and response to salicylic acid. *Plant Cell* 24, 4220–4235.

Catinot, J., Buchala, A., Abou-Mansour, E., and Métraux, J. P. (2008). Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett.* 582, 473–478.

Champigny, M. J., Shearer, H., Mohammad, A., Haines, K., Neumann, M., Thilmony, R., et al. (2011). Localization of DIR1 at the tissue, cellular and subcellular levels during Systemic Acquired Resistance in Arabidopsis using DIR1:GUS and DIR1:EGFP reporters. *BMC Plant Biol.* 11:125. doi: 10.1186/1471-2229-11-125

Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K.-T., Gao, Q.-M., et al. (2011). Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat. Genet.* 43, 421–427.

Chang, Y.-F. (1976). Pipecolic acid pathway: the major lysine metabolic route in the rat brain. *Biochem. Biophys. Res. Commun.* 69, 174–180.

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A., Roth, M. R., et al. (2008). Plastid ω-3 desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid. *Plant J.* 54, 106–117.

Chaturvedi, R., and Shah, J. (2007). "Salicylic acid in plant disease resistance," in *Salicylic Acid – A Plant Hormone*, eds S. Hayat and A. Ahmad (Dordrecht, The Netherlands: Springer), 335–370.

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172.

Chester, K. S. (1933). The problem of acquired physiological immunity in plants. *Q. Rev. Biol.* 8, 275–324.

Conrath, U. (2011). Molecular aspects of defence priming. *Trends Plant Sci.* 16, 524–531.

Dempsey, D. A., and Klessig, D. F. (2012). SOS – too many signals for systemic acquired resistance? *Trends Plant Sci.* 17, 538–545.

Despres, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290.

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209.

Forouhar, F., Yang, Y., Kumar, D., Chen, Y., Fridman, E., Park, S. W., et al. (2005). Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1773–1778.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232.

Fujioka, S., Sakurai, A., Yamaguchi, I., Murofushi, N., Takahashi, N., Kaihara, S., et al. (1987). Isolation and identification of L-pipecolic acid and nicotinamide as flower-inducing substances in *Lemna*. *Plant Cell Physiol.* 28, 995–1003.

Gessler, C., and Kuc, J. (1982). Induction of resistance to *Fusarium* wilt in cucumber by root and foliar pathogens. *Phytopathology* 72, 1439–1441.

Griebel, T., and Zeier, J. (2008). Light regulation and daytime dependency

of inducible plant defenses in Arabidopsis: phytochrome signaling controls systemic acquired resistance rather than local defense. *Plant Physiol.* 147, 790–801.

Griebel, T., and Zeier, J. (2010). A role for β-sitosterol to stigmasterol conversion in plant-pathogen interactions. *Plant J.* 63, 254–268.

Guedes, M. E. M., Richmond, S., and Kuc, J. (1980). Induced systemic resistance to anthracnose in cucumber as influenced by the location of the inducer inoculation with *Colletotrichum lagenarium* and the onset of flowering and fruiting. *Physiol. Plant Pathol.* 17, 229–233.

Hanson, J. R. (2009). Diterpenoids. *Nat. Prod. Rep.* 26, 1156–1171.

Heidel, A. J., Clarke, J. D., Antonovics, J., and Dong, X. (2004). Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. *Genetics* 168, 2197–2206.

Jaskiewicz, M., Conrath, U., and Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55.

Jenns, A., and Kuc, J. (1979). Graft transmission of systemic resistance of cucumber to anthracnose induced by *Colletotrichum lagenarium* and tobacco necrosis virus. *Phytopathology* 69, 753–756.

Jing, B., Xu, S., Xu, M., Li, Y., Li, S., Ding, J., et al. (2011). Brush and spray: a high-throughput systemic acquired resistance assay suitable for large-scale genetic screening. *Plant Physiol.* 157, 973–980.

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., et al. (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13583–13588.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91.

Katz, V. A., Thulke, O. U., and Conrath, U. (1998). A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. *Plant Physiol.* 117, 1333–1339.

Kesarwani, M., Yoo, J., and Dong, X. (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. *Plant Physiol.* 144, 336–346.

Kidd, B. N., Cahill, D. M., Manners, J. M., Schenk, P. M., and Kazan, K. (2011). Diverse roles of the Mediator complex in plants. *Semin. Cell Dev. Biol.* 22, 741–748.

Kiefer, I. W., and Slusarenko, A. J. (2003). The pattern of systemic acquired resistance induction within the Arabidopsis rosette in relation to the pattern of translocation. *Plant Physiol.* 132, 840–847.

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of *PR* gene expression. *Plant Cell* 12, 2339–2350.

Koch, M., Vorwerk, S., Masur, C., Sharifi-Sirchi, G., Olivieri, N., and Schlaich, N. L. (2006). A role for a flavin-containing monooxygenase in resistance against microbial pathogens in Arabidopsis. *Plant J.* 47, 629–639.

Kumar, D., Gustafsson, C., and Klessig, D. F. (2006). Validation of RNAi silencing specificity using synthetic genes: salicylic acid-binding protein 2 is required for innate immunity in plants. *Plant J.* 45, 863–868.

Lascombe, M.-B., Bakan, B., Buhot, N., Marion, D., Blein, J.-P., Larue, V., et al. (2008). The structure of "defective in induced resistance" protein of *Arabidopsis thaliana*, DIR1, reveals a new type of lipid transfer protein. *Protein Sci.* 17, 1522–1530.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant-Microbe Interact.* 8, 863–870.

Li, B., Gao, R., Cui, R., Lu, B., Li, X., Zhao, Y., et al. (2012). Tobacco TTG2 suppresses resistance to pathogens by sequestering NPR1 from the nucleus. *J. Cell Sci. 125*, 4913–4922.

Li, J., Hansen, B. G., Ober, J. A., Kliebenstein, D. J., and Halkier, B. A. (2008). Subclade of flavin-monooxygenases involved in aliphatic glucosinolate biosynthesis. *Plant Physiol.* 148, 1721–1733.

Liu, J., Maldonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, C. D., and Harrison, M. J. (2007). Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *Plant J.* 50, 529–544.

Liu, P. P., von Dahl, C. C., and Klessig, D. F. (2011a). The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. *Plant Physiol* 157, 2216–2226.

Liu, P. P., von Dahl, C. C., Park, S. W., and Klessig, D. F. (2011b). Interconnection between methyl salicylate and lipid-based long distance signaling during the development of systemic acquired resistance in Arabidopsis and tobacco. *Plant Physiol.* 144, 1762–1768.

Liu, P.-P., Yang, Y., Pichersky, E., and Klessig, D. F. (2010). Altering expression of *Benzoic acid/salicylic acid carboxyl methyltransferase 1* compromises systemic acquired resistance and PAMP-triggered immunity in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 23, 82–90.

Lorenc-Kukula, K., Chaturvedi, R., Roth, M., Welti, R., and Shah, J. (2012). Biochemical and molecular-genetic characterization of SFD1's involvement in lipid metabolism and defense signaling. *Front. Plant Sci.* 3:26. doi: 10.3389/fpls.2012.00026

Luna, E., Bruce, T. J. A., Roberts, M. R., Flors, V., and Ton, J. (2012). Next generation systemic acquired resistance. *Plant Physiol.* 158, 844–853.

Maier, F., Zwicker, S., Hückelhoven, A., Meissner, M., Funk, J., Pfitzner, A. J., et al. (2011). NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and some NPR1-related proteins are sensitive to salicylic acid. *Mol. Plant Pathol.* 12, 73–91.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004.

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., and Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic acquired resistance signalling in *Arabidopsis*. *Nature* 419, 399–403.

Manosalva, P. M., Park, S. W., Forouhar, F., Tong, L., Fry, W. E., and Klessig, D. F. (2010). *Methyl Esterase 1* (*StMES1*) is required for systemic acquired resistance in potato. *Mol. Plant-Microbe Interact.* 23, 1151–1163.

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006.

Mishina, T. E., and Zeier, J. (2006). The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* 141, 1666–1675.

Mishina, T. E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant J.* 50, 500–513.

Mishina, T. E., Griebel, T., Geuecke, M., Attaran, E., and Zeier, J. (2008). "New insights into the molecular events underlying systemic acquired resistance," in *Biology of Plant-Microbe Interactions, Vol. 6,* eds M. Lorito, S. L. Woo, and F. Scala (St. Paul, MN: International Society for Molecular Plant-Microbe Interactions), 81.

Morrison, R. I. (1953). The isolation of L-pipecolinic acid from *Trifolium repens*. *Biochem. J.* 53, 474–478.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944.

Moulin, M., Deleu, C., Larher, F., and Bouchereau, A. (2006). The lysine-ketoglutarate reductase-saccharopine dehydrogenase is involved in the osmo-induced synthesis of pipecolic acid in rapeseed leaf tissues. *Plant Physiol. Biochem.* 44, 474–482.

Nandi, A., Krothapalli, K., Buseman, C., Li, M., Welti, R., Enyedi, A., et al. (2003). The *Arabidopsis thaliana sfd* mutants affect plastidic lipid composition and suppress dwarfing, cell death and the enhanced disease resistance phenotypes resulting from the deficiency of a fatty acid desaturase. *Plant Cell* 15, 2383–2398.

Nandi, A., Welti, R., and Shah, J. (2004). The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene *SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1* is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* 16, 465–477.

Návarová, H., Bernsdorff, F., Döring, A.-C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24, 5123–5141.

Nawrath, C., and Métraux, J.-P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11, 1393–1404.

Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., et al. (2012). Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in Arabidopsis. *Plant Cell* 24, 3795–3804.

Olszak, B., Malinovsky, F. G., Brodersen, P., Grell, M., Giese, H., Petersen, M., et al. (2006). A putative flavin-containing monooxygenase as a marker for certain defense and cell death pathways. *Plant Sci.* 170, 614–623.

Pajerowska-Mukhtar, K. M., Wang, W., Tada, Y., Oka, N., Tucker, C. L., Fonesca, J. P., et al. (2012). The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. *Curr. Biol.* 22, 103–112.

Pálfi, G., and Dézsi, L. (1968). Pipecolic acid as an indicator of abnormal protein metabolism in diseased plants. *Plant Soil* 29, 285–291.

Pallas, J. A., Paiva, N. L., Lamb, C., and Dixon, R. A. (1996). Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant J.* 10, 281–293.

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116.

Park, S.-W., Liu, P.-P., Forouhar, F., Vlot, A. C., Tong, L., Tietjen, K., et al. (2009). Use of a synthetic salicylic acid analog to investigate the roles of methyl salicylate and its esterases in plant disease resistance. *J. Biol. Chem.* 284, 7307–7317.

Pavet, V., Quintero, C., Cecchini, N. M., Rosa, A. L., and Alvarez, M. E. (2006). Arabidopsis displays centromeric DNA hypomethylation and cytological alterations of heterochromatin upon attack by *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 19, 577–587.

Rasmann, S., De Vos, M., Casteel, C. L., Tian, D., Halitschke, R., Sun, J. Y., et al. (2012). Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol.* 158, 854–863.

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., and Després, C. (2006). The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18, 3670–3685.

Ross, A. F. (1966). "Systemic effects of local lesion formation," in *Viruses of Plants*, eds A. B. R. Beemster and J. Dijkstra (Amsterdam: North-Holland Publishing), 127–150.

Schlaich, N. L. (2007). Flavin-containing monooxygenases in plants: looking beyond detox. *Trends Plant Sci.* 12, 412–418.

Shah, J. (2009). Plants under attack: systemic signals in defence. *Curr. Opin. Plant Biol.* 12, 459–464.

Shah, J., Kachroo, P. K., Nandi, A., and Klessig, D. F. (2001). A recessive mutation in the Arabidopsis *SSI2* gene confers SA- and NPR1-independent expression of *PR* genes and resistance against bacterial and oomycete pathogens. *Plant J.* 25, 563–574.

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43.

Shulaev, V., Silverman, P., and Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385, 718–721.

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B., and Mauch-Mani, B. (2012). Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant Physiol.* 158, 835–843.

Snoeren, T. A. L., Mumm, R., Poelman, E. H., Yang, Y., Pichersky, E., and Dicke, M. (2010). The herbivore-induced plant volatile methyl salicylate negatively affects attraction of the parasitoid *Diadegma semiclausum*. *J. Chem. Ecol.* 36, 479–489.

Song, J., Lu, H., and Greenberg, J. (2004a). Divergent roles in *Arabidopsis thaliana* development and defense of two homologous genes, *ABERRANT GROWTH AND DEATH2* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1*, encoding novel aminotransferases. *Plant Cell* 16, 353–366.

Song, J., Lu, H., McDowell, J. M., and Greenberg, J. (2004b). A key role for *ALD1* in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40, 200–212.

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100.

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual role in regulating plant immunity. *Cell* 317, 860–872.

Sticher, L., Mauch-Mani, B., and Métraux, J. P. (1997). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 35, 235–270.

Tahiri-Alaoui, A., Dumas-Gaudot, E., Gianinazzi, S., and Antoniw, J. F. (1993). Expression of the PR-b1 gene in roots of two *Nicotiana* species and their amphidiploid hybrid infected with virulent and avirulent races of *Chalara elegans*. *Plant. Pathol.* 42, 728–736.

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr. Opin. Plant Biol.* 9, 297–304.

Thulke, O., and Conrath, U. (1998). Salicylic acid has a dual role in the activation of defence-related genes in parsley. *Plant J.* 14, 35–42.

Trapp, S., and Croteau, R. (2001). Defensive resin biosynthesis in conifers. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* 52, 689–724.

Traw, M. B., Kniskern, J. M., and Bergelson, J. (2007). SAR increases fitness of *Arabidopsis thaliana* in the presence of natural bacterial pathogens. *Evolution* 61, 2444–2449.

Tuzun, S., and Kuc, J. (1985). Movement of a factor in tobacco infected with *Peranospora tabacina* Adam which systemically protects against blue mold. *Physiol. Plant. Pathol.* 26, 321–330.

van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *Eur. J. Plant Pathol.* 119, 243–354.

Van Poecke, R. M. P., and Dicke, M. (2002). Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway. *J. Exp. Bot.* 53, 1793–1799.

van Wees, S. C. M., de Swart, E. A. M., van Pelt, J. A., van Loon, L. C., and Pieterse, C. M. J. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8711–8716.

Verhoeven, K. J. F., Jansen, J. J., van Dijk, P. J., and Biere, A. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol.* 185, 1108–1118.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., et al. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance. *Plant Cell* 6, 959–965.

Vicente, J., Cascón, T., Vicedo, B., Garcia-Agustin, P., Hamberg, M., and Castresana, C. (2012). Role of 9-Lipoxygenase and α-dioxygenase oxylipin pathways as modulators of local and systemic defense. *Mol. Plant.* 5, 914–928.

Vlot, A. C., Liu, P.-P., Cameron, R. K., Park, S.-W., Yang, Y., Kumar, D., et al. (2008). Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* 56, 445–456.

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308, 1036–1040.

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L. V., Katagiri, F., et al. (2011). CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *Plant J.* 67, 1029–1041.

Ward, J. L., Forcat, S., Beckmann, M., Bennett, M., Miller, S. J., Baker, J. M., et al. (2010). The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. *Plant J.* 63, 443–457.

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565.

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports* 1, 639–647.

Yatsu, L., and Boynton, D. (1959). Pipecolic acid in leaves of strawberry plant as influenced by treatments affecting growth. *Science* 130, 864–865.

Zeier, J., Pink, B., Mueller, M. J., and Berger, S. (2004). Light conditions influence specific defence responses in incompatible plant-pathogen interactions uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta* 219, 673–683.

Zhang, S., Yang, X., Sun, M., Sun, F., Deng, S., and Dong, H. (2009). Riboflavin-induced priming for pathogen defense in *Arabidopsis thaliana*. *J. Integr. Plant Biol.* 51, 167–174.

Zhang, X., and Mou, Z. (2009). Extracellular pyridine nucleotides induce *PR* gene expression and disease resistance in Arabidopsis. *Plant J.* 57, 302–312.

Zhang, X., Wang, C., Zhang, Y., Sun, Y., and Mou, Z. (2012). The Arabidopsis mediator complex subunit16 positively regulates salicylate-mediated systemic acquired resistance and jasmonate/ethylene-induced defense pathways. *Plant Cell* 24, 4294–4309.

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15, 2647–2653.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl Acad. Sci. U.S.A.* 107, 18220–18225.

Zhao, Y., Christensen, S. K., Fankhauser, C., Cashman, J. R., Cohen, J. D., Weigel, D., et al. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* 291, 306–309.

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., and Glazebrook, J. (1998). *PAD4* functions upstream from salicylic acid to control defence responses in Arabidopsis. *Plant Cell* 10, 1021–1030.

Zimmerli, L., Jakab, G., Métraux, J.-P., and Mauch-Mani, B. (2000). Potentiation of pathogen-specific defense mechanisms in Arabidopsis by β-aminobutyric acid. *Proc. Natl. Acad. Sci. U.S.A.* 97, 12920–12925.

Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., et al. (2012). Lipid profiling of the Arabidopsis hypersensitive response reveals specific lipid peroxidation and fragmentation processes: biogenesis of pimelic and azelaic acid. *Plant Physiol.* 160, 365–378.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 December 2012; paper pending published: 28 January 2013; accepted: 06 February 2013; published online: 22 February 2013.

Citation: Shah J and Zeier J (2013) Long-distance communication and signal amplification in systemic acquired resistance. Front. Plant Sci. ***4****:30. doi: 10.3389/fpls.2013.00030*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Shah and Zeier. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 15 July 2013
doi: 10.3389/fpls.2013.00252

Reprogramming of plants during systemic acquired resistance

Katrin Gruner[1], Thomas Griebel[2], Hana Návarová[1], Elham Attaran[3] and Jürgen Zeier[1]*

[1] Department of Biology, Heinrich Heine University, Düsseldorf, Germany
[2] Department of Plant-Microbe Interactions, Max Planck Institute for Plant Breeding Research, Cologne, Germany
[3] Department of Plant Biology, Michigan State University, East Lansing, MI, USA

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Peer Schenk, The University of Queensland, Australia
Jyoti Shah, University of North Texas, USA
Jean-Pierre Metraux, Université de Fribourg, Switzerland

***Correspondence:**
Jürgen Zeier, Department of Biology, Heinrich Heine University, Universitätsstraße 1, 40225 Düsseldorf, Germany
e-mail: Juergen.Zeier@uni-duesseldorf.de

Genome-wide microarray analyses revealed that during biological activation of systemic acquired resistance (SAR) in Arabidopsis, the transcript levels of several hundred plant genes were consistently up- (SAR^+ genes) or down-regulated (SAR^- genes) in systemic, non-inoculated leaf tissue. This transcriptional reprogramming fully depended on the SAR regulator FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1). Functional gene categorization showed that genes associated with salicylic acid (SA)-associated defenses, signal transduction, transport, and the secretory machinery are overrepresented in the group of SAR^+ genes, and that the group of SAR^- genes is enriched in genes activated via the jasmonate (JA)/ethylene (ET)-defense pathway, as well as in genes associated with cell wall remodeling and biosynthesis of constitutively produced secondary metabolites. This suggests that SAR-induced plants reallocate part of their physiological activity from vegetative growth towards SA-related defense activation. Alignment of the SAR expression data with other microarray information allowed us to define three clusters of SAR^+ genes. Cluster I consists of genes tightly regulated by SA. Cluster II genes can be expressed independently of SA, and this group is moderately enriched in H_2O_2- and abscisic acid (ABA)-responsive genes. The expression of the cluster III SAR^+ genes is partly SA-dependent. We propose that SA-independent signaling events in early stages of SAR activation enable the biosynthesis of SA and thus initiate SA-dependent SAR signaling. Both SA-independent and SA-dependent events tightly co-operate to realize SAR. SAR^+ genes function in the establishment of diverse resistance layers, in the direct execution of resistance against different (hemi-)biotrophic pathogen types, in suppression of the JA- and ABA-signaling pathways, in redox homeostasis, and in the containment of defense response activation. Our data further indicated that SAR-associated defense priming can be realized by partial pre-activation of particular defense pathways.

Keywords: systemic acquired resistance, transcriptional profiling, salicylic acid, gene classification, gene regulation, defense priming

INTRODUCTION

Plants are equipped with a multi-layered immune system that employs constitutive and inducible defense strategies to antagonize colonization by pathogenic microbes (Spoel and Dong, 2012). In pathogen-inoculated plant tissue, conserved microbial structures (pathogen-associated molecular patterns, PAMPs) elicit PAMP-triggered immunity (PTI), a basal resistance response that contains the extent of infection by compatible pathogen isolates. Following recognition of race-specific pathogen effectors by plant immune receptors ("resistance proteins"), plants are able to activate effector-triggered immunity (ETI) that usually results in a hypersensitive response (HR) at inoculation sites and provides effective local resistance to pathogens with a biotrophic or hemibiotrophic lifestyle (Jones and Dangl, 2006). Although ETI is associated with stronger local responses than PTI, the signaling networks underlying both resistance forms partially overlap (Tsuda et al., 2009).

A localized microbial infection of a single or a few leaves can also immunize the rest of the foliage to subsequent infection, a phenomenon known as systemic acquired resistance (SAR) (Fu and Dong, 2013; Shah and Zeier, 2013). Once activated, SAR provides enhanced resistance to a broad range of (hemi) biotrophic fungal, bacterial, and viral pathogens (Sticher et al., 1997). The SAR response is initiated by microbes eliciting PTI or ETI at inoculation sites, and can also be triggered by localized leaf treatment with purified PAMPs. The mechanistic principles leading to SAR induction by different types of bacterial pathogens and the resulting systemic immunization patterns are highly overlapping (Mishina and Zeier, 2007; Jing et al., 2011). For instance, compatible, PTI-inducing *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) and ETI-inducing *Psm avrRpm1* elicit highly similar systemic responses in *Arabidopsis thaliana* (Arabidopsis) plants, including systemic accumulation of the SAR immune signals pipecolic acid (Pip) and salicylic acid (SA), and

enhanced systemic expression of a variety of classical SAR marker genes such as *PATHOGENESIS-RELATED GENE 1 (PR1)*, *PR2*, and *PR5* (Mishina and Zeier, 2006; Návarová et al., 2012).

Effective long-distance communication between inoculated (1°) leaves and distant (2°, "systemic") leaves is required for the activation of SAR. Several plant-derived substances have been proposed to participate in SAR long-distance signaling (Dempsey and Klessig, 2012; Shah and Zeier, 2013). These involve the putative lipid transfer protein DEFECTIVE IN INDUCED RESISTANCE1 (DIR1), the methyl ester of SA (MeSA), glycerol-3-phosphate (G3P), the diterpenoid dehydroabietinal, the dicarboxylic acid azelaic acid, and the Lys catabolite Pip (Maldonado et al., 2002; Park et al., 2007; Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Návarová et al., 2012). The importance of several of these candidate signals for SAR induction in plants appears to depend on external parameters such as the light environment (Attaran et al., 2009; Liu et al., 2011; Návarová et al., 2012).

At the onset of SAR, the long-distance information released from 1° leaves is supposed to be perceived in 2° leaf tissue (Shah and Zeier, 2013), and a feedback amplification mechanism in 2° leaves that involves Pip and SA then ensures the activation of SAR (Návarová et al., 2012). The Lys aminotransferase AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1), whose expression is intensified in 1 and 2° leaves during SAR, is required for SAR activation (Song et al., 2004a,b). ALD1 generates the non-protein amino acid Pip, which has recently been identified as a critical metabolic SAR signal (Návarová et al., 2012). Pip-mediated resistance requires FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1), another indispensable SAR module (Mishina and Zeier, 2006; Návarová et al., 2012). Pip enhances both its own biosynthesis and downstream signaling in SAR via intensification of *ALD1* and *FMO1* expression, and systemic accumulation of the amino acid is required for the de novo synthesis of SA in 2° leaf tissue (Návarová et al., 2012). SA is a second critical SAR metabolite that is produced in plants from chorismate by ISOCHORISMATE SYNTHASE1 (ICS1) (Wildermuth et al., 2001; Métraux, 2002). SA induces SAR-related gene expression via the downstream regulator NON-EXPRESSER OF PR GENES1 (NPR1), a transcriptional co-activator and bona fide SA receptor (Wu et al., 2012; Fu and Dong, 2013). SAR-induced plants therefore exhibit increased expression of a number of *PR* genes which is presumed to directly contribute to their state of increased disease resistance (Sticher et al., 1997). Additionally, SAR confers defense priming, which enables plants to more effectively respond to future pathogen encounter (Jung et al., 2009; Návarová et al., 2012).

The interaction between Arabidopsis and *P. syringae* represents a useful model system to elucidate the molecular principles underlying inducible plant immunity (Katagiri et al., 2002). Large scale transcriptional profiling in Arabidopsis has been used to better understand PTI- and ETI-associated defense networks that are activated at sites of bacterial inoculation, and the mode of action of bacterial effectors to promote disease (Thilmony et al., 2006; Truman et al., 2006; Wang et al., 2008; Tsuda et al., 2009). Moreover, genome-wide microarray analyses have been employed to characterize Arabidopsis transcriptional responses induced by the synthetic resistance activator S-methyl-1,2,3-benzothiadiazole-7-carbothioate (BTH), which is often considered as a functional SA analogue (Wang et al., 2006). A DNA microarray representing about 25–30 % of the Arabidopsis genes has also been used to monitor and analyse gene expression changes under different SAR-inducing conditions (Maleck et al., 2000).

In the current study, we aimed to characterize biologically-induced SAR in Arabidopsis at the whole genome level, classify SAR-regulated genes according to their function and regulation of expression, derive molecular and physiological characteristics of the SAR-induced state, and further clarify the role of *FMO1* in SAR. We therefore analysed the transcriptional changes that occur in upper 2° leaf tissue upon SAR induction with *Psm* bacteria in lower 1° leaves by use of ATH1 microarray chip analyses in the Arabidopsis wild-type and *fmo1* mutant plants. These analyses revealed that SAR is associated with massive transcriptional reprogramming in systemic tissue that virtually fully depends on *FMO1*. Alignment of the SAR expression data with publicly available microarray information from defense-, stress-, and hormone-related experiments allowed us to obtain information about the regulation of genes that are up- and down-regulated during SAR. For instance, within the group of genes up-regulated during SAR, subgroups consisting of SA-independent, SA-dependent, and partially SA-dependent genes could be discriminated. Moreover, our evaluation indicated that overlapping and contrasting regulatory principles exist for the induction of local resistance responses and SAR. Further, functional categorization of SAR-related genes suggested that, upon SAR induction, plants redirect some of their resources from vegetative growth towards defense.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana (L.) Heynh. ecotype Col-0 and *fmo1* mutant plants (T-DNA insertion line SALK_026163; Mishina and Zeier, 2006) were grown in individual pots on an autoclaved mixture of soil (Klasmann, Beetpflanzensubstrat Typ R.H.P.16), vermiculite and sand (10:0.5:0.5) in a controlled environmental chamber (J-66LQ4, Percival, Boone, IA) within 9 h day (9 AM to 6 PM; photon flux density 70 μmol m^{-2} s^{-1}) and 15 h night periods in a relative humidity of 70%. Growth temperatures during the day and night period were set to 21°C and 18°C, respectively. SAR experiments were performed with 5-6 week-old plants exhibiting a uniform appearance.

SAR EXPERIMENTS

Overnight cultures of *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) were cultivated as described (Mishina and Zeier, 2006). For SAR induction, plants were infiltrated between 10 AM and 11 AM into three lower (1°) leaves (typically leaf 7–9) with a suspension of *Psm* in 10 mM $MgCl_2$ [optical density at 600 nm (OD_{600}) = 0.005]. Infiltration with 10 mM $MgCl_2$ served as the mock-control treatment. Upper (2°) leaves (typically leaf 10–12) were harvested and shock-frozen in liquid nitrogen two days after the treatment of 1° leaves. Together, three biologically independent SAR experiments were performed for microarray analyses

(see below), and two further biological replicates were performed for qPCR-based expression analyses of selected genes (**Table 2**).

RNA ISOLATION

Total RNA was isolated from frozen leaves using QIAzol® Lysis Reagent (Qiagen, http://www.qiagen.com/) following the manufacturer's instructions. For each sample, two leaves from different plants that received the same treatment were used.

QUANTITATIVE REAL-TIME PCR ANALYSIS

RNA samples were reverse-transcribed with the Omniscript RT Kit (Qiagen) using 1 μg of total RNA. 2.5 μl of cDNA and 5 μl of SensiFAST™ SYBR No-ROX Kit (Bioline) were used in PCR reactions of 10 μl total reaction volume containing 0.75 μM gene-specific primers. The At4g26410 gene which is non-responsive to *P. syringae*-inoculation was used as a reference gene (Czechowski et al., 2005). The following primers were used to obtain the expression data for SAR experiments 4 and 5 (**Table 2**): *PR1-FORWARD*: 5′-GTGCTCTTGTTCTTCCCTCG-3′, *PR1-REVERSE*: 5′-GCCTGGTTGTGAACCCTTAG-3′, *PR2-FW*: 5′-TCAAGGAAGGTTCAGGGATG-3′, *PR2-RV*: 5′-GAGATTC ACGAGCAAGGGAG-3′, *PR5-FW*: 5′-ATCGGGAGATTGCA AATACG-3′, *PR5-RV*: 5′-ATGACCTTAAGCATGTCGGG-3′, *AGP5-FW*: 5′-CTACTGAATCTCCACCAGCTC-3′, *AGP5-RV*: 5′-GAGGGAGACTCTGCTAACTG-3′, *CALM3-FW*: 5′-GAC TGATGATAAATCGTTGGAG-3′, *CALM3-RV*: 5′-CCCAACA AACTAAGCATCCT-3′, *LTPa-FW*: 5′-GGTTCTACTTCTGACT CTCC-3′, *LTPa-RV*: 5′-GTCCGTCTCCTTCTCCT-3′, *PBS3-FW*: 5′-TGCCTGCTCGAGTCGCAACC-3′, *PBS3-RV*: 5′-TGGACTAA GCCACAGAGCAAATGGC-3′, *UGT76B1-FW*: 5′-CTTTACA AGAGACTAAGGCAG-3′, *UGT76B1-RV*: 5′-CACACCTATCT GTAACTTATCCC-3′, *2OGD1-FW*: 5′-ACCAAATGCAGGTCA TAAGC-3′, *2OGD1-RV*: 5′-TGAAGGGAAATAGAAAGTCGG-3′, *NIMIN-1-FW*: 5′-AGTAAGAGAAGACGAAGAAGAG-3′, *NIMIN-1-RV*: 5′-TCCGCCGTTAGATTTCCT-3′, At4g26410-*FW*: 5′-GAGCTGAAGTGGCTTCCATGAC-3′, At4g26410-*RV*: 5′-GGTCCGACATACCCATGACC-3′. The qPCR reaction was performed in triplicate in a Rotor-Gene Q apparatus (Qiagen) using the cycling program: 95°C for 2 min, followed by 40 cycles at 95°C for 7 s, 60°C for 25 s, and finally 72°C for 3 min. The data was analyzed using the Rotor-Gene Q 2.0.2 software, setting the threshold of the normalized fluorescence to 0.1, which corresponded to the exponential phase of the fluorescence signal. The resulting C_T and E values were used to calculate the relative mRNA abundance according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The values were normalized to those of the reference gene and expressed relative to the mock-control sample.

MICROARRAY ANALYSIS AND DATA EVALUATION

For each SAR microarray experiment, RNA samples from at least 7 replicates for a particular condition (Col-0/mock, Col-0/SAR, *fmo1*/mock, *fmo1*/SAR) were mixed. The pooled RNA samples were quality-checked and expression profiling performed with the GeneChip® Arabidopsis ATH1 Genome Array using the 3′ IVT express kit (Affymetrix) under accreditation conditions (DNAVision, Charleroi, Belgium). The quality of the used GeneChips was assessed and all the samples were hybridized, processed, and scanned in parallel, ensuring that samples could be directly compared to each other. Moreover, the raw microarray data was normalized using the RMA algorithm (Irizarry et al., 2003a,b).

Together, three biologically independent SAR microarray experiments were performed. Statistical analyses of the normalized expression values of the three biological replicates were performed using a two-sided Student's *t*-test. The large scale evaluation of the microarray data was performed using Microsoft Excel® data sheets. Ratios of normalized expression values for *Psm*- and mock samples were calculated ($[P/M]_{SAR}$), and genes were arranged and grouped according to the size of their averaged $[P/M]_{SAR}$ values with the Excel® data sort function. Similarly, mean gene expression values gathered from other publicly available microarray experiments were determined for each gene, and stimulus-to-mock ratios ($[S/M]_{stimulus}$) and ratios of local expression values in Col-0 and mutant leaves following *Psm* inoculation ($[Col/mutant]_{Psm}$) were calculated thereof (**Table 6**). The $[P/M]_{SAR}$ ratios for each gene were aligned with the corresponding $[S/M]_{stimulus}$ and $[Col/mutant]_{Psm}$ ratios using the "merge" function of FIRe, an Excel® macro designed for rapid microarray data analysis (Garcion et al., 2006). The detailed selection criteria for the categorization of genes are described in the main text. The Arabidopsis Information Resource (TAIR) functional categorization tool was used to classify the genes according to Gene Ontology (GO) descriptions (http://www.arabidopsis.org/tools/bulk/go/index.jsp). The SAR microarray data were deposited in the NASCArrays database (NASCARRAYS-703).

DETERMINATION OF CAMALEXIN, ABSCISIC ACID, AND JASMONIC ACID LEVELS

For the time course analyses of the local and systemic levels of camalexin, JA, and ABA (**Figure 8**), three 1° leaves of Arabidopsis Col-0 plants were treated with *Psm* ($OD_{600} = 0.005$) or 10 mM $MgCl_2$. At the indicated times after treatment, the treated (1°) leaves and three upper, non-treated (2°) leaves were separately harvested and frozen in liquid nitrogen. Individual samples consisted of 6 leaves from two plants. The determination of the leaf metabolite levels was performed by vapor-phase extraction and subsequent GC/MS analysis as described by Návarová et al. (2012). For quantification, metabolite peaks originating from selected ion chromatograms were integrated: camalexin (m/z 200), ABA (m/z 190), and JA (m/z 224). The area of a substance peak was related to the peak area of dihydrojasmonic acid (m/z 156) or indole-3-propionic acid (m/z 130) for internal standardization. Experimentally determined correction factors were considered.

RESULTS AND DISCUSSION

FUNCTIONAL CHARACTERIZATION OF THE SAR STATE

The bacterial phytopathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) triggers a classical and robust SAR response in vegetatively growing *Arabidopsis thaliana* plants (Mishina and Zeier, 2006; Jing et al., 2011). SAR induced in Arabidopsis by compatible *Psm* or HR-inducing *Psm avrRpm1* develops between day 1 and day 2 in the whole foliage after a localized leaf inoculation has occurred, and the SAR response is fully established

2 days post inoculation (dpi) (Mishina et al., 2008). We aimed to broaden our understanding of the nature of the SAR state by gathering and analyzing the transcriptional changes that occur upon SAR establishment on the whole Arabidopsis genome level. Therefore, we inoculated lower (1°) rosette leaves of 5 week-old Arabidopsis vegetatively growing Col-0 plants with a suspension of *Psm* ($OD_{600} = 0.005$) and harvested upper, non-treated (2°) leaves two days after inoculation for RNA extraction. A mock-infiltration of 1° leaves with 10 mM $MgCl_2$ served as a control treatment. Affymetrix expression analysis (GeneChip® Arabidopsis ATH1 Genome Array) was then used to compare the expression profiles in 2° leaves of mock- and *Psm*-treated plants. Considering the above-mentioned kinetics of SAR induction in the *Psm*-Col-0 pathosystem, the selected time point at 2 dpi allowed us to characterize the transcriptional reprogramming events when SAR has just fully established, but potentially not the earliest transcriptional events at the very onset of the response. We also included the *fmo1* mutant into the study which is fully compromised in SAR, systemic accumulation of SA, and systemic expression of characteristic SAR marker genes (Mishina and Zeier, 2006).

In sum, we performed three independent SAR experiments that were conducted in the same growth chamber under identical light, temperature and humidity settings at different time periods with distinct batches of plants and pathogens. Each of the independent SAR experiments yielded one pooled RNA sample for both the mock-control and the SAR-induced state that was used for microarray analysis. The pooled RNA samples were derived from at least seven biological replicates within each SAR experiment and every biological replicate consisted of two 2° leaves from distinct plants. Thus, the gene expression samples resulting from an individual SAR experiment exhibited a high intrinsic statistical validity. The 12 pooled RNA samples for microarray analysis (3 Col-0/mock, 3 Col-0/SAR, 3 *fmo1*/mock, 3 *fmo1*/SAR) were quality-checked and expression profiling performed using the 3′ IVT express kit (Affymetrix) under accreditation conditions (DNAVision, Charleroi, Belgium). The raw ATH1 microarray data was normalized using the RMA algorithm and normalized expression values obtained (Irizarry et al., 2003a,b).

To define genes systemically up-regulated during the SAR state in Col-0 plants (SAR^+ genes), we first calculated the ratios of the normalized expression values for the *Psm*- and mock-samples from individual SAR experiments for each gene $[P/M]_{SAR}$, determined the mean values for the 3 ratios from different experiments for each gene, and selected those genes that were up-regulated by a factor of at least 3 on average. We further applied a two-sided *t*-test between the normalized expression values of the *Psm*- and those of the mock-samples for each gene and excluded those genes from our list with a *P*-value > 0.05. These two selection criteria yielded 305 genes out of the 22810 genes represented on the ATH1 GeneChip that were up-regulated by a factor of at least 3 in a statistically significant manner among the 3 individual SAR experiments (**Table 1A**). The number of genes up-regulated upon SAR induction on average by a factor of 5 and 10 amounted to 149 and 67, respectively. For the *fmo1* mutant, not a single SAR^+ gene existed based on these criteria (**Table 1B**), corroborating our previous findings that functional *FMO1* is critical for the activation of systemic defense responses and SAR (Mishina and Zeier, 2006; Návarová et al., 2012). We also recognized that expression of several genes was consistently suppressed following SAR establishment in Col-0 plants (SAR^- genes), although the overall degree of gene down-regulation was lower than the degree of up-regulation (**Tables 1A**,**B**). For instance, 17 and 276 genes were significantly down-regulated by a factor of at least 4 ($[P/M]_{SAR} < 0.25$) and 2 ($[P/M]_{SAR} < 0.5$), respectively. Again, not a single SAR^- gene was differently expressed in 2° leaves of *Psm*- and mock-treated *fmo1* plants when following these selection criteria (**Tables 1A**,**B**).

Table 1 | Number of genes up- (SAR^+ genes) and down-regulated (SAR^- genes) upon *Psm*-induced SAR in Arabidopsis Col-0 and *fmo1*.

A	**SAR^+ (up-regulated)**		**Col-0**	***fmo1***	**B**	**SAR^- (down-regulated)**		**Col-0**	***fmo1***
	$[P/M]_{SAR}$	>10	67	0		$[P/M]_{SAR}$	<0.1	0	0
		>5	149	0			<0.25	17	0
		>3	305	0			<0.5	276	0
C	**SAR^+ (up-regulated)**		**Col-0**	***fmo1***	**D**	**SAR^- (down-regulated)**		**Col-0**	***fmo1***
	$[P/M]_{SAR}$	>10	145	0		$[P/M]_{SAR}$	<0.1	2	0
		>5	295	1			<0.25	50	0
		>3	**547**	**4**			**>0.5**	**700**	**0**

(A,B) *$[P/M]_{SAR}$ symbolizes the mean value over SAR experiments 1, 2 and 3 of the ratios of the normalized expression values for Psm-samples divided by those of the mock samples. A two-sided t-test comparing the normalized expression values of the Psm- and the mock-samples was performed, and genes with $P > 0.05$ were excluded. On the right of each table, the number of resulting* ***(A)*** *SAR^+ genes (for $[P/M]_{SAR} > 10$, 5, or 3) and* ***(B)*** *SAR^- genes ($[P/M]_{SAR} < 0.1$, 0.25 or 0.5) are given.* ***(C,D)*** *Final classification of SAR^+ genes and SAR^- genes. The data from the untypical SAR experiment 3 was excluded.* ***(C)*** *Only genes with mean $[P/M]_{SAR} > 3$ from SAR experiments 1 and 2 were considered for the SAR^+ gene cluster. Genes whose individual $[P/M]_{SAR}$ ratios in either experiment 1 or 2 were below 2 were excluded. These selection criteria were taken as a basis for the final classification of genes into the SAR^+ gene cluster (number of SAR^+ genes in bold). Number of genes with mean $[P/M]_{SAR} > 5$ and > 10 are also given.* ***(D)*** *Only genes with mean $[P/M]_{SAR} < 0.5$ from SAR experiments 1 and 2 were considered for the SAR^- gene cluster. Genes whose individual $[P/M]_{SAR}$ ratios in either experiment 1 or 2 were above 0.67 were excluded (number of SAR^- genes in bold). Number of genes with mean $[P/M]_{SAR} < 0.25$ and < 0.1 are also given.*

We next examined the $[P/M]_{SAR}$ expression ratios of individual genes from the distinct SAR experiments more closely. Remarkably, many genes that on average belonged to the most prominently SAR-induced genes were not up-regulated in a statistically significant manner ($P > 0.05$) or only barely exhibited significantly increased expression values over all 3 independent experiments. **Table 2** lists 10 representative genes from this group. Among them are genes such as *PR1*, *PR2*, *PR5*, and *PBS3*, which belong, according to previous results from other groups and our own findings (Sticher et al., 1997; Maldonado et al., 2002; Mishina and Zeier, 2006; Lee et al., 2007), to the most characteristic SAR^+ genes. We recognized that many of these genes were highly induced in SAR experiments 1 and 2, but showed only a modest or low degree of up-regulation in experiment 3 (**Table 2**). For instance, *PR1* expression was induced by factors of 137, 183, and 7 in SAR experiments 1, 2, and 3, respectively. Thus, albeit markedly up-regulated in each of the individual SAR experiments, the high quantitative differences between expression values of experiment 3 compared to the two other experiments resulted in a P value larger than 0.05 for *PR1*. A similar trend was obvious for most of the other genes listed in **Table 2**. We consequently performed another two independent SAR experiments (experiments 4 and 5) and examined the expression characteristics of the 10 genes listed in **Table 2** by quantitative real-time PCR analyses. Strikingly, in both newly conducted experiments, virtually all of the examined genes exhibited $[P/M]_{SAR}$ ratio quantitatively similar to experiments 1 and 2. Furthermore, the $[P/M]_{SAR}$ values derived from experiments 4 and 5 for the genes *PR1*, *AGP5*, *UGT76B1*, *LTP-like*, *CALM3*, and *PBS3* were quantitatively much higher than the $[P/M]_{SAR}$ values obtained from experiment 3. On the basis of this data and previous findings, we concluded that the bacterial inoculation in experiment 3 only provoked a modest SAR response on the transcriptional level that is not representative for the SAR response that is generally observed.

A subsequent systematic search for differently expressed genes in the control samples of experiments 1, 2, and 3 identified about 50 genes that differed in their normalized expression values by a factor of 3 or more between experiment 3 and both other experiments. Interestingly, many of these genes represent central flavonoid pathway genes (**Table 3**). For instance, the normalized expression values of two main transcriptional regulators of anthocyanin biosynthesis, *MYB90* and *MYB75* (Borevitz et al., 2000), were about two and one order of magnitude, respectively, higher for the experiment 3-samples than for the samples from experiment 1 or 2. Similar quantitative expression patterns existed for *DFR* and *ANS*, encoding two key enzymes of the anthocyanidin biosynthesis pathway, dihydroflavonol reductase and anthocyanidin synthase, respectively, for the anthocyanin-5-O-glucosyltransferase gene *UGT75C1*, for the anthocyanin coumaroyltransferase gene *A3GlcCouT* and for the glutathione-S-transferase gene *GSTF12* that is involved in anthocyanin accumulation (Saito et al., 2013). Therefore, the biosynthesis of anthocyanins apparently was activated in the plants employed for experiment 3 to a markedly higher extent than in the plants from the other two experiments. Anthocyanin production in leaves is a characteristic response of plants to unfavorable environmental conditions such as drought, nitrogen deficiency or high

Table 2 | Normalized expression values and $[P/M]_{SAR}$ ratios (bold) for individual genes of the SAR experiments 1, 2 and 3, as determined by ATH1 microarray analyses.

		Experiment 1 (microarray)			Experiment 2 (microarray)			Experiment 3 (microarray)			*t-test (P- value)*	Experiment 4 (qPCR)	Experiment 5 (qPCR)
		Expression value		Ratio	Expression value		Ratio	Expression value		Ratio	[P1-P3] / [M1-M3]	Ratio	Ratio
Locus	Name	Mock 1	*Psm* 1	$[P/M]_{SAR1}$	Mock 2	*Psm* 2	$[P/M]_{SAR2}$	Mock 3	*Psm* 3	$[P/M]_{SAR3}$		$[P/M]_{SAR4}$	$[P/M]_{SAR5}$
At2g14610	***PR1***	12.4	1703.2	**137.3**	16.1	2948.6	**183.1**	10.5	75.7	**7.2**	*0.133*	**95.9**	**194.8**
At1g02450	***NIMIN-1***	10.8	2007.9	**186.4**	13.8	1119.0	**81.3**	8.6	90.9	**10.5**	*0.128*	**75.3**	**70.2**
At4g10500	***2OGD1***	12.6	1786.1	**141.9**	14.6	1725.1	**118.0**	15.8	404.4	**25.5**	*0.046*	**78.3**	**19.5**
At1g35230	***AGP5***	9.0	956.5	**105.8**	10.4	1109.6	**106.3**	11.4	60.1	**5.3**	*0.100*	**162.3**	**26.3**
At3g11340	***UGT76B1***	7.4	798.7	**108.4**	7.6	586.1	**76.7**	6.9	14.9	**2.2**	*0.121*	**352.0**	**64.9**
At3g22600	***LTPa***	14.1	1073.8	**76.3**	14.3	1461.4	**101.9**	15.3	45.3	**3.0**	*0.116*	**78.3**	**379.5**
At3g01830	***CALM3***	7.5	544.0	**72.8**	8.7	521.2	**59.7**	7.3	51.0	**7.0**	*0.086*	**46.2**	**20.6**
At3g57260	***PR2***	48.1	1657.4	**34.5**	59.0	3412.1	**57.8**	43.2	942.1	**21.8**	*0.056*	**64.1**	**19.1**
At1g75040	***PR5***	40.3	1737.9	**43.1**	96.4	3252.2	**33.7**	29.8	367.9	**12.4**	*0.106*	**39.0**	**24.2**
At5g13320	***PBS3***	9.3	425.9	**46.1**	14.5	434.3	**29.9**	12.1	18.7	**1.5**	*0.110*	**68.6**	**35.6**

P-values resulting from a two-sided t-test between the normalized expression values of the Psm- and the mock-samples of experiments 1 to 3 are given (italic). In addition, $[P/M]_{SAR}$ ratios of two additional, biologically independent SAR experiments that were determined by quantitative real-time PCR analyses (qPCR) are depicted.

Table 3 | Several central anthocyanin biosynthesis genes exhibit high normalized expression values in plants of SAR experiment 3 compared with experiments 1 and 2 (M1-3: mock treatment experiment 1-3; *P*1-3: *Psm* treatment experiment 1-3).

Locus	*Name*	Gene description	Expression value						*Ratio*	
			M1	M2	M3	*P*1	*P*2	*P*3	M3/M1	M3/M2
At1g66390	*MYB90/PAP2*	*PRODUCTION OF ANTHOCYANIN PIGMENT 2*	11.1	17.3	1281.1	14.9	12.5	539.1	*115.4*	*74.1*
At5g17220	*GSTF12*	Glutathione transferase, anthocyanin biosynthesis	14.4	19.6	721.3	15.1	54.3	284.1	*50.1*	*36.7*
At5g42800	*DFR*	Dihydroflavonol reductase	12.0	12.7	497.6	12.1	21.7	117.9	*41.4*	*39.2*
At4g22880	*ANS*	Anthocyanidin synthase, leucoanthocyanidin dioxygenase	17.5	31.4	687.7	17.8	46.1	134.4	*39.3*	*21.9*
At4g32940	γ-*VPE*	Vacuolar cysteine proteinase	220.0	315.7	3362.8	247.3	315.2	2297.1	*15.3*	*10.7*
At5g54060	*UF3GT*	*UDP-GLC-FLAVONOID 3-O-GLUCOSYLTRANSFERASE*	15.2	16.4	192.1	19.3	18.3	47.8	*12.7*	*11.7*
At1g03495	*A3GlcCouT*	Anthocyanin coumaroyltransferase	9.9	15.4	142.3	10.4	15.2	45.6	*14.3*	*9.2*
At4g14090	*UGT75C1*	Anthocyanidin-5-O-glucosyltransferase	10.6	9.1	86.7	10.5	9.2	28.2	*8.1*	*9.5*
At1g56650	*MYB75/PAP1*	*PRODUCTION OF ANTHOCYANIN PIGMENT 1*	109.7	117.4	803.9	28.7	26.1	481.8	*7.3*	*6.8*
At1g62710	β-*VPE*	Vacuolar cysteine proteinase	25.5	36.4	188.4	17.0	32.8	104.5	*7.4*	*5.2*
At3g51240	*F3'H*	Flavanone 3-hydroxylase	155.6	338.4	1473.2	128.6	271.5	544.6	*9.5*	*4.4*
At5g13930	*CHS*	Chalcone synthase (*TRANSPARENT TESTA 4, TT4*)	26.8	410.9	1146.6	93.6	280.5	334.9	*42.7*	*2.8*
At1g65060	*4CL3*	4-coumarate-CoA ligase	15.1	31.9	102.7	22.0	27.3	22.0	*6.8*	*3.2*
At5g05270	*CHI*	Chalcone isomerase	10.8	39.3	84.3	20.0	23.4	23.1	*7.8*	*2.1*

light stress (Misyura et al., 2013). Although the plants used in experiment 3 did not exhibit a macroscopically obvious stress phenotype, it is likely that they had suffered an unexpected stress exposure prior to or in the course of the SAR experiment that resulted in the activation of anthocyanin biosynthesis. Mutual influences between pathogen defense signaling and abiotic stress pathways or the anthocyanin biosynthesis pathway do exist (Fan et al., 2009; Saijo et al., 2009), and it is possible that the moderate SAR reaction observed in experiment 3 was a consequence of such cross-talk events.

Since the transcriptional data obtained from SAR experiment 3 markedly differed from those resulting from the other four experiments, we decided to exclude this data set from further analyses. We defined new selection criteria to classify the SAR^+ genes and only considered genes whose mean $[P/M]_{SAR}$ ratios from experiments 1 and 2 were larger than 3, and whose individual $[P/M]_{SAR}$ ratios in either experiment was at least 2. We thus ensured that a marked up-regulation of the selected SAR^+ genes had taken place in each of the experiments 1 and 2. When following this procedure, all the characteristically SAR up-regulated genes listed in **Table 2** fell into the category "SAR^+ genes" which altogether consisted of 547 genes for the Col-0 wild-type (**Table 1C**). Thereof, 295 and 145 SAR^+ genes showed average $[P/M]_{SAR}$ ratios larger than 5 and 10, respectively (**Table 1C**). To classify genes down-regulated during SAR, we selected genes with average $[P/M]_{SAR}$ ratios lower than 0.5, whereby genes with rations higher than 0.67 in either experiment 1 or 2 were excluded. This procedure yielded a group of 700 SAR^- genes, from which 50 genes had average $[P/M]_{SAR}$ ratios lower than 0.25 and 2 genes exhibited average $[P/M]_{SAR}$ ratios lower than 0.1 (**Table 1D**). The new selection criteria pinpointed only 4 genes systemically up-regulated in *fmo1* by a factor of at least 3 after *Psm*-infection. Moreover, not a single down-regulated gene was assigned for *fmo1* (**Tables 1C**,**D**), again highlighting that *fmo1* is virtually non-responsive to pathogen stimuli at the systemic plant level.

As a first step to functionally characterize the two clusters of SAR^+ and SAR^- genes, we used the The Arabidopsis Information Resource (TAIR) functional categorization tool to classify the genes according to Gene Ontology (GO) descriptions which discriminates the three main classes "cellular component", "biological process" and "molecular function" (http://www.arabidopsis.org/tools/bulk/go/index.jsp). We thereby compared the SAR^+ (547) and the SAR^- (700) gene cluster with all the genes represented on the ATH1 Genechip (22810) (**Tables 1C**,**D**). In the class "cellular component", the functional categories "Golgi", "endoplasmatic reticulum (ER)", and "plasma membrane" were strongly overrepresented in the group of SAR^+ genes. Moreover, the categories "cell wall", "extracellular", and "cytosol" were moderately overrepresented among the SAR^+ genes, whereas the categories "plastid", "nucleus", and "ribosome" were underrepresented (**Figure 1A**). For the group of SAR^- genes, it became apparent that "chloroplast", "plastid", "cell wall", and "extracellular" were highly represented categories (**Figure 1A**). These tendencies might indicate that cellular processes associated with the secretory apparatus and extracellular defenses are activated during SAR, and that certain activities occurring in chloroplasts and

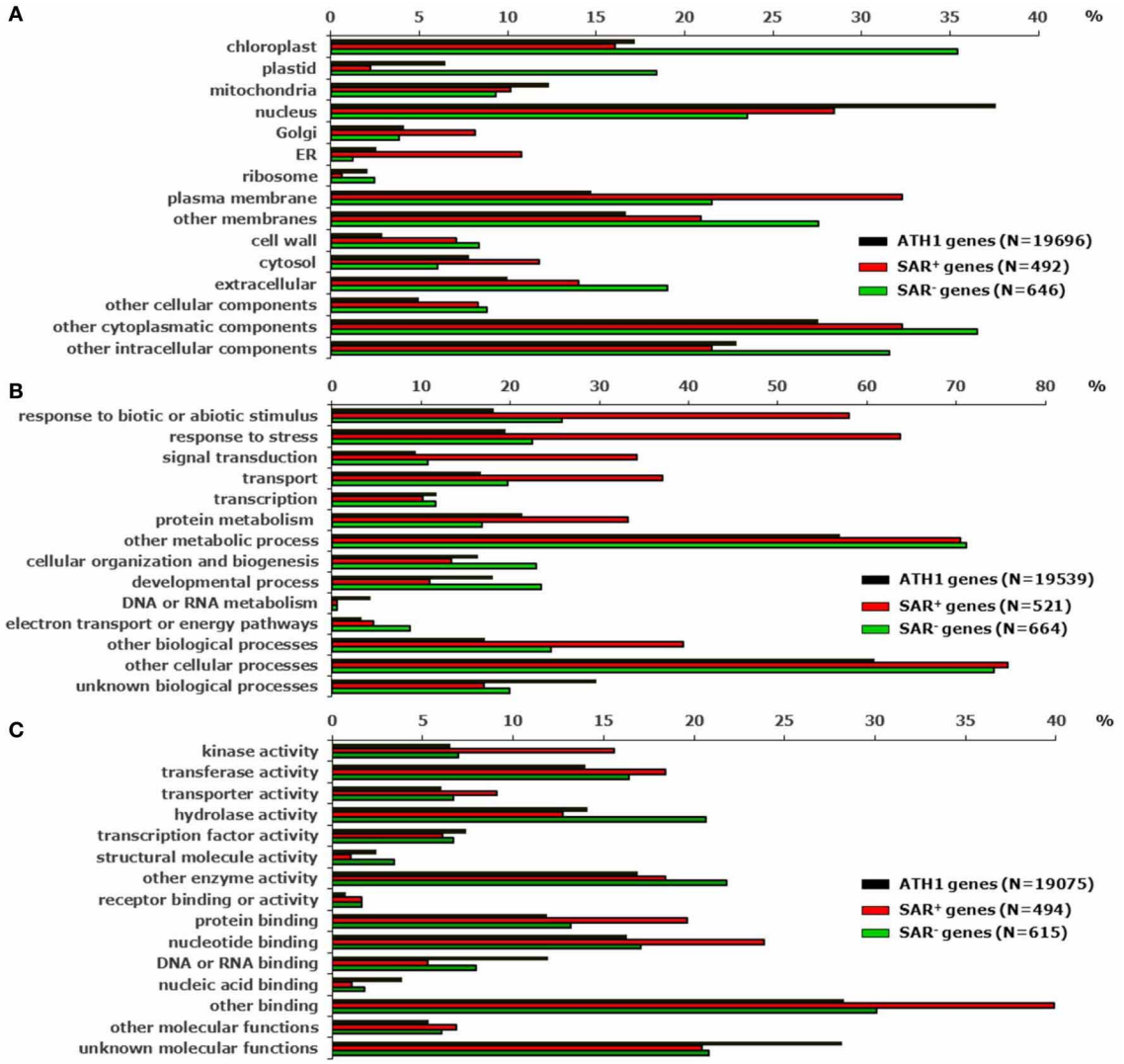


FIGURE 1 | Functional categorization of SAR$^+$ and SAR$^-$ genes according to Gene Ontology (GO) descriptions (http://www.arabidopsis.org/tools/bulk/go/index.jsp). The 547 genes up-regulated during SAR by $[P/M]_{SAR} > 3$ (SAR$^+$ genes) (**Table 1C**), the 700 genes down-regulated during SAR by $[P/M]_{SAR} < 0.5$ (SAR$^-$ genes) (**Table 1D**), and all the genes (22810) represented on the ATH1 chip were set as input lists for the categorization algorithm. The depicted value on each of the x axes represents the quotient of "the number of genes annotated to terms of the respective categorization class" divided by "the total number of genes from the input list annotated to any term in this ontology (N)" in %. **(A)** Categorization class "cellular component". **(B)** Categorization class "biological process". **(C)** Categorization class "molecular function".

plastids are reduced. Not unexpectedly, in the class "biological process", genes with GO annotations "response to (a)biotic stimuli" and "response to stress" were typically found in the group of SAR up-regulated genes (**Figure 1B**). Furthermore, the functional categories "signal transduction", "transport", and "protein metabolism" were highly represented in the group of SAR$^+$ genes, whereas the categories "developmental processes" and "DNA or RNA metabolism" were underrepresented. The category "electron transport and energy pathways" was relatively prominent among the SAR$^-$ genes (**Figure 1B**). Thus, the relative distributions of GO categories from the class "biological processes" indicated that stimulus- and stress-related signal transduction events, transport processes and protein metabolism are prominent features of the SAR state, whereas certain developmental, nucleic acid metabolic, and energy-related events might be reduced in SAR-induced plants. From the GO categories grouped into the class "molecular function", "kinase activity", "protein binding", "nucleotide binding", "receptor binding", and "transporter activity" were overrepresented in the SAR$^+$ gene cluster, whereas "DNA or RNA/nucleic acid binding" was poorly represented (**Figure 1C**). Again, this might emphasize the importance of signal transduction events such as protein phosphorylation and protein-ligand interactions for SAR.

In the cluster of SAR$^-$ genes, the relatively high abundance of the GO annotation "hydrolase activity" was one of the most obvious features (**Figure 1C**). Moreover, when examining the

specific functional annotation of genes in this cluster, two other trends became apparent. First, several genes belonging to the xyloglucan endotransglucosylase/hydrolase (XTH), the (fasciclin-like) arabinogalactan protein (AGP), the expansin-like protein, the extensin-like protein, and the polygalacturonase families were among the genes most strongly down-regulated during SAR (**Table 4**). Members of these gene families encode proteins associated with the extracellular matrix and/or the cell wall and have important functions in the rearrangement of cell wall components, wall loosening, cell elongation, and cell growth. For instance, members of the XTH family are involved in xyloglucan endotransglucosylation and/or in xyloglucan hydrolysis. These enzymatic activities can contribute to primary cell growth by restructuring and loosening the xyloglucan network, thereby enabling cell expansion (Rose et al., 2002). Two of the four XTH genes markedly down-regulated during SAR, *XTH4* and *XTH31* (**Table 4**), code for proteins that have been experimentally identified as constituents of the cell wall proteome in Arabidopsis hypocotyls (Irshad et al., 2008). The strongly SAR down-regulated *EXLA1* gene belongs to the expansin multigene family. The presence of EXLA1 protein in the Arabidopsis cell wall has also been experimentally verified (Irshad et al., 2008). Expansins directly modify the mechanical properties of plant cell walls leading to turgor-driven cell extension (Li et al., 2002). Another class of extracellular proteins implicated in plant growth and development are the hydroxyproline-rich and highly glycosylated AGPs (Schultz et al., 2002). Several AGPs, among them the three fasciclin-like AGPs (FLAs) *FLA8*, *FLA9*, and *FLA13*, belong to the genes most highly down-regulated following SAR induction (**Table 4**). FLAs have, in addition to predicted AGP-like glycosylated regions, putative cell adhesion domains known as fasciclin domains (Johnson et al., 2003). Together, these examples indicate that the SAR state is associated with a marked down-regulation of various genes involved in cell wall modification, cell growth and development (**Table 4**).

The cluster of SAR$^-$ genes also contained several genes involved in glucosinolate and sinapoylester production (**Table 5**). These include genes encoding regulatory components or enzymes of indolic glucosinolate biosynthesis such as the MYB transcription factor MYB34 and the cytochrome P450 CYP79B3 that converts Trp to the indolic glucosinolate precursor indole-3-acetaldoxime (Glawischnig et al., 2004; Celenza et al., 2005), and of aliphatic glucosinolate biosynthesis such as the flavin-dependent monooxygenases FMO_{GS-OX1} and FMO_{GS-OX3} that oxidize Met-derived methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates (Li et al., 2008), or the 2-oxoglutarate-dependent dioxygenase AOP2 involved in the conversion of methylsulfinylalkyl to alkenyl glucosinolates (**Table 5A**) (Neal et al., 2010). Moreover, among the most prominently SAR down-regulated genes are *SCPL8*, *SCPL10*, and *SCPL13* (**Table 5B**), encoding serine carboxypeptidase-like proteins that act as sinapoyltransferases to generate sinapoylmalate, sinapoylanthocyanins, and 1,2-disinapoyl-glucose derivatives, respectively (Stehle et al., 2009; Fraser and Chapple, 2011). Sinapoylesters and glucosinolates are among the most abundant secondary metabolites produced in Arabidopsis in the course of normal growth and development (Stehle et al., 2009; Sønderby et al., 2010). The reduced expression of genes involved in the constitutive production of major secondary metabolites in SAR-induced compared with control plants again supports the hypothesis that SAR represents a state of diminished vegetative growth.

Together, the marked down-regulation of genes with presumed roles in cell wall modification, cell growth and the constitutive production of secondary metabolites (**Tables 4**, **5**), the overrepresentation of annotated chloroplast functions among SAR down-regulated genes (**Figure 1A**), and the strong up-regulation of stimulus-, stress- and defense-related genes during SAR (**Figure 1B**) indicate that, compared with control plants, SAR-induced plants reallocate a part of their physiological activity from vegetative growth towards particular defense-associated

Table 4 | Genes associated with cell wall remodelling, cell extension, and growth belong to the most strongly down-regulated genes during SAR.

Locus	*Name*	Gene description	$[P/M]_{SAR}$	Rank among down-regulated genes
At3g45970	*EXLA1*	Expansin-like A1*	0.14	11
At1g03870	*FLA9*	Fasciclin-like arabinogalactan-protein 9	0.17	13
At2g45470	*FLA8*	Fasciclin-like arabinogalactan-protein 8	0.18	15
At2g06850	*XTH4*	Xyloglucan endotransglucosylase/hydrolase*	0.18	16
At3g06770	–	polygalacturonase (pectinase)*	0.20	17
At1g55330	*AGP21*	Arabinogalactan protein 21	0.21	22
At2g14890	*AGP9*	Arabinogalactan protein 9 /putative proline-rich protein	0.21	23
At2g47930	*AGP26*	Arabinogalactan protein 26	0.22	26
At1g12090	*ELP*	Extensin-like protein	0.23	32
At5g44130	*FLA13*	Fasciclin-like arabinogalactan-protein 13	0.24	39
At3g44990	*XTH31*	Xyloglucan endotransglucosylase/hydrolase*	0.24	43
At4g37800	*XTH7*	Xyloglucan endotransglucosylase/hydrolase	0.25	52

Genes with asterisks encode proteins experimentally verified as cell wall constituents.

Table 5 | Genes involved in the biosynthesis of major constitutively produced secondary metabolites are down-regulated during SAR.

Locus	*Name*	Gene description	*P*/M	Rank among down-regulated genes
A				
At5g60890	*MYB34/ATR1*	MYB transcription factor, ALTERED TRYPTOPHAN REGULATION 1, regulates indole glucosinolate biosynthesis	0.25	51
At4g03060	*AOP2*	2-oxoglutarate-dependent dioxygenase, ALKENYL HYDROXALKYL PRODUCING 2, aliphatic glucosinolate biosynthesis, conversion of methylsulfinylalkyl glucosinolates to alkenyl glucosinolates, not functional in Col-0	0.27	72
At1g65860	*FMO GS-OX1*	FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 1, aliphatic glucosinolate biosynthesis, conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates	0.34	163
At1g62560	*FMO GS-OX3*	FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 3, aliphatic glucosinolate biosynthesis, conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates	0.36	215
At4g03070	*AOP1*	2-oxoglutarate-dependent dioxygenase, similar to AOP2, possibly involved in aliphatic glucosinolate biosynthesis	0.40	339
At2g22330	*CYP79B3*	Cytochrome P450 monooxygenase, converts Trp to indole-3-acetaldoxime (IAOx), a precursor to IAA and indole glucosinolates	0.41	370
B				
At2g22980	*SCPL13*	Serine carboxypeptidase-like (SCPL) protein; sinapoylglucose:sinapoylglucose sinapoyltransferase	0.11	5
At2g23000	*SCPL10*	SCPL protein; anthocyanin sinapoyltransferase	0.22	28
At2g22990	*SCPL8/SNG1*	SCPL protein; sinapoylglucose:malate sinapoyltransferase	0.30	100

(A) *Genes associated with glucosinolate biosynthesis.* ***(B)*** *Sinapoyltransferase genes involved in sinapoylester biosynthesis.*

processes that confer broad-spectrum disease resistance (see below).

REGULATORY PRINCIPLES UNDERLYING SAR GENE EXPRESSION

Having categorized defined groups of SAR up- and down-regulated genes, we next aimed to combine our SAR expression data with further transcriptional information to elucidate regulatory principles that govern the SAR response. We used the FIRe software, an Excel® macro designed for rapid microarray data analysis (Garcion et al., 2006), to assemble the SAR expression data with expression data from other, publicly available microarray experiments describing the impact of various defense-, stress-, and hormone-related stimuli on gene expression in Arabidopsis plants. Information about the employed microarray data, the experimenters, and the experimental setup underlying each data set are summarized in **Table 6**.

The information drawn from these microarray experiments is based on two distinct types of comparisons. In most experiments, Arabidopsis wild-type plants were exogenously treated with a chemical stimulus and the gene expression values of stimulus-treated plants or leaf tissue was compared to the values resulting from an adequate mock-treatment. In this way, we could acquire information about the impact of exogenous application of defense and stress hormones or their derivatives [SA, methyl jasmonate (MeJA), abscisic acid (ABA)], oxidative stress (H_2O_2), the resistance-enhancing chemical BTH, which is often considered as an SA analogue (Lawton et al., 1996; Wang et al., 2006; Canet et al., 2010), and flg22-treatment, a 22mer peptide corresponding to the elicitor-active domain of the bacterial PAMP flagellin (Gomez-Gomez et al., 1999), on gene expression (**Table 6**, **Figures 2–7**; microarray designations "BTH", "SA", "MeJA", "H_2O_2", "ABA", "flg22"). Similarly, one experiment investigated the impact of *Psm* inoculation on gene expression (designation "*Psm*"). In contrast to our SAR microarray data (designation "SAR") that describes systemic changes in 2°, non-inoculated leaves at 48 hours post inoculation (hpi), this experiment yielded information about the local changes in gene expression at the site of pathogen inoculation (1° leaves) at 24 hpi. For all microarray experiments, we calculated the means of normalized expression values from the stimulus replicates and divided them by the means of the respective mock-values. This yielded, in analogy to the $[P/M]_{SAR}$ ratios for the SAR experiment, stimulus to mock ratios $[S/M]_{stimulus}$ that quantitatively indicated by which factors genes were differently expressed following application of the exogenous stimulus compared with the mock-control in wild-type Col-0 plants (**Figures 2–7**).

The microarray experiment 2 (**Table 6**) that investigated the impact of *Psm* leaf inoculation on local gene expression yielded two kinds of information: the ratio *Psm*/mock in Col-0 ($[S/M]_{Psm}$; microarray 2a), and expression ratios of *Psm*-treated wild-type samples to different *Psm*-treated mutant samples ($[Col/mutant]_{Psm}$; microarray 2b). We evaluated the expression data from *sid2* which is defective in *ICS1* and, consequently, pathogen-induced SA production (Nawrath and Métraux, 1999;

Table 6 | Publicly available microarray data sets ("microarrays 1–7") used in this study.

Microarray	Name	Experimenter	Experiment description	Designation	Depicted value
0	NASCARRAYS-703	Griebel, Attaran, Zeier	Biological SAR, syringe infiltration of lower leaves of 5 week-old Col-0 plants with *Psm* (OD 0.005), upper (non-treated) leaves harvested 2 d later, plants grown in soil under a 9/15-h light/dark cycle at 21/18 °C	"SAR"	*Psm*/mock = $[P/M]_{SAR}$
1	NASCARRAYS-392	Wang, Dong (Wang et al., 2006)	60 μM BTH, spray-treatment of 4 week-old Col-0 plants grown on soil under a 16/8-h light/dark cycle at 22°C, samples 24 h post treatment were considered	"BTH"	BTH/mock = $[S/M]_{BTH}$
2a	NASCARRAYS-454	Mitra, Glazebrook (Wang et al., 2008)	Leaf inoculation (syringe infiltration) of 4-5 week-old Col-0 plants with *Psm* (OD 0.002), inoculated leaves harvested 24 hpi, plants grown in soil under a 12/12-h light/dark cycle at 22 °C	"*Psm*"	Col-0-*Psm*/Col-0-mock = $[S/M]_{Psm}$
2b	NASCARRAYS-454	Mitra, Glazebrook (Wang et al., 2008)	Leaf inoculation (syringe infiltration) of 4-5 week-old Col-0 or mutant plants with *Psm* (OD 0.002), inoculated leaves harvested 24 hpi, plants grown in soil under a 12/12-h light/dark cycle at 22 °C	"Col / *mutant*"	Col-0-*Psm*/*mutant*-*Psm* = $[Col/mutant]_{Psm}$
3	E-GEOD-3984	Thibaud-Nissen (Thibaud-Nissen et al., 2006)	1 mM SA in 0.01 % Silwet, spray-treatment of 3-4 week-old, non-flowering Col-0 plants, leaf samples harvested 2 h post treatment	"SA"	SA/mock = $[S/M]_{SA}$
4	NASCARRAYS-174	Goda, Yoshida, Shimada (Goda et al., 2008)	7 day-old Col-0 seedlings grown in MS liquid medium under constant light at 22°C were treated with 10 μM MeJA, leaf samples at 3 h post treatment were considered	"JA"	JA/mock = $[S/M]_{JA}$
5	NASCARRAYS-338	Mittler (Davletova et al., 2005)	Application of 20 mM H_2O_2 to 5 day-old Col-0 seedlings grown on MS agar plates under constant light at 21-22° C	"H_2O_2"	H_2O_2/mock = $[S/M]_{H2O2}$
6	NASCARRAYS-176	Goda, Yoshida, Shimada (Goda et al., 2008)	7 day-old Col-0 seedlings grown in MS liquid medium under constant light at 22° C were treated with 10 μM ABA, leaf samples at 3 h post treatment were considered	"ABA"	ABA/mock = $[S/M]_{ABA}$
7	NASCARRAYS-123	Scheel, Brunner, Westphal	Surface-treatment of leaves of 5 week-old Col-0 plants with 1 mM flg22 peptide, plants grown on soil at 22°C under a 8/16 hour light/dark regime, leaf samples 4 h post treatment were considered	"flg22"	flg22/mock = $[S/M]_{flg22}$

Sources, experimenters, relevant literature citations, and experimental descriptions are given.

*The designation of each experiment and the value depicted in the **Figures 2–7** are also indicated.*

Wildermuth et al., 2001), SA insensitive *npr1* defective in the transcriptional co-activator and SA receptor NPR1 (Durrant and Dong, 2004; Wu et al., 2012), *pad4* defective in the lipase-like defense regulator PHYTOALEXIN-DEFICIENT4 (Jirage et al., 1999), ethylene-insensitive *ein2* (Alonso et al., 1999), and JA insensitive *coi1* defective in the JA receptor CORONATINE INSENSITIVE1 (Katsir et al., 2008). The $[Col/mutant]_{Psm}$ ratios could be used to assess at the genetic level whether *Psm*-induced gene expression was dependent on SA accumulation (*sid2*), SA perception (*npr1*), JA perception (*coi1*), and ET perception (*ein2*) (**Figures 2–7**).

We now assembled the $[P/M]_{SAR}$ ratios for each gene from the SAR experiment with the corresponding [S/M] or $[Col/mutant]_{Psm}$ ratios from the other microarray experiments

microarray-experiment		0	1	2a	2b	2b	3	2b	2b	2b	4	5	6	7
A gene cluster	# genes	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/*sid2*]	*Psm* [Col/*npr1*]	SA [S/M]	*Psm* [Col/*pad4*]	*Psm* [Col/*ein2*]	*Psm* [Col/*coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]
SAR$^+$ genes	524	10.5	6.5	6.5	3.0	2.5	2.5	2.6	1.1	1.1	1.1	2.5	1.5	2.9
SAR$^-$ genes	670	0.4	0.4	1.0	0.7	0.6	0.8	0.4	0.7	5.6	2.0	0.9	1.1	0.6
rest	20575	1.0	1.0	1.6	1.2	1.2	1.0	1.0	1.1	1.5	1.1	1.2	1.5	1.3
B SAR$^+$ genes	# genes	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/*sid2*]	*Psm* [Col/*npr1*]	SA [S/M]	*Psm* [Col/*pad4*]	*Psm* [Col/*ein2*]	*Psm* [Col/*coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]
cluster I: SA-dep.	100	14.0	9.1	2.9	7.9	6.4	4.9	2.4	0.8	0.8	0.9	1.5	1.0	3.7
cluster II: SA-indep.	156	10.3	6.6	11.8	1.1	1.4	2.1	2.8	1.3	1.4	1.3	3.9	2.4	2.9
cluster III: rest	268	9.4	5.5	4.6	1.6	1.7	1.8	2.6	1.0	1.0	1.1	2.1	1.2	2.6
C SAR$^-$ genes	# genes	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/*sid2*]	*Psm* [Col/*npr1*]	SA [S/M]	*Psm* [Col/*pad4*]	*Psm* [Col/*ein2*]	*Psm* [Col/*coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]
JA-activated	76	0.4	0.4	6.6	0.7	0.5	0.8	0.4	1.1	43.9	10.3	1.0	1.5	0.8
JA-repressed	190	0.4	0.4	0.1	0.6	0.5	0.8	0.3	0.5	0.3	0.7	0.8	1.3	0.6
JA-independent	404	0.4	0.4	0.3	0.8	0.7	0.8	0.4	0.8	0.9	1.1	0.9	1.0	0.6
D gene category	# genes	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/*sid2*]	*Psm* [Col/*npr1*]	SA [S/M]	*Psm* [Col/*pad4*]	*Psm* [Col/*ein2*]	*Psm* [Col/*coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]
systemically up 48 hpi (SAR$^+$ genes)	524	10.5	6.5	6.5	3.0	2.5	2.5	2.6	1.1	1.1	1.1	2.5	1.5	2.9
locally up 24hpi	1921	2.9	2.7	10.9	1.9	1.9	1.5	2.5	1.7	6.4	1.8	2.5	3.3	2.8
systemically down 48 hpi (SAR$^-$ genes)	670	0.4	0.4	1.0	0.7	0.6	0.8	0.4	0.7	5.6	2.0	0.9	1.1	0.6
locally down 24 hpi	7452	0.9	0.8	0.3	0.9	0.8	0.9	0.5	0.7	0.9	1.0	1.0	1.1	1.0

ratio: > 20 | 10 to 20 | 5 to 10 | 2.5 to 5 | 1.5 to 2.5 | 0.7 to 1.5 | 0.4 to 0.7 | 0.2 to 0.4 | < 0.2

FIGURE 2 | Mean values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and [Col/*mutant*]$_{Psm}$ ratios over groups of differently categorized genes. The numerical values are embedded in a heat map, and the legend on the right hand side depicts the value range assigned to each color of the heat map. The top row indicates the origin of the microarray data (**Table 6**). The selection criteria for the categorization of genes are detailed in the main text. **(A)** SAR$^+$ genes, SAR$^-$ genes, and remaining ATH1 genes. Gene probes not unequivocally assignable to a single gene ("s_at"-probes) were removed so that the number of genes was slightly reduced in each group (**Tables 1C**,**D**). **(B)** SA-dependent (cluster I), SA-independent (cluster II), and remaining (cluster III) SAR$^+$ genes. **(C)** JA-activated, JA-repressed, and JA-independent SAR$^-$ genes. **(D)** Comparison of expression characteristics of SAR$^+$ genes (row 1) and SAR$^-$ genes (row 3) with locally up-regulated genes ($[S/M]_{Psm} > 3$, row 2) and locally down-regulated genes ($[S/M]_{Psm} < 0.5$, row 4).

using the "merge" macro of the FIRe program (Garcion et al., 2006). This yielded an Excel® table in which the gene expression information from all the experiments listed in **Table 6** for the ATH1 genes was brought together. Some Affymetrix probes (labeled "s_at") hybridize to two or more related genes (Redman et al., 2004). These non-gene specific gene probes had been eliminated in some of the public microarray data used, and we consequently also deleted them from our merged Excel® list. The genes from our list were then grouped into different categories according to evaluation criteria outlined below (**Figure 2**), and excerpts of the Excel® data set relating to these categories are depicted in **Figures 3–7**. In addition to $[P/M]_{SAR}$ ratios, the normalized expression values of the genes depicted in **Figures 3–7** for each of the three replicate SAR experiments is provided in an accompanying Excel® data file (Supplemental material).

Before considering above-mentioned gene categories, however, we discuss expression information of a specific example, the classical SAR marker gene *PR1* (**Figure 3**, top row), to illustrate the gene regulatory information that we have drawn from the merged data set. *PR1* is the most prominently up-regulated SAR$^+$ gene ($[P/M]_{SAR} = 160.2$), and its expression is, as reported in previous studies (Delaney et al., 1995; Lawton et al., 1996), enhanced by exogenous BTH ($[S/M]_{BTH} = 46.1$) and SA ($[S/M]_{SA} = 5.6$) (**Figure 3**). Local inoculation with *Psm* also increases *PR1* expression at 24 hpi, leading to an ($[S/M]_{Psm}$ ratio of 5.5 in the "*Psm*" experiment. The $[Col/sid2]_{Psm}$ (5.3) and the $[Col/npr1]_{Psm}$ (5.6) ratios reveal that *PR1* expression upon *Psm*-inoculation in the two mutants does not exceed *PR1* expression in Col-0 mock-control plants, indicating that *P. syringae*-induced *PR1* expression fully depends on ICS1-mediated SA biosynthesis

gene locus	gene name	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/*sid2*]	*Psm* [Col/*npr1*]	SA [S/M]	*Psm* [Col/*pad4*]	*Psm* [Col/*ein2*]	*Psm* [Col/*coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]	gene description
At2g14610	*PR1*	160.2	46.1	5.5	5.3	5.6	5.6	2.3	0.8	1.0	1.2	0.9	1.1	1.1	PATHOGENESIS-RELATED GENE 1
At1g02450	*NIMIN-1*	133.9	26.6	2.0	25.0	18.0	32.9	2.0	0.6	0.5	0.4	0.7	1.1	1.3	NIM1-INTERACTING 1
At3g11340	*UGT76B1*	92.6	21.8	15.1	16.4	6.6	80.4	2.3	1.3	2.0	2.6	1.0	0.8	7.3	UDP-glycosyltransferase, conjugates isoleucic acid
At5g22570	*WRKY38*	63.1	29.0	4.2	43.7	85.2	50.6	0.8	0.4	1.8	1.7	0.9	0.7	2.2	WRKY transcription factor
At2g26400	*ARD3*	48.6	41.0	19.4	180.8	110.8	15.7	7.1	1.0	0.9	0.8	1.2	0.7	0.9	acireductone dioxygenase (ARD) family
At1g03850	*GRXS13*	40.1	32.2	7.5	6.7	6.7	30.7	6.6	0.9	0.6	0.9	1.9	0.5	1.1	GLUTAREDOXIN 13
At2g04450	*NUDX6*	38.7	26.8	5.5	3.8	3.4	2.6	5.3	0.5	0.6	0.6	2.2	0.7	0.9	NUDIX HYDROLASE HOMOLOG 6, NADH pyrophosphatase activity
At4g23810	*WRKY53*	26.8	18.2	3.3	4.3	3.2	2.9	1.9	0.5	0.4	0.5	2.2	0.7	4.5	WRKY transcription factor
At4g35180	*LHT7*	23.6	41.1	11.4	30.8	7.2	17.3	2.0	0.7	1.3	1.7	5.0	1.5	20.0	lysine/histidine-like transporter 7
At3g28510	-	17.9	133.3	9.6	52.1	40.2	11.4	9.6	1.1	0.8	0.2	1.2	0.2	1.5	P-loop containing nucleoside triphosphate hydrolase
At2g47130	*SDR3*	9.6	6.9	3.6	3.5	3.4	3.1	3.2	1.0	0.9	0.7	1.2	0.5	2.0	short chain dehydrogenase/reductase
At3g56400	*WRKY70*	9.1	2.8	3.4	3.9	2.1	3.3	2.0	1.0	1.0	0.9	2.8	0.8	0.9	WRKY transcription factor
At4g39830	-	4.5	22.5	5.7	5.2	7.0	2.9	5.1	1.0	0.6	1.0	1.4	0.7	6.6	cupredoxin superfamily protein, putative L-ascorbate oxidase
At1g11310	*MLO2*	4.5	1.8	3.7	4.5	3.6	3.1	3.6	1.0	0.9	0.8	2.8	0.7	2.0	MILDEW RESISTANCE LOCUS O 2/ POWDERY MILDEW RESISTANT 2
At3g53150	*UGT73D1*	3.7	12.2	9.6	26.3	11.4	3.0	10.5	0.8	0.7	0.8	1.1	1.2	12.0	UDP-glycosyltransferase
At2g19190	*FRK1*	3.5	21.7	9.8	20.0	23.4	3.0	4.9	0.9	0.5	0.2	0.6	2.2	92.2	FLG22-INDUCED RECEPTOR-LIKE KINASE 1
At1g64280	*NPR1*	2.6	2.2	2.2	2.2	1.9	1.8	1.7	1.2	1.0	0.9	1.8	0.8	1.3	NONEXPRESSER OF PR GENES 1, SA signal transduction, SA receptor

FIGURE 3 | Values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for individual genes of the group of SA-dependent (cluster I) SAR$^+$ genes (Figure 2B). The data for the *NPR1* gene is also included into the list. The gene names of genes indispensable for SAR are depicted in red. The legend for the heat map representation is depicted in **Figure 2**.

and on NPR1-mediated downstream signaling. Thus, exogenous SA is sufficient and endogenous SA accumulation following bacterial inoculation is necessary to induce *PR1* expression. By contrast, induction of *PR1* expression is independent of JA signaling, because exogenous MeJA ($[S/M]_{JA} = 1.2$) does not elevate *PR1* levels, and the $[Col/coi1]_{Psm}$ ratio equals 1.0, indicating identical *P. syringae*-induced expression of the gene in the Col-0 wild-type and in JA-insensitive *coi1*. Further, the $[Col/pad4]_{Psm}$ ratio equals 2.3, indicating an attenuated but not a fully compromised *Psm*-induced expression of *PR1* in *pad4* and thus a partial PAD4 dependency (**Figure 3**). Finally, *PR1* expression occurs virtually independently of ET signaling ($[Col/ein2]_{Psm} = 0.8$) and is not stimulated by exogenous H_2O_2 ($[S/M]_{H2O2} = 0.9$), ABA ($[S/M]_{ABA} = 1.1$), or flg22 ($[S/M]_{flg22} = 1.1$) (**Figure 3**).

To draw information about regulatory principles of gene expression in the clusters of SAR up-regulated, SAR down-regulated, and remaining ATH1 genes, we first determined the mean values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for all the genes from each category. Compared with the rest of the ATH1 genes, the SAR$^+$ genes exhibited, in addition to a strong average expression in 2° leaves of SAR-induced plants (mean $[P/M]_{SAR} = 10.5$), a marked average up-regulation in leaves of BTH-treated plants and in *Psm*-inoculated leaves (**Figure 2A**). To a lesser extent, the average expression of these genes was stimulated by the SA pathway, H_2O_2, and flg22, and positively influenced by functional *PAD4*. Moreover, a small average inducing stimulus of ABA on the expression of SAR$^+$ genes was obvious, and the JA- and ET- pathways had virtually no influence on the average SAR$^+$ gene expression patterns. By contrast, the genes down-regulated in SAR (mean $[P/M]_{SAR} = 0.4$) exhibited a completely different regulatory pattern. On average, these genes were markedly down-regulated by BTH and PAD4, and to lesser extent, by SA signaling, ET-signaling, and flg22-treatment. Remarkably, the average expression of the SAR$^-$ genes was strongly stimulated by JA signaling ($[Col/coi1]_{Psm} = 5.6$) (**Figure 2A**).

The positive effect of BTH, SA signaling, and PAD4 on the average expression of SAR$^+$ genes is consistent with the facts that BTH induces plant resistance patterns similar to SAR (Lawton et al., 1996), that SA is a central signal for SAR (Wildermuth et al., 2001; Mishina and Zeier, 2006), and that the PAD4 defense regulator is required for SAR establishment (Mishina and Zeier, 2006; Jing et al., 2011). One of the hallmarks of SAR is systemic SA accumulation at the onset of SAR (Métraux et al., 1990; Shulaev et al., 1995; Attaran et al., 2009), and increased levels of SA in 2° leaves upon SAR induction is closely associated with increased expression of SAR-related genes (Shulaev et al., 1995; Mishina and Zeier, 2006). We therefore determined whether all or only a sub-fraction of the SAR$^+$ genes are indeed up-regulated by SA. To categorize SA-regulated genes, we aimed at selecting only

gene locus	gene name	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/ *sid2*]	*Psm* [Col/ *npr1*]	SA [S/M]	*Psm* [Col/ *pad4*]	*Psm* [Col/ *ein2*]	*Psm* [Col/ *coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]	gene description
At3g22600	-	89.1	12.8	8.8	1.2	1.5	1.2	2.6	1.1	1.0	0.7	0.8	3.0	2.4	lipid-transfer protein/seed storage 2S albumin superfamily protein
At2g43570	*CHI*	42.5	9.1	8.1	1.2	1.5	1.1	2.5	0.9	1.0	1.2	0.8	22.3	2.8	putative chitinase
At5g13320	*PBS3*	38.0	21.9	40.7	1.4	1,6	1.4	6.2	1.1	1.3	1.8	0.9	3.2	7.4	GH3 family protein, p-hydroxybenzoyl amino acid synthetase activity
At1g57630	-	35.1	36.9	25.1	1.2	1.4	2.0	4.9	1.4	1.3	0.9	1.7	3.3	10.8	Toll-Interleukin-Resistance (TIR) domain family protein
At5g52640	*HSP90-1*	29.8	2.7	7.9	1.2	1.6	1.2	1.9	1.3	1.2	0.5	11.9	0.8	3.1	HEAT SHOCK PROTEIN 90-1
At2g29460	*GST22*	27.4	14.2	38.8	0.8	0.8	3.0	1.4	2.8	6.4	9.2	3.6	12.9	6.3	GLUTATHIONE S-TRANSFERASE 22
At5g39670	-	21.1	22.6	11.8	1.1	1.3	1.3	5.8	1.0	1.0	0.7	6.3	0.5	1.8	Calcium-binding EF-hand family protein
At5g20230	*BCB*	20.7	13.2	5.7	0.8	1.3	1.6	1.4	0.8	1.0	1.3	13.3	2.4	1.7	BLUE-COPPER-BINDING PROTEIN, SENESCENCE ASSOCIATED GENE 14
At3g26830	*PAD3*	20.0	16.2	58.0	0.7	0.9	4.1	2.4	1.9	4.2	1.9	3.4	0.7	1.8	cytochrome P450 CYP71B15, camalexin biosynthesis
At4g37370	*CYP81D8*	15.7	5.4	12.0	1.4	2.0	7.9	3.3	1.2	1.0	0.3	28.2	1.7	2.5	cytochrome P450 family protein
At3g12580	*HSP70*	12.5	2.2	4.8	1.0	1.2	1.0	1.3	1.3	1.4	1.4	14.3	6.1	2.3	HEAT SHOCK PROTEIN 70/ HEAT SHOCK COGNATE 70-4 (HSC70-4)
At1g19250	*FMO1*	12.0	3.5	81.8	0.9	1.2	1.4	5.5	3.0	2.7	1.2	0.6	0.9	1.9	FLAVIN-DEPENDENT MONOOXYGENASE 1
At1g15520	*PDR12*	10.3	2.8	77.6	1.3	1.6	2.8	6.4	3.4	3.6	1.6	2.2	1.0	1.9	ATP binding cassette transporter ABCG40, abscisic acid transport
At1g74710	*ICS1*	9.8	9.9	13.1	-	1.0	1.0	5.4	0.9	0.9	0.7	1.1	0.9	1.6	ISOCHORISMATE SYNTHASE 1, salicylic acid biosynthesis
At2g38470	*WRKY33*	9.3	5.6	7.9	1.3	1.4	2.0	2.9	1.1	1.0	0.9	13.6	1.0	2.8	WRKY transcription factor, regulates camalexin biosynthesis
At5g59820	*ZAT12*	6.6	6.7	15.6	1.4	1.9	1.4	2.7	1.5	1.6	1.1	5.1	2.0	4.6	putative zinc finger transcription factor, RESPONSIVE TO HIGH LIGHT 41
At2g29350	*SAG13*	5.5	6.8	19.9	0.9	1.0	0.8	2.3	1.3	1.7	1.1	1.4	17.1	1.9	senescence-associated gene SAG13, short-chain alcohol dehydrogenase
At2g13810	*ALD1*	4.8	6.0	57.1	1.3	1.3	1.6	8.6	1.1	1.8	0.6	1.0	1.0	1.9	AGD2-like defense response protein 1, lysine aminotransferase, pipecolic acid biosynthesis
At2g30770	*CYP71A13*	4.6	13.3	107.3	0.9	1.2	1.6	3.9	2.7	4.2	1.8	0.9	1.0	0.9	cytochrome P450, indoleacetaldoxime dehydratase, camalexin biosynhtesis
At3g28930	*AIG2*	3.6	3.2	8.1	1.0	0.7	1.0	1.6	3.0	7.4	2.1	3.8	0.6	0.9	AvrRpt2-induced gene

FIGURE 4 | Values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for individual genes of the group of SA-independent (cluster II) SAR$^+$ genes (Figure 2B). The gene names of genes indispensable for SAR are depicted in red. The legend for the heat map representation is depicted in **Figure 2**.

those genes whose induced local expression upon *Psm*-treatment was severely compromised in *sid2*. We therefore had to consider genes with increased $[Col/sid2]_{Psm}$ ratios and first selected genes with $[Col/sid2]_{Psm}$ ratios > 2. However, this criterion alone was not sufficient for selection because the genes strongly varied in their $[S/M]_{Psm}$-ratios and thus their *Psm*-responsiveness. For instance, genes strongly *Psm*-up-regulated in Col-0 still exhibit considerable *Psm*-induced up-regulation in *sid2* with the criterion $[Col/sid2]_{Psm}$ ratios > 2 and thus are only weakly SA dependent. We consequently coupled the $[Col/sid2]_{Psm}$ ratio to the degree of *Psm*-responsiveness and defined that only genes with quotients of $[Col/sid2]_{Psm}$ / $[S/M]_{Psm}$ > 0.67 were taken. The combination of these two selection criteria provided a set of 100 genes out of 541 SAR$^+$ genes whose expression was locally *Psm*-inducible in a modest to strong manner and whose *Psm*-induced up-regulation was largely dependent on *SID2/ICS1* and thus on endogenous SA. Moreover, most of these genes were also up-regulated by exogenous SA (**Figures 2B**, **3**). To categorize SAR$^+$ genes independently expressed from SA, we assembled all the genes with a low $[Col/sid2]_{Psm}$ ratio ($[Col/sid2]_{Psm}$ < 1.5) that were up-regulated upon *Psm* inoculation by at least a factor of 3. This selection yielded 156 SA-independent SAR$^+$ genes (**Figures 2B**, **4**). The remaining SAR$^+$ genes (268) were grouped into a third category that mainly consisted of genes partly requiring *SID2/ICS1* for *Psm*-induced expression (partly SA-dependent genes), or of genes not locally up-regulated by *Psm* at 24 hpi (**Figures 2B**, **5**). Therefore, SAR$^+$ genes were categorized into three groups according to their SA-dependent expression: strictly SA-dependent genes (cluster I), SA-independent genes (cluster II), and cluster III genes predominantly consisting of genes with partial SA-dependency. Irrespective of their SA responsiveness, the vast majority of SAR$^+$ genes were also up-regulated by exogenous BTH, indicating that the action of the so-called "SA analogue" BTH on gene transcription is significantly broader than the action of SA itself (**Figures 2B**, **3**, **4**, **5**). Similarly, genes down-regulated during biological SAR were generally down-regulated by exogenous BTH (**Figures 2**, **6**). These tendencies indicate extensive overlap between the biologically-induced SAR state and the state of enhanced disease resistance after BTH application. Nevertheless, some differences between biological SAR and BTH-treatment existed for the transcription levels of individual genes not affected in biological SAR (**Figure 2A**, "rest"). Hereunder, 1.8 % of genes were positively ($[S/M]_{BTH}$ > 3) and 15.8 % negatively ($[S/M]_{BTH}$ < 2) regulated by BTH (specific examples are *MAPKKK19*, *CYP94C1*, *UGT76E1*, *ACS2*, *DXL1*, *PR3*; **Figure 7**).

gene locus	gene name	SAR [P/M]	BTH [S/M]	Psm [S/M]	Psm [Col/sid2]	Psm [Col/npr1]	SA [S/M]	Psm [Col/pad4]	Psm [Col/ein2]	Psm [Col/coi1]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]	gene description
At1g35230	*AGP5*	106.0	16.5	8.1	3.8	4.3	2.4	7.7	0.9	0.7	0.5	1.2	2.7	1.4	arabinogalactan protein 5
At3g01830	-	66.3	58.2	28.7	1.9	2.1	3.8	4.6	1.0	1.5	0.1	11.9	1.8	14.9	Calcium-binding EF-hand family protein
At3g57260	*PR2*	46.1	13.7	1.0	1.1	0.9	1.1	0.6	0.3	1.3	1.5	1.5	1.0	1.2	PATHOGENESIS-RELATED GENE 2, β-1,3-glucanase
At4g11890	*ARCK1*	40.4	13.6	4.6	1.9	2.0	2.8	3.5	1.0	0.8	0.5	2.0	1.6	7.5	protein kinase superfamily protein
At5g22570	*PR5*	38.4	18.8	1.7	1.1	1.5	1.4	3.3	1.4	1.5	1.1	1.1	1.1	0.8	PATHOGENESIS-RELATED GENE 5, osmotin / thaumatin-like protein
At4g00700	-	29.8	25.2	8.9	3.1	3.2	1.6	8.5	0.9	0.9	0.7	0.7	1.4	1.1	C2 calcium/lipid-binding plant phosphoribosyltransferase family protein
At2g46400	*WRKY46*	17.4	11.3	5.4	1.7	2.0	3.5	3.5	0.7	0.7	0.8	1.9	0.9	2.6	WRKY transcription factor
At1g80840	*WRKY40*	15.6	11.4	17.5	1.7	1.4	6.3	2.3	1.4	1.8	4.2	22.4	1.4	3.6	WRKY transcription factor
At3g52430	*PAD4*	13.5	10.3	11.1	2.2	2.2	2.5	-	1.1	1.0	0.9	1.4	0.4	1.1	PHYTOALEXIN-DEFICIENT 4, α/β-hydrolases superfamily protein
At1g28480	*GRX480*	12.7	19.4	19.5	2.5	1.3	10.4	1.6	1.2	14.0	3.2	1.8	0.6	2.3	glutaredoxin family protein
At3g48090	*EDS1*	11.1	7.1	2.1	1.5	1.5	2.5	2.7	0.7	0.6	0.5	1.3	0.9	1.4	ENHANCED DISEASE SUSCEPTIBILITY 1, α/β-hydrolases superfamily protein
At4g31800	*WRKY18*	10.7	23.7	7.6	3.5	2.0	9.5	1.5	0.8	1.1	2.8	3.8	0.6	2.2	WRKY transcription factor
At5g60900	*RLK1*	7.9	3.4	1.8	1.8	1.3	2.7	1.4	0.9	1.0	1.3	1.0	1.3	1.2	receptor-like protein kinase 1
At5g26920	*CBP60g*	6.5	9.3	17.0	2.1	2.6	1.5	11.7	1.0	0.7	1.4	1.7	0.7	17.0	calmodulin binding protein 60-like.g
At3g11820	*PEN1*	6.4	3.9	2.7	1.9	1.9	1.9	2.8	1.0	0.9	0.7	6.9	0.9	3.1	PENETRATION1, syntaxin of plants 121 (SYP121)
At3g52400	*SYP122*	5.3	8.5	8.2	1.7	2.9	1.5	6.1	1.3	0.9	1.2	1.4	0.9	5.5	syntaxin of plants 122
At5g45110	*NPR3*	4.6	3.8	4.8	2.6	1.7	6.1	3.1	0.9	0.9	1.3	5.2	0.6	2.0	NPR1-like protein 3, SA receptor
At5g14930	*SAG101*	4.0	3.3	5.0	1.6	1.9	1.5	4.3	0.9	0.9	0.7	1.1	0.6	2.0	senescence-associated gene 101, acyl hydrolase
At3g45640	*MPK3*	3.5	2.5	2.1	1.3	1.3	1.3	3.0	1.0	0.9	1.0	4.5	0.2	1.5	mitogen-activated protein kinase 3
At1g73805	*SARD1*	3.0	9.3	7.6	3.5	2.5	4.8	5.5	1.1	0.9	0.6	1.1	0.7	1.1	calmodulin-binding protein-like

FIGURE 5 | Values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for individual genes of the group of partially SA-dependent (cluster III) SAR^+ genes (Figure 2B). The gene names of genes indispensable for SAR are depicted in red. The legend for the heat map representation is depicted in **Figure 2**.

On average, the $[Col/sid2]_{Psm}$ ratios for the SA-dependent SAR^+ genes, the SA-independent SAR^+ genes, and the remaining genes amounted to 7.9, 1.1, and 1.6, respectively, reflecting strong, virtually absent, and moderate SA regulation of the respective genes (**Figure 2B**). The $[Col/npr1]_{Psm}$ ratios (6.4, 1.4, 1.7) paralleled the $[Col/sid2]_{Psm}$ ratios, confirming above-mentioned influences of the SA pathway on the regulation of the different gene groups (**Figure 2B**). The $[S/M]_{SA}$ values exhibited a similar tendency as well (4.9 for SA-dependent, 2.1 for SA-independent, and 1.8. for remaining genes), although some genes of the SA-independent gene cluster showed a moderate responsiveness to exogenous SA (**Figure 2B**). *PAD3* is a typical example of a gene grouped into the SA-independent gene cluster which responded to exogenous SA (**Figure 4**). We reasoned, however, that in such cases, a $[Col/sid2]_{Psm}$ ratio close to 1 would provide a more meaningful criterion for SA-independency than an elevated $[S/M]_{SA}$ value, because the $[Col/sid2]_{Psm}$ ratio results from physiological differences in SA rather than from artificial SA differences caused by exogenous treatment. In general, however, the $[Col/sid2]_{Psm}$ values paralleled the $[S/M]_{SA}$ values remarkably well: SA-dependent genes generally exhibited high values for both parameters (**Figure 3**), and the majority of SA-independent genes, as exemplified by *CHI*, *FMO1*, and *SAG13*, showed both $[Col/sid2]_{Psm}$ and $[S/M]_{SA}$ values close to 1 (**Figure 4**).

Noticeably, the average $[P/M]_{SAR}$ ratios and the $[S/M]_{flg22}$ ratios were higher for the SA-dependent than for the SA-independent SAR^+ genes, indicating a comparable high degree of up-regulation of SA-regulated genes upon both SAR induction and flg22-treatment (**Figure 2B**). When examining the expression patterns of individual SA-dependent SAR^+ genes, it became apparent that several genes such as *FRK*, *LHT7*, or *UGT73D1* are strongly flg22-responsive whereas others are not at all (**Figure 3**). In addition, the average induction of SA-independent SAR^+ genes was higher than the induction of SA-dependent SAR^+ genes in *Psm*-inoculated tissue, in H_2O_2-treated tissue, and in ABA-treated tissue (**Figure 2B**), indicating a more prominent stimulatory capacity of H_2O_2- and ABA-signaling on the expression of individual members of the SA-independent compared with the SA-dependent gene cluster (**Figures 2B**, **3**, **4**).

SAR^+ GENES EXHIBITING TIGHT SA REGULATION (SAR^+ GENE CLUSTER I)

Representative examples of SAR^+ genes tightly regulated by SA are *PR1*, the classical marker gene for SA-dependent defense gene activation (Nawrath and Métraux, 1999), *NIMIN-1*, *UGT76B1*, *WRKY38*, *GRXS13*, *NUDX6*, *SDR3*, *WRKY70*, and *MLO2* (**Figure 3**). *NPR1*, encoding a critical regulator of SAR that functions in SA perception and transcriptional activation of

gene locus	gene name	SAR [P/M]	BTH [S/M]	Psm [S/M]	Psm [Col/sid2]	Psm [Col/npr1]	SA [S/M]	Psm [Col/pad4]	Psm [Col/ein2]	Psm [Col/coi1]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]	gene description
At3g55970	*JRG21*	0.4	0.2	56.0	1.0	1.1	0.8	1.1	1.5	760.4	32.5	1.6	0.8	3.9	JASMONATE-REGULATED GENE 21, 2-oxoglutarate-Fe(II) oxygenase
At5g05600	-	0.4	1.1	14.5	0.8	0.9	1.0	0.9	1.6	464.6	17.3	1.1	1.0	1.5	2-oxoglutarate-Fe(II) oxygenase
At3g11480	*BSMT1*	0.4	0.7	293.2	0.8	0.6	0.7	0.6	3.1	448.4	7.4	0.5	1.4	2.0	benzoic acid/salicylic acid methyl transferase
At2g39030	*NATA1*	0.4	0.4	6.4	0.6	0.7	0.4	0.5	1.1	407.1	48.1	0.9	6.1	1.1	ornithine N-delta-acetyltransferase
At1g19670	*COR1*	0.4	0.3	0.3	0.6	0.2	0.9	0.1	0.5	303.3	7.6	1.0	0.8	0.7	CORONATINE-INDUCED PROTEIN 1, CHLOROPHYLLASE 1 (CLH1)
At1g17380	*JAZ5*	0.4	2.8	31.7	1.0	0.8	1.1	1.0	1.0	129.1	19.2	1.3	0.8	2.1	JASMONATE-ZIM-DOMAIN PROTEIN 5
At3g28220	-	0.2	0.1	0.3	0.5	0.2	0.6	0.1	0.7	52.4	59.7	0.8	2.5	0.6	TRAF-like family protein
At1g52400	*BGL1*	0.4	0.3	0.3	0.6	0.2	0.9	0.1	0.5	46.4	7.8	1.0	1.0	0.7	glycosyl hydrolase family 1 protein
At1g44350	*ILL6*	0.4	0.5	10.2	0.8	0.7	1.3	0.7	1.1	45.8	10.1	0.9	0.9	1.4	IAA-LEUCINE RESISTANT-LIKE GENE 6, similar to IAA amino acid conjugate hydrolase
At4g23600	*JR2*	0.3	0.1	2.6	0.7	0.6	0.8	0.4	0.9	45.4	4.4	1.1	2.0	0.8	JASMONIC ACID RESPONSIVE 2, CORONATINE INDUCED 1 (CORI3), cystine lyase
At2g43530	-	0.3	0.4	1.0	0.5	0.4	0.7	0.2	0.5	44.4	8.5	0.8	1.0	0.9	defensin-like (DEFL) family protein
At1g52000	-	0.2	0.2	10.2	0.5	0.2	0.5	0.1	0.5	44.3	35.4	0.9	1.4	0.7	mannose-binding lectin superfamily protein
At3g16470	*JR1*	0.4	0.3	1.0	0.4	0.3	0.8	0.2	0.5	41.4	8.1	0.6	1.3	0.9	JASMONATE RESPONSIVE 1, JACALIN-RELATED LECTIN 35 (JAL35)
At5g47240	*NUDX8*	0.2	0.7	2.2	0.3	0.2	0.5	0.2	1.1	34.9	12.6	0.7	1.2	0.9	NUDIX HYDROLASE HOMOLOG 8
At4g15440	*CYP74B2*	0.3	0.4	2.2	0.9	0.5	0.4	0.3	0.8	33.7	17.0	1.1	1.7	0.7	cytochrome P450, hydroperoxide lyase (non-functional in ecotype Col-0)
At1g54020	-	0.5	0.1	8.5	0.3	0.2	0.7	0.1	2.7	28.6	45.0	1.3	1.0	0.9	GDSL-like lipase/acylhydrolase superfamily protein
At1g70700	*JAZ9*	0.3	0.8	4.9	0.7	0.7	0.8	0.7	1.2	28.0	26.3	1.0	1.0	0.9	JASMONATE-ZIM-DOMAIN PROTEIN 5
At5g06870	*PGIP2*	0.4	0.3	2.4	0.6	0.6	1.2	0.6	0.9	26.2	17.0	1.8	1.0	0.9	POLYGALACTURONASE INHIBITING PROTEIN 2
At1g56650	*MYB75*	0.2	0.2	4.1	0.3	0.3	0.3	0.2	1.2	25.0	5.6	0.8	4.3	0.5	MYB domain containing transcription factor, regulates anthocyanin biosynthesis
At5g42650	*AOS*	0.4	0.5	1.3	0.6	0.5	0.8	0.3	0.8	18.0	13.5	1.8	0.8	1.2	ALLENE OXIDE SYNTHASE, CYP74A, DELAYED DEHISCENCE 2, JA biosynthesis
At2g34930	-	0.4	0.2	1.0	0.6	0.4	1.3	0.2	0.6	16.1	4.5	2.6	1.3	0.9	disease resistance family protein / leucine-rich repeat domain
At3g45140	*LOX2*	0.5	0.4	2.2	1.0	0.9	0.9	0.7	0.7	15.7	1.8	1.1	0.9	0.9	LIPOXYGENASE 2, wound-induced JA biosynthesis
At2g43550	-	0.2	0.1	0.2	0.4	0.2	0.9	0.04	0.5	13.6	7.2	0.6	0.9	0.7	defensin-like (DEFL) family protein
At5g44420	*PDF1.2*	0.04	0.1	0.1	0.02	0.1	0.4	0.1	1.6	11.5	1.1	0.4	0.7	0.8	PLANT DEFENSIN 1.2
At5g60890	*MYB34*	0.3	0.03	0.4	0.4	0.2	0.5	0.1	0.4	7.4	6.3	0.4	2.0	0.6	MYB domain cont. transcription factor, ALTERED TRYPTOPHAN REGULATION 1

FIGURE 6 | Values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for individual genes of the group of JA-activated SAR$^-$ genes (Figure 2C). The legend for the heat map representation is depicted in **Figure 2**.

downstream genes, is only moderately up-regulated in 1° and 2° leaf tissue upon inoculation ($[P/M]_{SAR} = 2.6$) and thus not assigned to the group of SAR$^+$ genes. Nevertheless, its modest local up-regulation is also SA-dependent (**Figure 3**).

Increased expression of *PR1* upon SAR induction might directly contribute to resistance execution following fungal and oomycete pathogen attack, because PR1 proteins isolated from tobacco and tomato possess *in vitro* antifungal activity (Niderman et al., 1995). Moreover, overexpression of *PR1* in tobacco increases resistance to infection by the oomycetes *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* but has no protective effect on tobacco mosaic virus or *P. syringae* infection (Linthorst et al., 1989; Alexander et al., 1993; Rayapuram et al., 2008). Other SA-dependent SAR$^+$ genes have a proven role in the activation of SA-associated defense responses that confers resistance to (hemi)biotrophic pathogens. For instance, the transcription factor WRKY70 has been recognized as a regulatory node that positively regulates SA-related plant defenses and suppresses JA-mediated responses. Overexpression of *WRKY70* increases basal resistance to *P. syringae* and to the powdery mildew *Erysiphe cichoracearum*, and results in the constitutive expression of SAR-related genes such as *PR1*, *PR2*, and *PR5*. Conversely, antisense suppression of *WRKY70* or insertional inactivation leads to enhanced expression of JA-responsive genes and compromises *E. cichoracearum* resistance (Li et al., 2004, 2006). In addition, *WRKY70* acts in concert with *WRKY53* and *WRK46*, two other SAR$^+$ genes that belong to the SA-dependent and the partial SA-dependent gene cluster, respectively (**Figures 3**, **5**). This is reflected by the finding that *wrky46/53/70* triple but not *wrky70* single mutants exhibit attenuated basal resistance towards *P. syringae* (Hu et al., 2012). Another SA-regulated SAR component that positive regulates *PR1* expression and is required for full basal resistance to *P. syringae* is the short chain dehydrogenase/reductase SDR3. The metabolic function of SDR3 has not been elucidated yet (Hwang et al., 2012). Moreover, NUDX6, a member of the Nudix (nucleoside diphosphate linked to moiety X)

gene locus	gene name	SAR [P/M]	BTH [S/M]	*Psm* [S/M]	*Psm* [Col/ *sid2*]	*Psm* [Col/ *npr1*]	SA [S/M]	*Psm* [Col/ *pad4*]	*Psm* [Col/ *ein2*]	*Psm* [Col/ *coi1*]	MeJA [S/M]	H_2O_2 [S/M]	ABA [S/M]	flg22 [S/M]	gene description
At3g46660	*UGT76E12*	1.0	0.7	1216.7	0.5	0.6	2.7	1.1	4.4	118.9	5.0	10.2	8.9	5.2	UDP-glucosyl transferase 76E12
At3g11480	*BSMT1*	0.4	0.7	293.2	0.8	0.6	0.7	0.6	3.1	448.4	7.4	0.5	1.4	2.0	benzoic acid/salicylic acid methyl transferase
At5g67080	*MAPKKK19*	1.0	6.1	195.6	0.8	0.9	1.5	3.1	7.0	256.1	17.8	5.2	2.0	3.2	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 19
At1g01480	*ACS2*	1.1	0.1	167.2	1.0	1.2	1.1	3.3	0.9	2.4	0.3	3.0	2.3	8.1	1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2, ethylene biosynthesis
At3g21500	*DXL1*	1.0	0.1	166.7	0.4	0.3	0.9	3.2	6.9	232.7	1.3	1.0	0.5	1.4	1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE-LIKE 1
At1g61120	*TPS4*	0.9	1.1	163.7	0.7	0.7	1.6	1.9	2.2	765.8	83.1	1.0	2.4	9.6	TERPENE SYNTHASE 04, geranyllinalool synthase
At3g01420	*DOX1*	1.1	0.3	130.1	1.0	2.7	1.1	7.6	5.0	3.2	1.1	0.8	7.0	3.4	ALPHA-DIOXYGENASE 1, fatty acid oxygenation
At1g30135	*JAZ8*	0.9	1.6	84.5	0.5	0.5	1.3	0.9	2.9	77.0	13.0	0.8	1.5	3.2	JASMONATE-ZIM-DOMAIN PROTEIN 8
At1g52890	*ANAC019*	0.7	0.5	50.7	0.9	1.0	0.5	1.2	3.4	6.9	16.0	1.0	210.7	5.2	NAC transcription factor family
At2g27690	*CYP94C1*	0.6	7.4	49.1	2.1	2.2	3.4	3.1	1.3	39.4	16.6	1.3	2.1	2.3	cytochrome P450 family protein; oxidation of 12-OH-JA-Ile to 12-COOH-JA-Ile
At2g34500	*CYP710A1*	1.1	1.0	44.0	0.9	1.3	5.3	3.7	2.5	2.8	1.0	2.0	2.8	1.0	cytochrome P450 family protein, desaturation of β-sitosterol to stigmasterol
At3g12500	*PR3*	1.0	0.1	38.3	1.5	0.8	1.5	6.5	2.9	3.9	1.4	0.9	3.1	0.5	PATHOGENESIS-RELATED GENE 3, basic chitinase, CHI-B
At1g05680	*UGT74E2*	1.3	1.2	32.6	0.6	0.6	3.1	1.4	2.3	1.9	1.6	8.6	5.0	1.3	UDP-glucosyltransferase 74E2, acts on indole-3-butyric acid
At3g25180	*CYP82G1*	0.6	1.0	27.4	0.4	0.5	0.9	3.2	2.3	342.4	2.1	1.3	1.3	8.6	cytochrome P450 family protein, biosynthesis of the homoterpene TMTT
At5g59580	*UGT76E1*	1.3	5.0	27.3	6.1	2.6	1.5	1.8	1.4	83.3	1.2	1.0	0.9	1.8	UDP-glucosyltransferase 76E1
At3g48520	*CYP94B3*	0.8	0.5	22.9	0.5	0.5	1.3	0.6	2.3	22.6	0.9	1.0	6.2	3.1	cytochrome P450 family protein; hydroxylation of JA-Ile to 12-OH-JA-Ile
At4g39950	*CYP79B2*	0.6	1.4	10.3	1.0	1.4	1.2	1.7	1.6	2.0	4.6	2.1	0.5	0.7	cytochrome P450 family protein; conversion of Trp to indole-3-acetaldoxime

FIGURE 7 | Values of $[P/M]_{SAR}$, $[S/M]_{stimulus}$, and $[Col/mutant]_{Psm}$ ratios for individual genes strongly up-regulated at inoculation sites (24 hpi) but not in distal tissue (48 hpi) (Figure 2D). The legend for the heat map representation is depicted in **Figure 2**.

hydrolase family that catalyze the hydrolysis of several nucleoside diphosphate derivatives, not only acts in NADH metabolism in response to SA but also positively regulate SA-related defense responses (Ishikawa et al., 2010). *MILDEW RESISTANCE LOCUS O 2* (*MLO2*) belongs to a plant-specific family of genes coding for membrane proteins that contain seven transmembrane domains. *MLO2* contributes to Arabidopsis resistance towards attack by the necrotrophic fungus *Botrytis cinerea* and participates together with other components to non-host resistance of Arabidopsis to the rice blast pathogen *Magnaporthe oryzae* (Humphry et al., 2010; Nakao et al., 2011). Interestingly, MLO2 has been recognized as target of the *P. syringae* type III effector HopZ2 which physically interacts with MLO2. A *mlo2* insertion line exhibits increased resistance to *P. syringae*, suggesting that the MLO2/HopZ2-interaction is required for HopZ2-associated virulence (Lewis et al., 2012).

NUDX6 and *GRXS13* are two examples of SA-dependent SAR^+ genes that appear to function in redox homeostasis during SAR. *GRXS13* codes for a plant glutaredoxin which facilitates infection of Arabidopsis by *B. cinerea* (La Camera et al., 2011). Moreover, *GRXS13* expression is critical to limit basal and photooxidative stress-induced ROS production (Laporte et al., 2012). A redox-related function might also exist for At4g39830 which encodes a putative ascorbate oxidase (Yamamoto et al., 2005). Somewhat surprisingly, several SA-regulated SAR^+ components obviously reduce SA accumulation and/or SA signaling and therefore appear to function in the containment of defense response activation during SAR establishment (**Figure 3**). For example, NIMIN-1 interacts with the SA receptor NPR1 in yeast-2-hybrid assays and functions as a negative regulator of SA-induced *PR1* expression (Weigel et al., 2005). The UDP-dependent glycosyltransferase UGT76B1 can glycosylate the Ile catabolite isoleucic acid and thereby negatively influences SA accumulation (von Saint Paul et al., 2011). Beyond that, UGT76B1 exhibits in vitro glycosylating activity towards SA, and conversion of free, signaling active SA to glycosylated derivatives is supposed to attenuate SA signaling (Noutoshi et al., 2012). Finally, the transcription factor WRKY38 negatively affects SA sensitivity and basal resistance to *P. syringae* (Kim et al., 2008).

Together, SA-dependent SAR^+ genes can have distinct roles in the activation of defenses and resistance execution against different pathogen types (e.g. *PR1*, *WRKY70*, *WRKY53*, *SDR3*, *NUDX6*, *MLO2*), down-regulation of the JA pathway (*WRKY70*), redox homeostasis (*GRXS13*, *NUDX6*), and containment of defense response activation after SAR establishment (*NIMIN-1*, *UGT76B1*, *WRKY38*) (**Figure 9**). The specific functions of several other SA-regulated SAR^+ genes still remain to be clarified.

SAR^+ GENES THAT CAN BE EXPRESSED INDEPENDENTLY OF SA INCLUDE CRITICAL SAR ACTIVATORS (SAR^+ GENE CLUSTER II)

The group of SA-independent SAR^+ genes contains at least three genes whose functions are necessary for SAR establishment: *ALD1*, *FMO1*, and *ICS1* (**Figures 4**, **9**). ALD1 encodes a Lys

aminotransferase (Song et al., 2004b) that mediates the biosynthesis of the Lys catabolite Pip, a critical SAR regulator (Návarová et al., 2012). Pip accumulation in 2° leaves of SAR-induced plants timely precedes SA accumulation, and Pip signaling requires the flavin-dependent monooxygenase FMO1 for SAR induction. The function of Pip as a metabolic amplifier of defense signaling is crucial for the ICS1-mediated accumulation of SA in 2° leaves (Mishina and Zeier, 2006; Návarová et al., 2012). Therefore, at the onset of SAR, SA-independent signaling events obviously trigger the expression of the key SAR^+ genes *ALD1*, *FMO1*, and *ICS1* that are required for systemic SA accumulation (**Figure 9**). *ICS1* can be regarded as a "bridge" between SA-independent and SA-dependent SAR signaling events because its up-regulation results in the de novo biosynthesis of SA (Wildermuth et al., 2001). It is important to note that a feedback amplification mechanism in 2° leaves that involves ALD1, Pip, FMO1, ICS1, SA, and NPR1 exists to ensure full SAR establishment (Návarová et al., 2012; Shah and Zeier, 2013). Therefore, in the context of SAR signaling and establishment in 2° leaf tissue, SA-independent and SA-dependent signaling process cannot be regarded as separately acting unities. For instance, although the SA-deficient *sid2* mutant is able to accumulate wild-type like Pip levels in 1° leaves upon *P. syringae* inoculation, it accumulates markedly reduced Pip levels in 2° leaves (Návarová et al., 2012). Thus, Pip accumulation does occur independently from the capacity of SA biosynthesis at inoculation sites, but SA synthesis is required within the above-mentioned amplification cycle for the full accumulation of Pip in systemic tissue (**Figure 9**). Similarly, the systemic up-regulation of SA-independent genes might be substantially reduced in *sid2*, as has previously been shown for *FMO1* (Mishina and Zeier, 2006). Thus, the classification of SAR^+ genes as "SA-independent" is based on expression characteristics in 1° leaves (**Table 6**) and does not consider the necessity of SA production for SAR-associated signal amplification in 2° leaves.

Another SAR^+ gene up-regulated at inoculation sites in an SA-independent fashion is *PBS3* (alias GH3.12, GDG1, and WIN3) (**Figure 4**). Null mutants of *pbs3* exhibit severe defects in the induction of local resistance to bacterial infection and are significantly but not fully compromised in SAR (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007). *PBS3* acts upstream of SA in the induction of immune responses and encodes a GH3 acyl adenylase that is able to conjugate 4-substituted benzoic acid derivatives to amino acids in vitro (Okrent et al., 2009). *HSP90-1* and *HSP70* are SA-independent SAR^+ genes strongly up-regulated by H_2O_2 (**Figure 4**). *HSP90-1* encodes a cytosolic isoform of the heat shock protein HSP90 that associates with the co-chaperones SGT1 and RAR1 to mediate the maturation of various nucleotide-binding domain and leucine-rich repeat containing (NLR)-type of resistance proteins. Gene knockouts of *RAR1*, *SGT1* or *HSP90* compromise resistance against various bacterial, fungal and viral pathogens (Kadota and Shirasu, 2012). Arabidopsis *SGT1a* but not *SGT1b* or *RAR1* falls into the category of (SA-dependent) SAR^+ genes (**Figure 9**). HSP70 (alias HSC70-4) represents one of four cytosolic HSC70 isoforms that, similarly to HSP90, interact with the co-chaperone SGT1. Knockout of individual cytosolic *HSC70* genes has no defense phenotype, but *HSC70-1* overexpression compromises resistance to virulent and avirulent pathogens (Noël et al., 2007).

A typical local response of Arabidopis leaves to infection with necrotrophic or (hemi)biotrophic pathogens is the accumulation of the indolic phytoalexin camalexin (Glawischnig, 2007). In *Psm*-inoculated plants, camalexin is heavily produced in locally infected tissue but the phytoalexin does not accumulate systemically in 2° leaves (**Figures 8A**,**B**). However, SAR-induced plants are conditioned to more vigorously synthesize camalexin in response to subsequent pathogen encounter, and this priming effect is mediated by Pip (Návarová et al., 2012). The induction of camalexin biosynthesis at pathogen inoculation sites is associated with a modest activation of genes coding for enzymes of the Trp biosynthetic pathway and strong induction of genes encoding cytochrome P450 monooxygenases involved in Trp catabolism towards camalexin: *CYP79B2/3*, *CYP71A13*, and *CYP71B15* (alias *PAD3*) (Ren et al., 2008). In 2° leaves of SAR-induced plants, *PAD3* expression is strongly and *CYP71A13* expression is moderately up-regulated, and both genes belong to the SA-independent cluster (**Figure 4**). By contrast, *Psm*-inoculation does only lead to a local but not a systemic up-regulation of *CYP79B2* (**Figure 7**), and *CYP79B3* is neither locally nor systemically up-regulated. This expression pattern is consistent with the observed lack of systemic camalexin accumulation because *CYP79B* expression and thus the metabolic step from Trp to indole-3-acetaldoxime (IAOx) are not activated in 2° leaves of SAR-induced plants. However, enhanced systemic expression of *CYP71A13* and *PAD3* pre-activates the pathway downstream of IAOx. This partial biosynthetic pathway activation can explain why camalexin accumulation is primed upon SAR induction and therefore can occur faster and more pronouncedly in challenged SAR-induced than in challenged control plants: key enzymes of the pathway are already expressed before the challenge inoculation takes place, and the ab initio expression of fewer components (for camalexin biosynthesis probably merely CYP79B2) is therefore required to provide the full enzymatic capacity for the biosynthesis of the metabolite (**Figure 9**). The WRK33 transcription factor controls the activation of camalexin biosynthetic genes and camalexin production, and WRKY33 activity is regulated via a MAP kinase cascade involving MPK3 and MPK6 (Mao et al., 2011). *WRKY33* and *MPK3* but not *MPK6* are up-regulated during SAR in a largely SA-independent manner (**Figures 4**, **5**).

As stated above, the average responsiveness to H_2O_2, ABA, and JA/ET is higher for SAR^+ genes assigned to the SA-independent cluster than for those assigned to the SA-dependent cluster (**Figure 2B**). This is based on the fact that individual SA-independent genes are strongly inducible by H_2O_2 (e.g. *CYP81D8*, *WRKY33*, *BCB*, *HSP70*, *HSP90-1*, *ZAT12*) or ABA (e.g. *CHI*, *SAG13*, *GST22*, *HSP70*), and that some genes are modestly inducible by JA/ET signaling (e.g. *AIG2*, *GST22*, *PDR12*, *FMO1*). Moreover, *PDR12* encodes an ATP-binding cassette (ABC) transporter that mediates cellular uptake of ABA (Kang et al., 2010), and *ZAT12* codes for a transcription factor implicated in ROS signaling (Davletova et al., 2005). This raises the question whether an early wave of ROS-, JA/ET-, and/or ABA-signaling would precede Pip- and SA-mediated establishment of SAR in 2° leaves (**Figure 9**). An earlier work on *P. syringae*-induced SAR

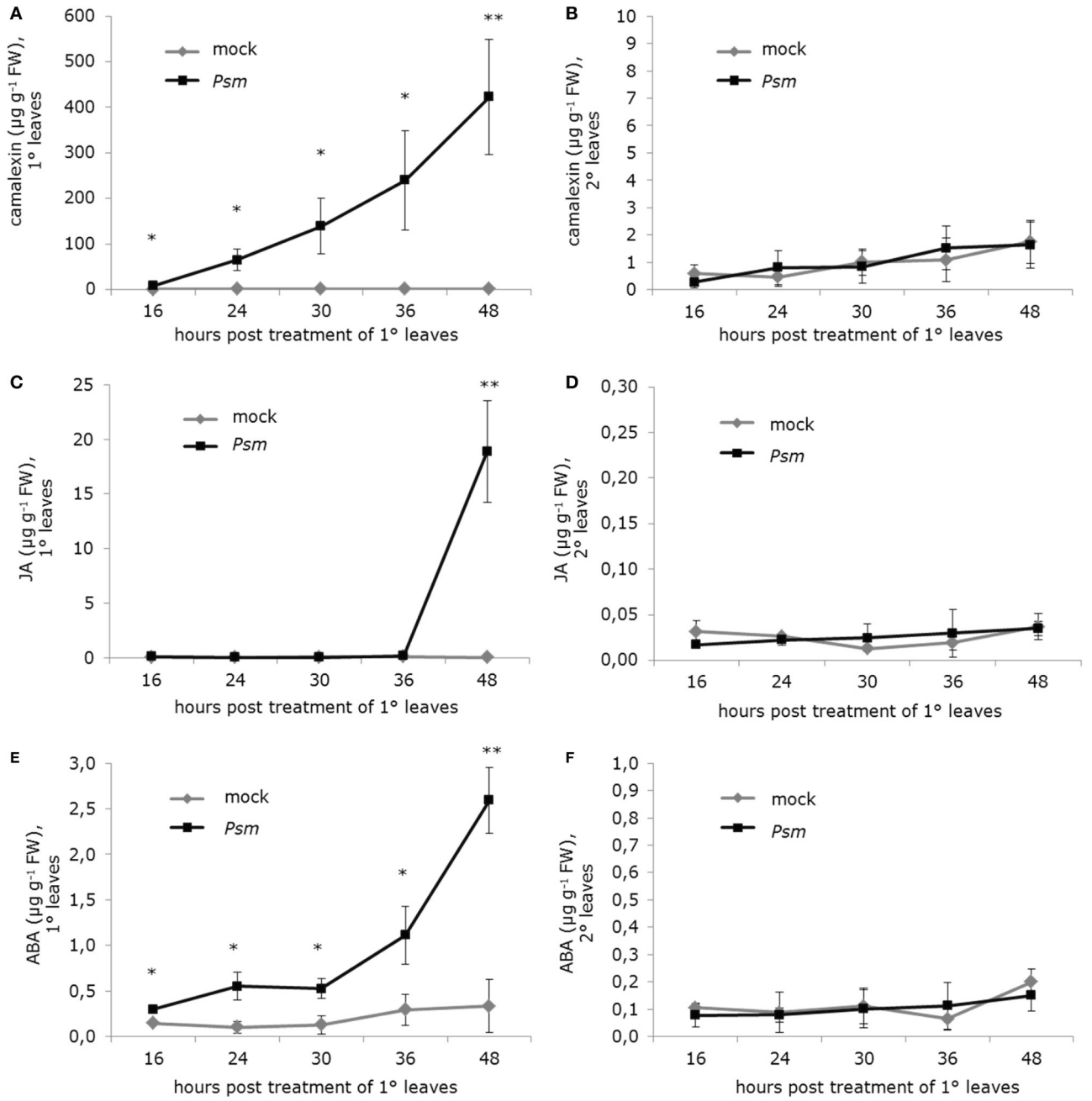


FIGURE 8 | Levels of camalexin, jasmonic acid (JA), and abscisic acid (ABA) in treated (1°) and non-treated distal (2°) leaves of Arabidopsis Col-0 plants inoculated with a suspension of *P. syringae* pv. *maculicola* (*Psm*; OD 0.005) or infiltrated with a 10 mM $MgCl_2$ solution (mock-treatment). Data represent the mean ± SD of at least three replicate samples. Asterisks denote statistically significant differences between *Psm*- and mock-samples (** $P < 0.01$; * $P < 0.05$; two-tailed *t*-test). **(A,B)** Camalexin levels at indicated times post treatment of 1° leaves in **(A)** 1° leaves and **(B)** 2° leaves. **(C,D)** JA levels at indicated times post treatment of 1° leaves in **(C)** 1° leaves and **(D)** 2° leaves. **(E)** and **(F)** ABA levels at indicated times post treatment of 1° leaves in **(E)** 1° leaves and **(F)** 2° leaves.

has reported the involvement of systemic ROS micro-bursts in the SAR regulatory network of 2° leaf tissue that requires an early oxidative burst in 1° inoculated leaves. These systemic microbursts were only observed in plants inoculated with avirulent but not with virulent *P. syringae* (Alvarez et al., 1998). However, the fact that both avirulent and virulent *P. syringae* are able to induce a typical SAR response argues against a critical role of microbursts in SAR activation (Mishina and Zeier, 2006, 2007; Attaran et al., 2009; Jing et al., 2011; Liu et al., 2011). Moreover, the occurrence of systemic microbursts during SAR has not been

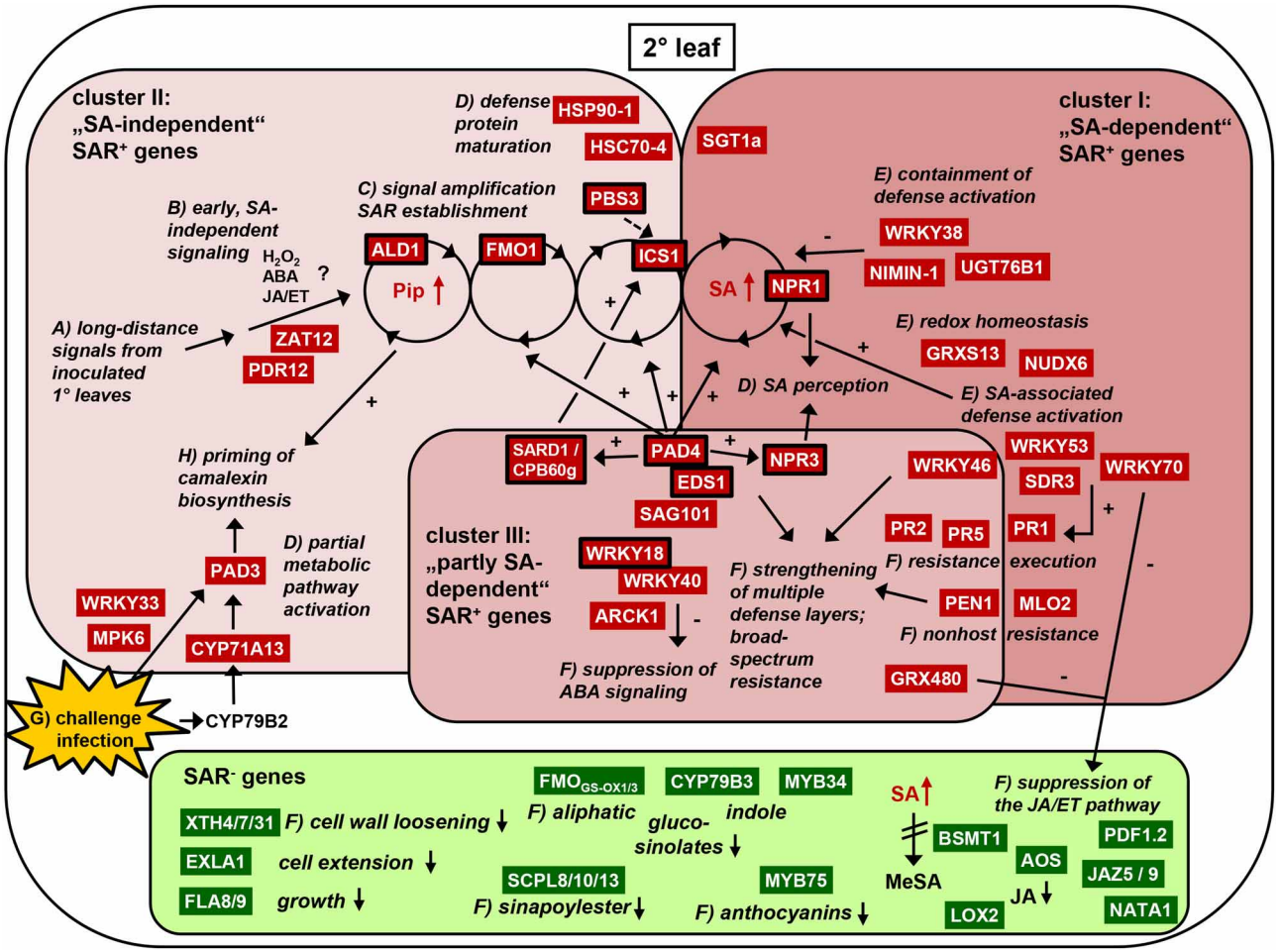


FIGURE 9 | Events occurring in distal (2°) leaves of Arabidopsis plants in which SAR has been biologically activated by *Psm* inoculation in 1° leaves. SAR up-regulated (SAR$^+$ genes) are depicted in red, SAR down-regulated genes (SAR$^-$ genes) depicted in green. Genes known to be indispensable for SAR activation are framed with a black line. The three clusters of SA-independent, SA-dependent, and partially SA-dependent SAR$^+$ genes are illustrated. Processes leading to SAR establishment and functions of individual SAR-related genes or groups of genes are italicized. The alphabetical labels indicate a hypothetical order of events. **(A)** First, long-distance signals derived from inoculated leaves activate initial SA-independent signaling events. **(B)** Possible contributions of ROS, ABA, or JA/ET to these initial events are hypothetic or even doubtful. **(C)** A feedback amplification cycle (depicted as interconnected wheels) that requires the accumulation and the action of the two critical SAR metabolites pipecolic acid (Pip) and salicylic acid (SA) as well as the function of the flavin-monooxygenase FMO1 establishes SAR. **(D–F)** Various events such as the activation of SA signaling, resistance induction, suppression of JA- and ABA-signaling, and partial pre-activation of camalexin biosynthesis occur. **(G,H)** SAR-induced plants are primed for early defense activation such as camalexin accumulation and defense gene expression. Small up arrows symbolize metabolite accumulation, small down arrows symbolize reduction of metabolite biosynthesis or of indicated physiological responses. Large arrows indicate the interconnection between the responses. Plus-signs symbolize activation, minus-sings repression. The indicated events are described in detail in the main text and summarized in the "Summary and conclusions" paragraph.

confirmed by other studies. However, wounding and different abiotic stresses can trigger systemic ROS signaling that is dependent on the NADPH oxidase RBOHD (Miller et al., 2009). Miller et al., reported that the RBOHD-dependent signal can translocate from wounded to systemic tissue within minutes. This rapid systemic distribution of the ROS-related signal is not in accordance with the relatively slow establishment of SAR that essentially takes place between day 1 and day 2 after 1° pathogen inoculation for the *Psm*-Arabidopsis interaction (Mishina et al., 2008), and it is thus not clear whether ROS signaling indeed contributes to SAR establishment.

Is the relatively high number of strongly ABA- and moderately JA/ET-responsive genes in the SA-independent group indicative for early ABA- and JA/ET-signaling events required for SAR establishment? Truman et al. (2007) have described an early JA response in 2° leaves of plants inoculated with very high titers [$OD_{600} = 0.2$] of avirulent *P. syringae* pv. *tomato* DC3000 (*Pst*) expressing the *avrRpm1* avirulence gene (*Pst avrRpm1*) that was associated with an increase of JA in petiole exudates collected from 1° leaves. These high initial titers of *Pst avrRpm1* induce a rapid HR in leaves, and the necrotic disruption of leaf tissue goes hand in hand with the accumulation of JA and other

oxylipins (Mishina and Zeier, 2007; Zoeller et al., 2012). By contrast, our experimental conditions include bacterial inoculations with lower densities ($OD_{600} = 0.005$) of compatible *Psm*, which are not associated with an HR and elevations of JA in petiole exudates (Návarová et al., 2012). Although JA levels rise in *Psm*-inoculated leaves at about 48 hpi, systemic rises of JA are not detected between 16 to 48 hpi (**Figures 8C,D**). Considering these data and the above-mentioned 24 hpi to 48 hpi time window in which SAR establishment takes place in *Psm*-inoculated Arabidopsis plants, it seems unlikely that JA signaling contributes to the up-regulation of the moderately JA/ET-responsive SAR^+ genes of the SA-independent gene cluster. Irrespective of the existence of an early wave of systemic JA signaling after bacterial inoculation, its influence on SAR induction is supposedly negligible, because different Arabidopsis mutants compromised in JA biosynthesis or downstream signaling are SAR-competent (Attaran et al., 2009).

The accumulation of ABA in *Psm*-inoculated leaves occurs more gradually and faster than the accumulation of JA (**Figure 8E**), and the kinetics of ABA production in leaves upon bacterial attack is similar for *Psm*- and *Pst*-inoculations (**Figure 8E**, Fan et al., 2009). Again, the accumulation of ABA, which negatively influences SA signaling and counteracts SAR (Fan et al., 2009), is confined to the site of bacterial inoculation and does not occur in 2° leaf tissue (**Figure 8F**). This metabolic data argues against a function for ABA as an initial trigger for the expression of SA-independent SAR^+ genes in 2° leaves during SAR.

THE SAR^+ GENE CLUSTER III CONTAINS GENES EXHIBITING PARTLY SA-DEPENDENT EXPRESSION

The third cluster of SAR up-regulated genes contains genes that are essential for the initiation of the SAR process as well (**Figures 5**, **9**). These include *PAD4*, *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *SARD1*, *CBP60g*, and *NPR3* (Mishina and Zeier, 2007; Truman et al., 2007; Zhang et al., 2010; Rietz et al., 2011; Fu et al., 2012). These genes are less tightly regulated by SA than the cluster I genes (**Figure 3**), because, unlike the latter, their $[S/M]_{Psm}$ ratios are (per definition) at least 1.5-fold higher than the corresponding $[Col/sid2]_{Psm}$ values (**Figure 5**). PAD4, EDS1, and another SAR^+ gene product from cluster III, SAG101 (**Figure 5**), constitute a family of plant-specific hydrolase proteins that are critical regulatory components of plant basal resistance to (hemi)biotrophic pathogens and ETI triggered by a subset of resistance proteins (Wiermer et al., 2005). Apart from forming homodimers, EDS1 can interact with PAD4 and SAG101, and the formation of different EDS1 homo- or heteromeric complexes is associated with distinct localization patterns in the cytoplasm and/or nucleus (Feys et al., 2005). Complex formation between EDS1 and PAD4 is required for the full establishment of SAR (Rietz et al., 2011). Moreover, the EDS1-PAD4-SAG101 signaling complex also plays an important function in Arabidopsis post-invasion nonhost resistance to non-adapted powdery mildew fungi (Lipka et al., 2005). The $[Col/pad4]_{Psm}$ ratios illustrate that expression of the predominant fractions of SAR^+ genes from all three distinguished clusters is more or less tightly regulated by PAD4, indicating an important function for PAD4 in overall SAR^+ gene transcription (**Figures 2B**, **3**, **4**, **5**, **9**). For instance, the expression of the critical SAR regulatory pathway genes *ALD1*, *FMO1*, and *ICS1* are all partially dependent on PAD4 (**Figure 5**; Song et al., 2004a), and consequently, accumulation of the SAR regulatory metabolites Pip and SA are both positively regulated by PAD4 (Zhou et al., 1998; Návarová et al., 2012). Moreover, PAD4 tightly regulates the *Psm*-induced expression of two members of the plant-specific transcription factor gene family ACBP60, *SAR-DEFICIENT1* (*SARD1*) and *CALMODULIN-BINDING PROTEIN60G (CBP60g)*, as well as the *NPR1* homologue *NPR3* (**Figure 5**). The SARD1 and CBP60g transcription factors are partly redundant in their function and activate pathogen-induced *ICS1* transcription resulting in SA accumulation (Zhang et al., 2010; Wang et al., 2011). A *sard1 cbpg60g* double mutant is therefore completely SAR defective (Zhang et al., 2010). NPR3 has been recently identified, besides NPR1 and NPR4, as a bona fide SA receptor (Fu et al., 2012; Wu et al., 2012). Therefore, the SAR^+ gene cluster III contains central elements of both SA biosynthesis and SA downstream signaling.

The SAR^+ gene cluster III also contains the two WRKY transcription factor genes *WRKY18* and *WRKY40* (**Figure 5**). WRKY18, WRKY40 and WRKY60 (*WRKY60* is not a SAR^+ gene) constitute a group of sequence-related WRKYs with complex and partly redundant roles in plant defense against different pathogen types. WRKY18 is required for biological SAR activation and mediates a subset of NPR1-mediated responses (Wang et al., 2006). Whereas overexpression of *WRKY18* alone increases resistance to *P. syringae*, simultaneous overexpression of *WRKY18* and *WRKY40* enhances susceptibility to the same pathogen (Xu et al., 2006). WRKY18/40/60 negatively regulate ABA signaling (Shang et al., 2010), and ABA signal transduction is also attenuated by *ARCK1*, another cluster III SAR^+ gene that encodes a receptor-like cytosolic protein kinase (Tanaka et al., 2012). Since ABA signaling can interfere with the SA pathway and thus attenuate plant defenses against (hemi)biotrophic pathogens (de Torres-Zabala et al., 2007; Fan et al., 2009), an impairment of the ABA pathway might ensure a robust SA response during SAR (**Figure 9**). Another cluster III SAR^+ gene is *GRX480* encoding for a glutaredoxin that interacts with TGA transcription factors and negatively affects JA and ET signaling. This results in the suppression of expression of typically JA/ET-regulated genes such as *PDF1.2* (Ndamukong et al., 2007; Zander et al., 2012).

The group III also contains the two SAR marker genes *PR2* and *PR5* which, similar to *PR1*, may function in the direct execution of disease resistance because their gene products exhibit antimicrobial activity. Plant *PR2* genes code for β-1,3-glucanases, and purified β-1,3-glucanases from pea have been shown to act synergistically with chitinases in the degradation of fungal cell walls (Mauch et al., 1988). The PR5 protein family includes the basic osmotins whose members are homologous to the sweet-tasting protein thaumatin. Osmotin and other *PR5* proteins have been shown to exhibit antifungal activity in vitro and in planta. For example, overexpression of tobacco osmotin in different plant species results in increased resistance to oomycete pathogens of the genus *Phytophtora* (Liu et al., 1994). Moreover, SAR^+ group III contains *PEN1*, a critical determinant of Arabidopsis pre-invasion nonhost resistance to non-adapted powdery mildew

fungi. *PEN1* codes for a plasma membrane-resident syntaxin which becomes recruited at sites of attempted fungal ingress and is implicated in a vesicle-associated resistance mechanism that prevents fungal penetration through epidermal cell walls (Collins et al., 2003; Bhat et al., 2005). The up-regulation of genes involved in non-host resistance, basal resistance to different pathogen types and ETI indicate that SAR simultaneously strengthens different defense layers that make up the plant immune system (Thordal-Christensen, 2003).

A SIGNIFICANT PORTION OF THE GENES DOWN-REGULATED DURING SAR EXHIBIT STRONG JA-RESPONSIVENESS

A remarkable feature of the SAR$^-$ genes is that their average expression is markedly activated via the JA signaling pathway (mean $[\text{Col}/coi1]_{\text{Psm}} = 5.6$; **Figure 2A**). Taken the $[\text{Col}/coi1]_{\text{Psm}}$ values as a basis, we divided the SAR$^-$ genes into three categories: JA-activated genes ($[\text{Col}/coi1]_{\text{Psm}} > 2$; 76 genes), JA-repressed genes ($[\text{Col}/coi1]_{\text{Psm}} < 0.5$; 190 genes), and JA-independent genes ($[\text{Col}/coi1]_{\text{Psm}} > 0.5$ and < 2; 404 genes) (**Figure 2C**). Although quantitatively the smallest group, the JA-activated genes most strongly influenced the average $[\text{Col}/coi1]_{\text{Psm}}$ ratio because most genes in this group have very high $[\text{Col}/coi1]_{\text{Psm}}$ ratios (average $[\text{Col}/coi1]_{\text{Psm}} = 43.9$). Apart from a few exceptions (e.g. *PDF1.2*), this is associated with high $[\text{S/M}]_{JA}$ ratios (average $[\text{S/M}]_{\text{JA}} = 10.3$) (**Figures 2C**, **6**). Thus, both the $[\text{Col}/coi1]_{\text{Psm}}$ and the $[\text{S/M}]_{\text{JA}}$ ratios indicate that the members of the JA-activated group of SAR$^-$ genes are highly responsive to JA signaling.

In fact, the JA-activated group of SAR$^-$ genes consist of a series of genes typically regarded as marker genes of the JA pathway (**Figure 6**). Among them are *JASMONATE-REGULATED 21* (*JRG21*) (Nickstadt et al., 2004), *JASMONATE ZIM-DOMAIN PROTEIN 5* (*JAZ5*), *JAZ9* (Thines et al., 2007), *BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE 1* (*BSMT1*) (Chen et al., 2003), *N-ACETYLTRANSFERASE ACTIVITY 1* (*NATA1*) (Adio et al., 2011), *CORONATINE-INDUCED PROTEIN 1* (*COR1*) (Benedetti et al., 1998), *JASMONIC ACID RESPONSIVE 1* (*JR1*), *JR2* (León et al., 1998), *POLYGALACTURONASE INHIBITING PROTEIN 2* (*PGIP2*) (Schenk et al., 2003), *ALLENE OXIDE SYNTHASE* (*AOS*) (Laudert and Weiler, 1998), *LIPOXYGENASE 2* (*LOX2*) (Bell and Mullet, 1993), and the plant defensin *PDF1.2* (Ndamukong et al., 2007). This indicates that JA defense signaling is significantly reduced in the SAR-induced state. One of the key genes involved in JA biosynthesis, *AOS* (Laudert and Weiler, 1998), is markedly down-regulated during SAR (**Figure 6**), suggesting that the JA pathway could be attenuated already at the level of JA biosynthesis. However, since the experimentally determined levels of JA are similarly low in 2° leaves of mock-control and *Psm*-inoculated plants (**Figure 8D**), it is more likely that signaling events downstream of JA production are negatively affected during SAR. SAR is characterized by activated SA signaling (**Figure 2A**), and the well-established negative cross-talk between the SA- and JA-pathways might be responsible for the attenuation of JA responses (Spoel et al., 2003). As discussed above, molecular players such as the SA-activated transcription factor WRKY70 and the glutaredoxin GRX480 could mediate the suppression of the JA pathway during SAR (Li et al., 2004, 2006; Ndamukong et al., 2007) (**Figure 9**). Moreover, the decreased expression of the SA methyltransferase BSMT1 that converts signaling active SA into inactive methyl salicylate (MeSA) supposedly counteracts deactivation of the SA signal (Attaran et al., 2009; Wu et al., 2012) (**Figure 9**). SAR is associated with a reduced biosynthesis of constitutively produced metabolites such as glucosinolates or sinapoylmalates, as illustrated by the down-regulation of *MYB34*, a JA-inducible transcription factor that activates indolic glucosinolate production (**Figures 6**, **9**) and by the decreased expression of several sinapoyltransferase genes (**Table 5**). Another group of secondary metabolites whose biosynthesis might be negatively affected upon SAR activation are anthocyanins, because *MYB75* involved in the transcriptional regulation of anthocyanin biosynthesis (Borevitz et al., 2000) also belongs to the group of SAR-repressed and JA-activated genes (**Figures 6**, **9**). It is likely that the production of inducible metabolites that negatively interfere with resistance to (hemi)biotrophic pathogens is also repressed during SAR. For instance, the JA-inducible acetyltransferase NATA1 mediates the formation of N-δ-acetylornithine from ornithine. Since *nata1* knockout lines are more resistant to *P. syringae* than the wild-type, N-δ-acetylornithine is supposed to negatively influence bacterial resistance (Adio et al., 2011). A reduced induction of *NATA1* expression during SAR (**Figure 6**) could therefore counteract the presumed negative effect of N-δ-acetylornithine on bacterial resistance. This would be consistent with the finding of Adio et al. (2011) that SA pre-treatment, which renders plants in an enhanced state of pathogen resistance, inhibits induced N-δ-acetylornithine formation.

SIMILARITIES AND DIFFERENCES OF DEFENSE ACTIVATION IN 1° AND 2° LEAF TISSUE UPON BACTERIAL INOCULATION

The signaling network underlying basal resistance to local infection exhibits overlapping features to the signaling events that activate SAR because both forms of resistance share similar regulatory factors such as ICS1, SA, NPR1, PAD4, EDS1, ALD1, Pip, and FMO1. ALD1-mediated Pip production and FMO1-dependent transduction of Pip signaling do occur in both *P. syringae*-inoculated and in systemic leaf tissue (Návarová et al., 2012). Pip functions as a mediator of defense amplification in plants, and this fortification of defense responses is indispensable for the activation of SAR (Návarová et al., 2012; Shah and Zeier, 2013). The Pip/FMO1-resistance pathway is also important for local resistance induction but the extent of its impact on basal resistance appears to vary with the attacking pathogen type (Song et al., 2004a; Bartsch et al., 2006; Mishina and Zeier, 2006; Návarová et al., 2012). Further, SA accumulation and downstream signaling are common processes induced in 1°-inoculated leaf tissue and in distant 2°-leaves, and the activation of the SA pathway is required for both basal resistance to (hemi)biotrophic pathogens and SAR (Wildermuth et al., 2001; Mishina and Zeier, 2006; Spoel and Dong, 2012). The existence of common immune regulatory metabolites in 1° and 2° leaf tissue and the fact that most SAR$^+$ genes are also up-regulated in inoculated tissue after pathogen encounter illustrates that overlapping signaling principles and defense mechanisms exist in inoculated 1° and in systemic 2° leaves (**Figures 2A**,**B**, **3**, **4**, **5**).

Are there characteristic differences at the levels of defense metabolite production and gene activation in 1° and 2° leaf tissue? A first difference is of quantitative nature: Pip and SA accumulate to markedly higher levels in 1° than in 2° leaf tissue (Mishina et al., 2008; Návarová et al., 2012). In addition, the levels of a significant higher number of metabolites increase in 1° than in 2° leaf tissue after *P. syringae* inoculation (Ward et al., 2010; Griebel and Zeier, 2010; Chanda et al., 2011; Návarová et al., 2012). As shown in **Figure 8**, the substances that strongly accumulate in 1° but not in 2° leaf tissue include JA, ABA, and camalexin. In addition, when comparing the number of genes up- or down-regulated in 1° and 2° leaves of *Psm*-treated plants in different experiments, it becomes obvious that the transcriptional changes at inoculation sites are much more pronounced than those in distant tissue (**Figure 2D**). From the 1921 Arabidopsis genes assigned to be locally up-regulated at 24 h post *Psm*-inoculation (**Figure 2D**), 299 and 19 belong to the groups of SAR^+ and SAR^- genes, respectively. This implies that the expression levels of about 15.5 % of genes locally up-regulated at 24 hpi do increase systemically after SAR establishment, but that the expression of the largest portion of locally induced genes essentially remains unchanged at the systemic level. Strikingly, the average $[\text{Col}/coi1]_{\text{Psm}}$ ratio in the group of locally up-regulated genes is high (6.4) compared to the same value for 2° leaves (1.1) (**Figure 2D**), indicating that JA signaling is strongly activated in 1° but not in 2° leaf tissue, in which, as discussed in the previous section (**Figures 2C**, **6**), the expression of many JA-responsive genes is even reduced. The stimulation of JA signaling in 1° leaves can be triggered by the *P. syringae* phytotoxin coronatine, a structural mimic of the signaling active JA derivative JA-Ile (Geng et al., 2012), and by endogenously produced JA. In phases of the plant-bacterial interaction during which endogenous JA levels remain low (e.g. until 36 hpi for the *Psm*-Arabidopsis inoculation experiment shown in **Figure 8C**), bacterial coronatine is presumably the major stimulus. For instance, Attaran et al. (2009) performed a comparative assessment of the formation of MeSA in Arabidopsis leaves induced by coronatine-producing and non-producing *Pst*. MeSA is generated by the BSMT1-catalysed methylation of SA, and the *BSMT1* gene is strongly JA-responsive (**Figure 7**). Until 24 hpi, only the coronatine-producing but not the coronatine-deficient *Pst* strain elicited a significant formation of MeSA in inoculated plants, indicating that bacterial-derived coronatine rather than endogenous JA triggers the metabolic response in earlier phases of the interaction (Attaran et al., 2009).

The strong activation of the JA pathway in inoculated leaves and the partial suppression of JA responses in distant leaves reflect a major difference between the hormonal status of 1° and 2° tissue, and this difference impacts the nature of defense responses in both tissue types. JA pathway activation at inoculation sites is at least partially causative for the more pronounced metabolite accumulation in 1° compared to 2° leaves because JA signaling induces the expression of a series of genes involved in metabolite biosynthesis. For example, the biosynthesis of many mono-, sesqui- and diterpenoids in plants is characteristically regulated via JA signaling (Arimura et al., 2008; Attaran et al., 2008). Indeed, among the most strongly up-regulated genes in *Psm*-inoculated tissue are JA-inducible genes involved in metabolism such as the SA methyl transferase *BSMT1*, the terpene synthase *TPS4*, several cytochrome P450 monooxygenases (e.g. *CYP82G1, CYP94C1, CYP94B3, CYP79B2*), and UDP-dependent glycosyltransferases (*UGT76E12*, *UGT76E1*) (**Figure 7**). *P. syringae*-inoculated Arabidopsis leaves produce the C_{16}–homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) in a *TPS4*-dependent manner (Attaran et al., 2008). TPS4 catalyses the first step of TMTT biosynthesis, i.e. the conversion of the diterpene precursor geranylgeranyl diphosphate to (E,E)-geranyllinalool (Herde et al., 2008). Subsequent formation of the C_{16}-homoterpene from (E,E)-geranyllinalool by an oxidative cleavage reaction catalyzed by CYP82G1 completes TMTT biosynthesis (Lee et al., 2010). The two cytochrome P450 enzymes CYP94B3 and CYP94C1 are involved in the catabolic turnover of the signaling active jasmonate JA-Ile. CYP94B3 mediates the hydroxylation of JA-Ile to 12-hydroxy-JA-Ile and thereby inactivates hormone function (Koo et al., 2011). CYP94C1 then converts 12hydroxy-JA-Ile to the corresponding 12-carboxy-derivative (Heitz et al., 2012). Another strongly *Psm*-up-regulated gene whose expression is only moderately affected by the JA signaling pathway is *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2 (ACS2)*, an ACS isoform involved in ethylene biosynthesis (Tsuchisaka et al., 2009). The activation of ET biosynthesis in *P. syringae*-inoculated leaves is consistent with the microarray gene expression data, because ET-dependent genes are expressed more prominently in 1° (mean $[\text{Col}/ein2]_{\text{Psm}} = 1.7$) than in 2° (mean $[\text{Col}/ein2]_{\text{Psm}} = 1.1$) leaves (**Figures 2D**, **7**). Examples of genes partially regulated via ET signaling are *ALPHA-DIOXYGENASE 1* (*DOX1*), encoding a fatty acid α-dioxygenases which converts linolenic acid and other fatty acids into their 2-hydroperoxy derivatives (Hamberg et al., 1999). Moreover, *PR3* encodes a basic chitinase that is up-regulated in 1° inoculated but not in 2° tissue and regulated by JA/ET signaling (**Figure 7**; Zander et al., 2010).

Another obvious difference between the transcriptional changes in 1° and 2° leaves following *Psm* inoculation is the stronger activation of ABA-responsive genes at inoculation sites compared to systemic tissue (mean $[\text{S/M}]_{\text{ABA}} = 3.3$ and 1.5 for 1° and 2° leaves, respectively; **Figures 2D**, **7**), which is consistent with the observation that ABA accumulation is restricted to bacterial inoculation sites (**Figure 8E**). The $[\text{S/M}]_{\text{ABA}}$ values indicate that ABA signaling in 1° leaves contributes to the induction of genes such as *ANAC019*, *DOX1*, *CYP94B3*, *UGT74E2*, and *CYP710A1* (**Figure 7**). The transcription factor ANAC019 binds to a drought-responsive cis-element in the *early responsive to dehydration stress 1* promoter, and overexpression of *ANAC019* in Arabidopsis provides increased drought tolerance (Tran et al., 2004). This is one example of the fact that the local transcriptional responses following compatible *P. syringae* inoculation show large overlap with those occurring after drought or osmotic stress (de Torres-Zabala et al., 2007), presumably because strong bacterial proliferation in leaf tissue is associated with water deprivation and tissue necrosis. Activation of ABA signaling in infected tissue has also been interpreted as an active, effector-triggered virulence strategy of the pathogen, because ABA negatively interferes with SA signaling and therefore weakens one of the major pathways of plant defense to (hemi)biotrophic pathogens (de Torres-Zabala

et al., 2007; Fan et al., 2009). Moreover, the ABA- and H_2O_2-responsive UDP-depedent glucosyltransferase *UGT74E2* that is also strongly up-regulated at inoculation sites has been implicated with the modulation of water stress responses. UGT74E2 glycosylates indole-3-butyric acid and therefore affects auxin homeostasis in plants (Tognetti et al., 2010), and modulated auxin signaling can result in disturbed plant immune responses (Truman et al., 2010). The pathogen-induced *CYP710A1* gene is also moderately ABA- and H_2O_2-responsive (**Figure 7**), and CYP710A1 mediates the desaturation of the most common phytosterol in Arabidopsis, β-sitosterol, to produce sitosterol (Morikawa et al., 2006), which strongly accumulates in *P. syringae*-inoculated leaves, integrates into cell plasma membranes, and negatively affects plant defense and resistance to bacteria (Griebel and Zeier, 2010).

SUMMARY AND CONCLUSIONS

Based on gene expression data, metabolite data, and literature information, the present study aimed to contribute to a better understanding of the characteristics of the SAR-induced state in plants. **Figure 9** summarizes main events occurring in 2° leaves of plants after biological SAR activation. These include:

(1) The establishment of SAR in Arabidopsis in response to a localized leaf inoculation with the bacterial pathogen *Psm* is associated with a major transcriptional reprogramming in distant leaf tissue. Thereby, several hundred genes that are systemically up- (SAR^+ genes) and down-regulated (SAR^- genes) can be distinguished. This extensive transcriptional reprogramming upon SAR induction is dependent on the SAR regulatory gene *FMO1*.

(2) Functional categorization on the basis of GO annotations indicates that the SAR^+ gene cluster is enriched in genes associated with stress responses, signal transduction, transport, and the cell secretory apparatus, whereas in the SAR^- gene cluster, genes associated with the chloroplast, cell wall loosening, cell extension, and the biosynthesis of constitutively formed secondary metabolites are over-represented. This suggests that, upon SAR induction, plants redirect some of their resources from vegetative growth towards defense-related processes.

(3) Alignment of the SAR expression data with publicly available microarray information has allowed us to classify the SAR-associated genes and analyse their expression characteristics. However, since the microarray data compared in our study originate from different laboratories, experimental parameters such as plant age, growth conditions, the kind of treatment or the timing of sample collection varied between experiments (Tab. 6). For instance, whereas soil grown, 4 to 5 week-old Arabidopsis plants were used for the *P. syringae*-, the flg22-, the SA-, and BTH-treatments, 5-7 day-old seedlings grown on MS medium were used for the JA-, ABA-, and H_2O_2-treatments. Although these experimental differences might have impact on the expression characteristics of individual genes, the predominant part of gene regulatory principles described in this work appears robust. This is exemplified by the findings that the JA- or SA-inducibility of genes was inferred from mutant analyses ($[Col/coi1]_{Psm}$ or $[Col/sid2]_{Psm}$) and exogenous treatment ($[S/M]_{JA}$ or $[S/M]_{SA}$) with considerable conformity (**Figures 3**, **6**). Moreover, the stimulus-dependent regulation of the (SAR-related) genes discussed in this study proved consistent with available literature data.

(4) Based on the expression patterns of SAR-related genes in *Psm*-inoculated wild-type and *sid2/ics1* leaves and on the responsiveness of those genes to exogenous SA, we have categorized the group of SAR^+ genes into clusters of SA-independent genes (cluster II), SA-dependent genes (cluster I), and partially SA-dependent genes (cluster III).

(5) Albeit not congruent, extensive similarities of the transcriptional responses of Arabidopsis plants following biological SAR induction and treatment with the synthetic resistance activator BTH do exist. The so-called "SA analogue" BTH exhibits a broader effect on SAR-related gene expression than the endogenous defense signal SA.

(6) The cluster of SA-independent SAR^+ genes contains the three critical SAR components *ALD1*, *FMO1*, and *ICS1*, which are indispensable for SAR establishment. *ALD1* and *FMO1* are required for the biosynthesis and downstream signaling, respectively, of the immune regulator pipecolic acid, which mediates SAR activation via signal amplification (Návarová et al., 2012). *ICS1* is involved in the de novo biosynthesis of SA (Wildermuth et al., 2001) and "connects" the SA-independent and SA-dependent phases of SAR.

(7) In the initial stages of SAR establishment in 2° leaves, SA-independent signaling might precede and then activate an SA-dependent phase of SAR. Since both phases are required for the full activation of SAR, "SA-independent" and "SA-dependent" signaling events cannot be regarded as separately acting units but tightly co-operate to realize SAR. The average responsiveness to H_2O_2, ABA, and JA/ET is higher for SAR^+ genes from the SA-independent than for those from the SA-dependent group. This might suggest a role of these stimuli in the early signaling stages of SAR in 2° leaves. However, metabolite and mutant analyses rather argue against this possibility.

(8) On average, SA-dependent SAR^+ genes exhibit a higher responsiveness to flg22 than SA-independent SAR^+ genes. (Partially) SA-dependent SAR^+ genes function in the activation or maintenance of distinct defense layers (non-host resistance, basal resistance, ETI), and in resistance execution against different pathogen types. This indicates that SAR heightens the plant immune system on several levels and illustrates the hallmark of SAR as a state of broad-spectrum resistance. Other SA-regulated SAR^+ genes are involved in redox homeostasis and in the containment of defense response activation after SAR establishment.

(9) Negative cross-talk between JA/ET- or ABA-signaling pathways on the SA defense pathway is well-documented (Spoel et al., 2003; Fan et al., 2009). Several SAR^+ genes are involved in the suppression of ABA- and JA/ET-signaling, suggesting that they can relieve inhibitory effects of these hormonal pathways on the SA pathway during SAR. Suppression of JA signaling during SAR also manifests itself

in the fact that many highly JA-responsive genes are among the most strongly down-regulated genes during SAR.

(10) Overlapping defense principles exist for the induction of local resistance responses and SAR. However, the transcriptional and metabolic responses at sites of bacterial inoculation are generally more pronounced than those in systemic tissue. A major difference between the 1° inoculated and the 2° leaves relates to the stress hormonal status: whereas SA, Pip, JA, and ABA are produced at inoculation sites to largely high levels, only Pip and SA moderately accumulate at the systemic level (**Figure 8**; Mishina et al., 2008; Návarová et al., 2012). Therefore, JA/ET- and ABA-triggered responses are strongly induced in 1° leaves, whereas these responses are not activated or even suppressed (see above) in the 2° leaves.

(11) Pip accumulation during SAR primes plants to more quickly and effectively activate defense responses to subsequent pathogen encounter. A strongly primed defense response in Arabidopsis in SAR-induced plants is the *Psm*-triggered accumulation of the phytoalexin camalexin (Návarová et al., 2012). SAR is associated with enhanced expression of the camalexin biosynthetic genes *CYP71A13* and *PAD3* but not *CYP79B2*, and therefore provides partial activation of camalexin production. This implicates that fewer components would have to be induced in a future pathogen challenge to activate the whole response. Partial pre-activation of defense pathways might thus be a general mechanistic principle by which SAR-induced plants manage to accelerate defense responses when challenge-infected.

(12) Varying environmental conditions can influence the magnitude and particular mechanistic aspects of the SAR response (Shah and Zeier, 2013). The quantitative differences we have observed for the transcriptional SAR responses of experiment 3 compared with the responses in experiments 1 and 2 were associated with higher overall leaf expression levels of major anthocyanin biosynthesis genes. Leaf anthocyanin accumulation is a characteristic response to unfavorable environmental conditions (Misyura et al., 2013). An important task for future SAR research will be to systematically investigate to what extent and how other environmental issues and stress parameters influence SAR establishment.

ACKNOWLEDGMENTS

We are indebted to Dong Wang, Xinnian Dong, Raka Mitra, Jane Glazebrook, Francoise Thibaud-Nissen, Hideki Goda, Shigeo Yoshida, Yukihisa Shimida, Ron Mittler, Dierk Scheel, Frederic Brunner, Lore Westphal, the NASC's International Affymetrix Service, and the ArrayExpress functional genomics database for making ATH1 microarray data publicly available. This work was supported by a grant to Jürgen Zeier from the Swiss National Science Foundation (SNF grant No. 3100A-125374).

REFERENCES

Adio, A. M., Casteel, C. L., De Vos, M., Kim, J. H., Joshi, V., Li, B., et al. (2011). Biosynthesis and defensive function of Nδ-acetylornithine, a jasmonate-induced Arabidopsis metabolite. *Plant Cell* 23, 3303–3318. doi: 10.1105/tpc.111.088989

Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., et al. (1993). Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7327–7331. doi: 10.1073/pnas.90.15.7327

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J. R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* 284, 2148–2152. doi: 10.1126/science.284.5423.2148

Alvarez, M. E., Pennell, R. I., Meijer, P.-J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92, 773–784. doi: 10.1016/S0092-8674(00)81405-1

Arimura, G. I., Garms, S., Maffei, M., Bossi, S., Schulze, B., Leitner, M., et al. (2008). Herbivore-induced terpenoid emission in *Medicago truncatula*: concerted action of jasmonate, ethylene and calcium signaling. *Planta* 227, 453–464. doi: 10.1007/s00425-007-0631-y

Attaran, E., Rostás, M., and Zeier, J. (2008). *Pseudomonas syringae* elicits emission of the terpenoid (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4. *Mol. Plant-Microbe Interact.* 21, 1482–1497. doi: 10.1094/MPMI-21-11-1482

Attaran, E., Zeier, T. E., Griebel, T., and Zeier, J. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. *Plant Cell* 21, 954–971. doi: 10.1105/tpc.108.063164

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J. L., Bautor, J., et al. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monoxygenase FMO1 and the nudix hydrolase NUDT7. *Plant Cell* 18, 1038–1051. doi: 10.1105/tpc.105.039982

Bell, E., and Mullet, J. E. (1993). Characterization of an Arabidopsis lipoxygenase gene responsive to methyl jasmonate and wounding. *Plant Physiol.* 103, 1133–1337. doi: 10.1104/pp.103.4.1133

Benedetti, C. E., Costa, C. L., Turcinelli, S. R., and Arruda, P. (1998). Differential expression of a novel gene in response to coronatine, methyl jasmonate, and wounding in the *Coi1* mutant of Arabidopsis. *Plant Physiol.* 116, 1037–1042. doi: 10.1104/pp.116.3.1037

Bhat, R. A., Miklis, M., Schmelzer, E., Schulze-Lefert, P., and Panstruga, R. (2005). Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3135–3140. doi: 10.1073/pnas.0500012102

Borevitz, J. O., Xia, Y., Blount, J., Dixon, R. A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 12, 2383–2394.

Canet, J. V., Dobón, A., Roig, A., and Tornero, P. (2010). Structure-function analysis of *npr1* alleles in Arabidopsis reveals a role for its paralogs in the perception of salicylic acid. *Plant Cell Environ.* 33, 1911–1922. doi: 10.1111/j.1365-3040.2010.02194.x

Celenza, J. L., Quiel, J. A., Smolen, G. A., Merrikh, H., Silvestro, A. R., Normanly, J., et al. (2005). The Arabidopsis ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. *Plant Physiol.* 137, 253–262. doi: 10.1104/pp.104.054395

Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K.-T., Gao, Q.-M., et al. (2011). Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat. Genet.* 43, 421–427.

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172. doi: 10.1111/j.1365-313X.2012.04981.x

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., et al. (2003). An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.* 36, 577–588. doi: 10.1046/j.1365-313X.2003.01902.x

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J. L., et al. (2003). SNARE-protein-mediated disease resistance

at the plant cell wall. *Nature* 425, 973–977. doi: 10.1038/nature02076

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., and Scheible, W. R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* 139, 5–17. doi: 10.1104/pp.105.063743

Davletova, S., Schlauch, K., Coutu, J., and Mittler, R. (2005). The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. *Plant Physiol.* 139, 847–856. doi: 10.1104/pp.105.068254

Delaney, T. P., Friedrich, L., and Ryals, J. A. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6602–6606. doi: 10.1073/pnas.92.14.6602

Dempsey, D. A., and Klessig, D. F. (2012). SOS - too many signals for systemic acquired resistance? *Trends Plant Sci.* 17, 538–545. doi: 10.1016/j.tplants.2012.05.011

de Torres-Zabala, M., Truman, W., Bennett, M. H., Lafforgue, G., Mansfield, J. W., Rodriguez Egea, P., et al. (2007). *Pseudomonas syringae* pv. *tomato* hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *EMBO J.* 26, 1434–1443. doi: 10.1038/sj.emboj.7601575

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Fan, J., Hill, L., Crooks, C., Doerner, P., and Lamb, C. (2009). Abscisic acid has a key role in modulating diverse plant-pathogen interactions. *Plant Physiol.* 150, 1750–1761. doi: 10.1104/pp.109.137943

Feys, B. J., Wiermer, M., Bhat, R. A., Moisan, L. J., Medina-Escobar, N., Neu, C., et al. (2005). Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* 17, 2601–2613. doi: 10.1105/tpc.105.033910

Fraser, C. M., and Chapple, C. (2011). The phenylpropanoid pathway in Arabidopsis. *Arabidopsis Book* 9:e0152. doi: 10.1199/tab.0152

Fu, Z. Q., and Dong, X. (2013). Systemic acquired resistance: Turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863. doi: 10.1146/annurev-arplant-042811-105606

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232.

Garcion, C., Baltensperger, R., Fournier, T., Pasquier, J., Schnetzer, M. A., Gabriel, J. P., et al. (2006). FiRe and microarrays: a fast answer to burning questions. *Trends Plant Sci.* 11, 320–322. doi: 10.1016/j.tplants.2006.05.009

Geng, X., Cheng, J., Gangadharan, A., and Mackey, D. (2012). The coronatine toxin of *Pseudomonas syringae* is a multifunctional suppressor of Arabidopsis defense. *Plant Cell* 24, 4763–4774. doi: 10.1105/tpc.112.105312

Glawischnig, E. (2007). Camalexin. *Phytochemistry* 68, 401–406. doi: 10.1016/j.phytochem.2006.12.005

Glawischnig, E., Hansen, B. G., Olsen, C. E., and Halkier, B. A. (2004). Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8245–8250. doi: 10.1073/pnas.0305876101

Goda, H., Sasaki, E., Akiyama, K., Maruyama-Nakashita, A., Nakabayashi, K., Li, W., et al. (2008). The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access. *Plant J.* 55, 526–542. doi: 10.1111/j.1365-313X.2008.03510.x

Gomez-Gomez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* 18, 277–284. doi: 10.1046/j.1365-313X.1999.00451.x

Griebel, T., and Zeier, J. (2010). A role for β-sitosterol to stigmasterol conversion in plant-pathogen interactions. *Plant J.* 63, 254–268. doi: 10.1111/j.1365-313X.2010.04235.x

Hamberg, M., Sanz, A., and Castresana, C. (1999). α-Oxidation of fatty acids in higher plants. Identification of a pathogen-inducible oxygenase (piox) as an α-dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. *J. Biol. Chem.* 274, 24503–24513. doi: 10.1074/jbc.274.35.24503

Heitz, T., Widemann, E., Lugan, R., Miesch, L., Ullmann, P., Désaubry, L., et al. (2012). Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone Jasmonoyl-isoleucine for catabolic turnover. *J. Biol. Chem.* 287, 6296–6306. doi: 10.1074/jbc.M111.316364

Herde, M., Gartner, K., Kollner, T. G., Fode, B., Boland, W., Gershenzon, J., et al. (2008). Identification and regulation of TPS04/GES, an Arabidopsis geranyllinalool synthase catalyzing the first step in the formation of the insect-induced volatile C-16-homoterpene TMTT. *Plant Cell* 20, 1152–1168. doi: 10.1105/tpc.106.049478

Hu, Y., Dong, Q., and Yu, D. (2012). *Arabidopsis* WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. *Plant Sci.* 185–186, 288–297. doi: 10.1016/j.plantsci.2011.12.003

Humphry, M., Bednarek, P., Kemmerling, B., Koh, S., Stein, M., Göbel, U., et al. (2010). A regulon conserved in monocot and dicot plants defines a functional module in antifungal plant immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21896–21901. doi: 10.1073/pnas.1003619107

Hwang, S. G., Lin, N. C., Hsiao, Y. Y., Kuo, C. H., Chang, P. F., Deng, W. L., et al. (2012). The Arabidopsis short-chain dehydrogenase/reductase 3, an ABSCISIC ACID DEFICIENT 2 homolog, is involved in plant defense responses but not in ABA biosynthesis. *Plant Physiol. Biochem.* 51, 63–73. doi: 10.1016/j.plaphy.2011.10.013

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003a). Summaries of affymetrix genechip probe level data. *Nucleic Acids Res.* 31:e15. doi: 10.1093/nar/gng015

Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., et al. (2003b). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4, 249–264. doi: 10.1093/biostatistics/4.2.249

Irshad, M., Canut, H., Borderies, G., Pont-Lezica, R., and Jamet, E. (2008). A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: Confirmed actors and newcomers. *BMC Plant Biol.* 8:94. doi: 10.1186/1471-2229-8-94

Ishikawa, K., Yoshimura, K., Harada, K., Fukusaki, E., Ogawa, T., Tamoi, M., et al. (2010). AtNUDX6, an ADP-ribose/NADH pyrophosphohydrolase in Arabidopsis, positively regulates NPR1-dependent salicylic acid signaling. *Plant Physiol.* 152, 2000–2012. doi: 10.1104/pp.110.153569

Jagadeeswaran, G., Raina, S., Acharya, B. R., Maqbool, S. B., Mosher, S. L., Appel, H. M., et al. (2007). Arabidopsis GH3-like defense gene 1 is required for accumulation of salicylic acid, activation of defense responses and resistance to *Pseudomonas syringae*. *Plant J.* 51, 234–246. doi: 10.1111/j.1365-313X.2007.03130.x

Jing, B., Xu, S., Xu, M., Li, Y., Li, S., Ding, J., et al. (2011). Brush and spray: a high-throughput systemic acquired resistance assay suitable for large-scale genetic screening. *Plant Physiol.* 157, 973–980. doi: 10.1104/pp.111.182089

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., et al. (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13583–13588. doi: 10.1073/pnas.96.23.13583

Johnson, K. L., Jones, B. J., Bacic, A., and Schultz, C. J. (2003). The fasciclin-like arabinogalactan proteins of Arabidopsis. A multigene family of putative cell adhesion molecules. *Plant Physiol.* 133, 1911–1925. doi: 10.1104/pp.103.031237

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Kadota, Y., and Shirasu, K. (2012). The HSP90 complex of plants. *Biochim. Biophys. Acta* 1823, 689–697. doi: 10.1016/j.bbamcr.2011.09.016

Kang, J., Hwang, J. U., Lee, M., Kim, Y. Y., Assmann, S. M., Martinoia, E., et al. (2010). PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2355–2360. doi: 10.1073/pnas.0909222107

Katagiri, F., Thilmony, R., and He, S. Y. (2002). The *Arabidopsis thaliana-Pseudomonas syringae* interaction. *Arabidopsis Book* 1:e0039. doi: 10.1199/tab.0039

Katsir, L., Schilmiller, A. L., Staswick, P. E., He, S. Y., and Howe, G. A. (2008). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7100–7105. doi: 10.1073/pnas.0802332105

Kim, K.-C., Lai, Z., Fan, B., and Chen, Z. (2008). Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone

deacetylase 19 in basal defense. *Plant Cell* 20, 2357–2371. doi: 10.1105/tpc.107.055566

Koo, A. J. K., Thomas, F., Cooke, T. F., and Howe, G. A. (2011). Cytochrome P450 CYP94B3 mediates catabolism and inactivation of the plant hormone jasmonoyl-L-isoleucine. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9298–9303. doi: 10.1073/pnas.1103542108

La Camera, S., L'Haridon, F., Astier, J., Zander, M., Abou-Mansour, E., Page, G., et al. (2011). The glutaredoxin ATGRXS13 is required to facilitate *Botrytis cinerea* infection of *Arabidopsis thaliana* plants. *Plant J.* 68, 507–519. doi: 10.1111/j.1365-313X.2011.04706.x

Laporte, D., Olate, E., Salinas, P., Salazar, M., Jordana, X., and Holuigue, L. (2012). Glutaredoxin GRXS13 plays a key role in protection against photooxidative stress in *Arabidopsis*. *J. Exp. Bot.* 68, 507–519.

Laudert, D., and Weiler, E. W. (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. *Plant J.* 15, 675–684. doi: 10.1046/j.1365-313x.1998.00245.x

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82. doi: 10.1046/j.1365-313X.1996.10010071.x

Lee, M. W., Lu, H., Jung, H. W., and Greenberg, J. T. (2007). A key role for the Arabidopsis WIN3 protein in disease resistance triggered by *Pseudomonas syringae* that secrete AvrRpt2. *Mol. Plant-Microbe Interact.* 20, 1192–1200. doi: 10.1094/MPMI-20-10-1192

Lee, S., Badieyan, S., Bevan, D. R., Herde, M., Gatz, C., and Tholl, D. (2010). Herbivore-induced and floral homoterpene volatiles are biosynthesized by a single P450 enzyme (CYP82G1) in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21205–21210. doi: 10.1073/pnas.1009975107

León, J., Rojo, E., Titarenko, E., and Sánchez-Serrano, J. J. (1998). Jasmonic acid-dependent and -independent wound signal transduction pathways are differentially regulated by Ca^{2+}/calmodulin in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 258, 412–419. doi: 10.1007/s004380050749

Lewis, J. D., Wan, J., Ford, R., Gong, Y., Fung, P., Nahal, H., et al. (2012). Quantitative Interactor Screening with next-generation Sequencing (QIS-Seq) identifies *Arabidopsis thaliana* MLO2 as a target of the *Pseudomonas syringae* type III effector HopZ2. *BMC Genomics* 13:8. doi: 10.1186/1471-2164-13-8

Li, J., Brader, G., Kariola, T., and Palva, E. T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* 46, 477–491. doi: 10.1111/j.1365-313X.2006.02712.x

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331. doi: 10.1105/tpc.016980

Li, J., Hansen, B. G., Ober, J. A., Kliebenstein, D. J., and Halkier, B. A. (2008). Subclade of flavin-monooxygenases involved in aliphatic glucosinolate biosynthesis. *Plant Physiol.* 148, 1721–1733. doi: 10.1104/pp.108.125757

Li, Y., Darley, C. P., Ongaro, V., Fleming, A., Schipper, O., Baldauf, S. L., et al. (2002). Plant expansins are a complex multigene family with an ancient evolutionary origin. *Plant Physiol.* 128, 854–864. doi: 10.1104/pp.010658

Linthorst, H. J., Meuwissen, R. L., Kauffmann, S., and Bol, J. F. (1989). Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. *Plant Cell* 1, 285–291.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., et al. (2005). Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. *Science* 310, 1180–1183. doi: 10.1126/science.1119409

Liu, D., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. (1994). Osmotin overexpression in potato delays development of disease symptoms. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1888–1892. doi: 10.1073/pnas.91.5.1888

Liu, P. P., von Dahl, C. C., and Klessig, D. F. (2011). The extent to which methyl salicylate is required for signaling systemic acquired resistance is dependent on exposure to light after infection. *Plant Physiol.* 157, 2216–2226.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta c}{}_{\mathrm{T}}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., and Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic acquired resistance signalling in *Arabidopsis*. *Nature* 419, 399–403. doi: 10.1038/nature00962

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K. A., et al. (2000). The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* 26, 403–410. doi: 10.1038/82521

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z., and Zhang, S. (2011). Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. *Plant Cell* 23, 1639–1653. doi: 10.1105/tpc.111.084996

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. *Plant Physiol.* 88, 936–942. doi: 10.1104/pp.88.3.936

Métraux, J.-P. (2002). Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends Plant Sci.* 7, 332–334. doi: 10.1016/S1360-1385(02)02313-0

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006. doi: 10.1126/science.250.4983.1004

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M. A., Shulaev, V., et al. (2009). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.* 2, ra45. doi: 10.1126/scisignal.2000448

Mishina, T. E., Griebel, T., Geuecke, M., Attaran, E., and Zeier, J. (2008). "New insights into the molecular events underlying systemic acquired resistance," in *Biology of Plant-Microbe Interactions* Vol. 6, eds. M. Lorito, S. L. Woo, and F. Scala (St. Paul, MN: International Society for Molecular Plant-Microbe Interactions), 81.

Mishina, T. E., and Zeier, J. (2006). The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* 141, 1666–1675. doi: 10.1104/pp.106.081257

Mishina, T. E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant J.* 50, 500–513. doi: 10.1111/j.1365-313X.2007.03067.x

Misyura, M., Colasanti, J., and Rothstein, S. J. (2013). Physiological and genetic analysis of *Arabidopsis thaliana* anthocyanin biosynthesis mutants under chronic adverse environmental conditions. *J. Exp. Bot.* 64, 229–240. doi: 10.1093/jxb/ers328

Morikawa, T., Mizutani, M., Aoki, N., Watanabe, B., Saga, H., Saito, S., et al. (2006). Cytochrome P450 *CYP710A* encodes the sterol C-22 desaturase in Arabidopsis and tomato. *Plant Cell* 18, 1008–1022. doi: 10.1105/tpc.105.036012

Nakao, M., Nakamura, R., Kita, K., Inukai, R., and Ishikawa, A. (2011). Non-host resistance to penetration and hyphal growth of *Magnaporthe oryzae* in Arabidopsis. *Sci. Rep.* 1, 171. doi: 10.1038/srep00171

Návarová, H., Bernsdorff, F., Döring, A.-C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24, 5123–5141. doi: 10.1105/tpc.112.103564

Nawrath, C., and Métraux, J.-P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11, 1393–1404. doi: 10.1111/j.1365-313X.2007.03039.x

Ndamukong, I., Abdallat, A. A., Thurow, C., Fode, B., Zander, M., Weigel, R., et al. (2007). SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription. *Plant J.* 50, 128–139.

Neal, C. S., Fredericks, D. P., Griffiths, C. A., and Neale, A. D. (2010). The characterisation of AOP2: a gene associated with the biosynthesis of aliphatic alkenyl glucosinolates in *Arabidopsis thaliana*. *BMC Plant Biol.* 10:170. doi: 10.1186/1471-2229-10-170

Nickstadt, A., Thomma, B. P. H. J., Feussner, I., Kangasjärvi, J., Zeier, J., Loeffler, C., et al. (2004). The jasmonate-insensitive mutant *jin1* shows increased resistance to biotrophic as well as necrotrophic pathogens. *Mol. Plant Pathol.* 5, 425–434. doi: 10.1111/j.1364-3703.2004.00242.x

Niderman, T., Genetet, I., Bruyère, T., Gees, R., Stintzi, A., Legrand, M., et al. (1995). Pathogenesis-related

PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. *Plant Physiol.* 108, 17–27. doi: 10.1104/pp.108.1.17

Nobuta, K., Okrent, R. A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M. C., et al. (2007). The GH3 acyl adenylase family member PBS3 regulates salicylic acid-dependent defense responses in Arabidopsis. *Plant Physiol.* 144, 1144–1156. doi: 10.1104/pp.107.097691

Noël, L. D., Cagna, G., Stuttmann, J., Wirthmüller, L., Betsuyaku, S., Witte, C. P., et al. (2007). Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. *Plant Cell* 19, 4061–4076. doi: 10.1105/tpc.107.051896

Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., et al. (2012). Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in Arabidopsis. *Plant Cell* 24, 3795–3804. doi: 10.1105/tpc.112.098343

Okrent, R. A., Brooks, M. D., and Wildermuth, M. C. (2009). Arabidopsis GH3.12 (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. *J. Biol. Chem.* 284, 9742–9754. doi: 10.1074/jbc.M806662200

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Rayapuram, C., Wu, J., Haas, C., and Baldwin, I. T. (2008). *PR-13/Thionin* but not *PR-1* mediates bacterial resistance in *Nicotiana attenuata* in nature, and neither influences herbivore resistance. *Mol. Plant-Microbe Interact.* 21, 988–1000. doi: 10.1094/MPMI-21-7-0988

Redman, J. C., Haas, B. J., Tanimoto, G., and Town, C. D. (2004). Development and evaluation of an Arabidopsis whole genome Affymetrix probe array. *Plant J.* 38, 545–561. doi: 10.1111/j.1365-313X.2004.02061.x

Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J., et al. (2008). A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5638–5643. doi: 10.1073/pnas.0711301105

Rietz, S., Stamm, A., Malonek, S., Wagner, S., Becker, D., Medina-Escobar, N., et al. (2011). Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. *New Phytol.* 191, 107–119. doi: 10.1111/j.1469-8137.2011.03675.x

Rose, J. K., Braam, J., Fry, S. C., and Nishitani, K. (2002). The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: Current perspectives and a new unifying nomenclature. *Plant Cell Physiol.* 43, 1421–1435. doi: 10.1093/pcp/pcf171

Saito, K., Yonekura-Sakakibara, K., Nakabayashi, R., Higashi, Y., Yamazaki, M., Tohge, T., et al. (2013). The flavonoid biosynthetic pathway in Arabidopsis: Structural and genetic diversity. *Plant Physiol. Biochem.* doi: 10.1016/j.plaphy.2013.02.001. [Epub ahead of print].

Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Häweker, H., et al. (2009). Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* 28, 3439–3449. doi: 10.1038/emboj.2009.263

Schenk, P. M., Kazan, K., Manners, J. M., Anderson, J. P., Simpson, R. S., Wilson, I. W., et al. (2003). Systemic gene expression in Arabidopsis during an incompatible interaction with *Alternaria brassicicola*. *Plant Physiol.* 132, 999–1010. doi: 10.1104/pp.103.021683

Schultz, C. J., Rumsewicz, M. P., Johnson, K. L., Jones, B. J., Gaspar, Y. M., and Bacic, A. (2002). Using genomic resources to guide research directions. The arabinogalactan protein gene family as a test case. *Plant Physiol.* 129, 1448–1463. doi: 10.1104/pp.003459

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci.* 4:30. doi: 10.3389/fpls.2013.00030

Shang, Y., Yan, L., Liu, Z. Q., Cao, Z., Mei, C., Xin, Q., et al. (2010). The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* 22, 1909–1935. doi: 10.1105/tpc.110.073874

Shulaev, V., Leon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *Plant Cell* 7, 1691–1701.

Sønderby, I. E., Geu-Flores, F., and Halkier, B. A. (2010). Biosynthesis of glucosinolates – gene discovery and beyond. *Trends Plant Sci.* 15, 283–290. doi: 10.1016/j.tplants.2010.02.005

Song, J., Lu, H., McDowell, J. M., and Greenberg, J. (2004a). A key role for *ALD1* in activation of local and systemic defenses in *Arabidopsis*. *Plant J.* 40, 200–212. doi: 10.1111/j.1365-313X.2004.02200.x

Song, J., Lu, H., and Greenberg, J. (2004b). Divergent roles in *Arabidopsis thaliana* development and defense of two homologous genes, *ABERRANT GROWTH AND DEATH2* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1*, encoding novel aminotransferases. *Plant Cell* 16, 353–366. doi: 10.1105/tpc.019372

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Spoel, S. H., Koornneef, A., Claessens, S. M., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159

Stehle, F., Brandt, W., Stubbs, M. T., Milkowski, C., and Strack, D. (2009). Sinapoyltransferases in the light of molecular evolution. *Phytochemistry* 70, 1652–1662. doi: 10.1016/j.phytochem.2009.07.023

Sticher, L., Mauch-Mani, B., and Métraux, J. P. (1997). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 35, 235–270.

Tanaka, H., Osakabe, Y., Katsura, S., Mizuno, S., Maruyama, K., Kusakabe, K., et al. (2012). Abiotic stress-inducible receptor-like kinases negatively control ABA signaling in Arabidopsis. *Plant J.* 70, 599–613. doi: 10.1111/j.1365-313X.2012.04901.x

Thibaud-Nissen, F., Wu, H., Richmond, T., Redman, J. C., Johnson, C., Green, R., et al. (2006). Development of Arabidopsis whole-genome microarrays and their application to the discovery of binding sites for the TGA2 transcription factor in salicylic acid-treated plants. *Plant J.* 47, 152–162. doi: 10.1111/j.1365-313X.2006.02770.x

Thilmony, R., Underwood, W., and He, S. Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* 46, 34–53. doi: 10.1111/j.1365-313X.2006.02725.x

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature* 448, 661–665. doi: 10.1038/nature05960

Thordal-Christensen, H. (2003). Fresh insights into processes of nonhost resistance. *Curr. Opin. Plant Biol.* 6, 351–357. doi: 10.1016/S1369-5266(03)00063-3

Tognetti, V. B., Van Aken, O., Morreel, K., Vandenbroucke, K., van de Cotte, B., De Clercq, I., et al. (2010). Perturbation of indole-3-butyric acid homeostasis by the UDP-glucosyltransferase UGT74E2 modulates Arabidopsis architecture and water stress tolerance. *Plant Cell* 22, 2660–2679. doi: 10.1105/tpc.109.071316

Tran, L. S., Nakashima, K., Sakuma, Y., Simpson, S. D., Fujita, Y., Maruyama, K., et al. (2004). Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the *early responsive to dehydration stress 1* promoter. *Plant Cell* 16, 2481–2498. doi: 10.1105/tpc.104.022699

Truman, W., Bennett, M. H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1075–1080. doi: 10.1073/pnas.0605423104

Truman, W., de Torres-Zabala, M., and Grant, M. (2006). Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *Plant J.* 46, 14–33. doi: 10.1111/j.1365-313X.2006.02672.x

Truman, W. M., Bennett, M. H., Turnbull, C. G., and Grant, M. R. (2010). Arabidopsis auxin mutants are compromised in systemic acquired resistance and exhibit aberrant accumulation of various indolic compounds. *Plant Physiol.* 152, 1562–1573. doi: 10.1104/pp.109.152173

Tsuchisaka, A., Yu, G., Jin, H., Alonso, J. M., Ecker, J. R., Zhang, X., et al. (2009). A combinatorial interplay among the

1-aminocyclopropane-1-carboxylate isoforms regulates ethylene biosynthesis in *Arabidopsis thaliana*. *Genetics* 183, 979–1003. doi: 10.1534/genetics.109.107102

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS Genet.* 5:e1000772. doi: 10.1371/journal.pgen.1000772

von Saint Paul, V., Zhang, W., Kanawati, B., Geist, B., Faus-Keßler, T., Schmitt-Kopplin, P., et al. (2011). The Arabidopsis glucosyltransferase UGT76B1 conjugates isoleucic acid and modulates plant defense and senescence. *Plant Cell* 23, 4124–4145. doi: 10.1105/tpc.111.088443

Ward, J. L., Forcat, S., Beckmann, M., Bennett, M., Miller, S. J., Baker, J. M., et al. (2010). The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. *tomato*. *Plant J.* 63, 443–457. doi: 10.1111/j.1365-313X.2010.04254.x

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* 2:e123. doi: 10.1371/journal.ppat.0020123

Wang, L., Mitra, R. M., Hasselmann, K. D., Sato, M., Lenarz-Wyatt, L., Cohen, J. D., et al. (2008). The genetic network controlling the Arabidopsis transcriptional response to *Pseudomonas syringae* pv. *maculicola*: roles of major regulators and the phytotoxin coronatine. *Mol. Plant-Microbe Interact.* 21, 1408–1420. doi: 10.1094/MPMI-21-11-1408

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L. V., Katagiri, F., et al. (2011). CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *Plant J.* 67, 1029–1041. doi: 10.1111/j.1365-313X.2011.04655.x

Weigel, R. R., Pfitzner, U. M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates *PR* gene expression in Arabidopsis. *Plant Cell* 17, 1279–1291. doi: 10.1105/tpc.104.027441

Wiermer, M., Feys, B. J., and Parker, J. E. (2005), Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* 8, 383–389. doi: 10.1016/j.pbi.2005.05.010

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565. doi: 10.1038/35107108

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647. doi: 10.1016/j.celrep.2012.05.008

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* 18, 1310–1326. doi: 10.1105/tpc.105.037523

Yamamoto, A., Bhuiyan, M. N., Waditee, R., Tanaka, Y., Esaka, M., Oba, K., et al. (2005). Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and *Arabidopsis* plants. *J. Exp. Bot.* 56, 1785–1796. doi: 10.1093/jxb/eri167

Zander, M., Chen, S., Imkampe, J., Thurow, C., and Gatz, C. (2012). Repression of the *Arabidopsis thaliana* jasmonic acid/ethylene-induced defense pathway by TGA-interacting glutaredoxins depends on their C-terminal ALWL motif. *Mol. Plant* 5, 831–840. doi: 10.1093/mp/ssr113

Zander, M., La Camera, S., Lamotte, O., Métraux, J.-P., and Gatz, C. (2010). *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210. doi: 10.1111/j.1365-313X.2009.04044.xc

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., et al. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl Acad. Sci. U.S.A.* 107, 18220–18225. doi: 10.1073/pnas.1005225107

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F., and Glazebrook, J. (1998). *PAD4* functions upstream from salicylic acid to control defence responses in Arabidopsis. *Plant Cell* 10, 1021–1030.

Zoeller, M., Stingl, N., Krischke, M., Fekete, A., Waller, F., Berger, S., et al. (2012). Lipid profiling of the Arabidopsis hypersensitive response reveals specific lipid peroxidation and fragmentation processes: biogenesis of pimelic and azelaic acid. *Plant Physiol.* 160, 365–378. doi: 10.1104/pp.112.202846

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 March 2013; accepted: 21 June 2013; published online: 15 July 2013.

Citation: Gruner K, Griebel T, Návarová H, Attaran E and Zeier J (2013) Reprogramming of plants during systemic acquired resistance. Front. Plant Sci. 4:252. doi: 10.3389/fpls.2013.00252

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Gruner, Griebel, Návarová, Attaran and Zeier. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 12 April 2013
doi: 10.3389/fpls.2013.00088

The *Arabidopsis* NIMIN proteins affect NPR1 differentially

Meike Hermann‡, Felix Maier‡, Ashir Masroor, Sofia Hirth†, Artur J. P. Pfitzner and Ursula M. Pfitzner*

FG Allgemeine Virologie, Institut für Genetik, Universität Hohenheim, Stuttgart, Germany

Edited by:
Saskia C. Van Wees, Utrecht University, Netherlands

Reviewed by:
Steven H. Spoel, University of Edinburgh, UK
Daguang Cai, Christian Albrechts University of Kiel, Germany

***Correspondence:**
Ursula M. Pfitzner, FG Allgemeine Virologie, Institut für Genetik, Universität Hohenheim, Emil-Wolff-Strasse 14, D-70593 Stuttgart, Germany.
e-mail: pfitzner@uni-hohenheim.de

†Present address:
Sofia Hirth, Molecular Cardiology, Department of Internal Medicine II, University Hospital Ulm, Albert-Einstein-Allee 23, D-89081 Ulm, Germany.

‡*Meike Hermann and Felix Maier have contributed equally to this work.*

NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is the central regulator of the pathogen defense reaction systemic acquired resistance (SAR). NPR1 acts by sensing the SAR signal molecule salicylic acid (SA) to induce expression of *PATHOGENESIS-RELATED* (*PR*) genes. Mechanistically, NPR1 is the core of a transcription complex interacting with TGA transcription factors and NIM1-INTERACTING (NIMIN) proteins. *Arabidopsis* NIMIN1 has been shown to suppress NPR1 activity in transgenic plants. The *Arabidopsis NIMIN* family comprises four structurally related, yet distinct members. Here, we show that *NIMIN1*, *NIMIN2*, and *NIMIN3* are expressed differentially, and that the encoded proteins affect expression of the SAR marker *PR-1* differentially. *NIMIN3* is expressed constitutively at a low level, but *NIMIN2* and *NIMIN1* are both responsive to SA. While *NIMIN2* is an immediate early SA-induced and *NPR1*-independent gene, *NIMIN1* is activated after *NIMIN2*, but clearly before *PR-1*. Notably, *NIMIN1*, like *PR-1*, depends on *NPR1*. In a transient assay system, NIMIN3 suppresses SA-induced *PR-1* expression, albeit to a lesser extent than NIMIN1, whereas NIMIN2 does not negatively affect *PR-1* gene activation. Furthermore, although binding to the same domain in the C-terminus, NIMIN1 and NIMIN2 interact differentially with NPR1, thus providing a molecular basis for their opposing effects on NPR1. Together, our data suggest that the *Arabidopsis* NIMIN proteins are regulators of the SAR response. We propose that NIMINs act in a strictly consecutive and SA-regulated manner on the SA sensor protein NPR1, enabling NPR1 to monitor progressing threat by pathogens and to promote appropriate defense gene activation at distinct stages of SAR. In this scenario, the defense gene *PR-1* is repressed at the onset of SAR by SA-induced, yet instable NIMIN1.

Keywords: NIM1-INTERACTING (NIMIN) proteins, *NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1* (*NPR1*), *PATHOGENESIS-RELATED GENE1* (*PR-1*), plant defense gene activation, protein–protein interaction, salicylic acid (SA), systemic acquired resistance (SAR)

INTRODUCTION

Plants have evolved different layers of defense to recognize and combat invading microbes (Jones and Dangl, 2006). The immune response systemic acquired resistance (SAR) is launched after primary infection and activation of effector-triggered immunity (ETI) accompanied by formation of necrosis at the sites of pathogen invasion. SAR becomes effective in non-infected plant tissue far away from the pathogen penetration sites (Ross, 1961). The response fends off secondary infections by diverse types of biotrophic pathogens and is long-lasting. The local signal to induce SAR in non-infected leaves is salicylic acid (SA; Vernooij et al., 1994). Levels of free and conjugated SA rise not only in infected necrotic tissue, but also systemically in non-infected leaves (Malamy et al., 1990; Métraux et al., 1990). This increase in SA concentration is paralleled by local and systemic induction of various *PATHOGENESIS-RELATED* (*PR*) genes (van Loon and van Kammen, 1970; Ward et al., 1991; van Loon et al., 2006). Some *PR* genes, e.g., *PR-1*, can be induced solely by exogenous application of SA or its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; White, 1979; Vernooij et al., 1995; Friedrich et al., 1996; Lawton et al., 1996). Furthermore, it has been shown that SA-treated tobacco and *Arabidopsis* plants expressing *PR-1* genes display SAR (White, 1979; Uknes et al., 1992, 1993). Thus, accumulation of *PR-1* transcripts and PR-1 proteins either in non-infected parts of plants exhibiting necrosis or in response to exogenous application of SA serves as marker for the SAR resistance reaction.

The central regulator of SAR is *NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1* (*NPR1*). The gene was identified from *Arabidopsis* mutants compromised in chemical induction of *PR* genes and in resistance to fungal infection (Cao et al., 1997; Ryals et al., 1997; Shah et al., 1997). Overexpression experiments strongly suggest that NPR1 is active only after SA induction (Cao et al., 1998; Friedrich et al., 2001). The *Arabidopsis* NPR1 family encompasses six members, NPR1 to NPR6, and recent evidence indicates that SA signals directly through some members. However, the mechanism of how SA acts on NPR1 family proteins is controversial. First, it has been demonstrated that NPR1 from *Arabidopsis* (At) and two NPR1 family members from tobacco (Nt) alter some of their biochemical capabilities in response to the SA signal molecule in a heterologous yeast system in absence of any other plant protein (Maier et al., 2011). For example, Nt NPR1 gains transcription activity, when SA is added to yeast growth medium. The data indicate that NPR1 family proteins are able to sense SA, and that they undergo

an alteration upon perception of SA. Consequently, *Arabidopsis* NPR1 family members have been found to bind SA *in vitro* (Fu et al., 2012; Wu et al., 2012), albeit with very different affinities. While NPR4 is a high affinity receptor and NPR3 is a lower affinity receptor, SA appears to bind only very weakly to NPR1. It has been proposed that *PR-1* gene activation in the course of SAR is regulated through availability of NPR1, which, in turn, is controlled by cytoplasmic oligomer–nuclear monomer shuttling and by differential interaction of NPR1 with SA-perceiving NPR4 and NPR3 in the nucleus (Mou et al., 2003; Fu et al., 2012). In two other models, SA perception during SAR has, however, been attributed to the NPR1 protein, itself. Wu et al. (2012) have suggested that NPR1 binds SA via the transition metal copper in a complex with two cysteine residues, Cys-521 and Cys-529, and that, upon SA binding, a C-terminal transactivation domain is released from the N-terminal autoinhibitory BTB/POZ (broad complex, tramtrack, and bric à brac/pox virus and zinc finger) domain. Curiously, only *Arabidopsis* NPR1 contains two closely spaced cysteine residues in its C-terminus. In a third model, based on biochemical evidence obtained in the heterologous yeast system, two distinct domains in the C-terminus of NPR1 proteins have been implicated in sensing the SA signal (Maier et al., 2011). These domains are highly conserved in NPR1 proteins from diverse species and they are also conserved in the NPR1 paralogs NPR2, NPR3, and NPR4 from *Arabidopsis* and in tobacco NPR3 (also known as NIM1-LIKE1). One domain comprises the penta-amino acid motif LENRV (amino acids 429–433). The LENRV motif imposes SA sensitivity on NPR1 proteins from *Arabidopsis* and tobacco in yeast. The signature is altered in the non-functional *nim1-4* mutant (R432K; Ryals et al., 1997). The latter model is corroborated by genetic evidence provided through an *en masse in planta* screen for *Arabidopsis* insensitive to the functional SA analog BTH (Canet et al., 2010). In this screen, dozens of *npr1* alleles were identified, and the mutants have been found to be clustered in the same two regions identified independently by biochemical dissection of NPR1 family proteins in yeast. The *nim1-4* mutant was isolated three times. On the contrary, Cys-521 and Cys-529 were not uncovered genetically.

The SA sensor protein NPR1 interacts with two groups of proteins. TGA transcription factors connect NPR1 with SA-responsive *as-1*-like *cis*-acting elements present in the promoters of *PR-1* genes from tobacco and *Arabidopsis* (Lebel et al., 1998; Strompen et al., 1998; Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). This finding is consistent with several reports showing that NPR1 proteins from *Arabidopsis*, tobacco, and rice promote transcription activation in diverse systems (Rochon et al., 2006; Maier et al., 2011; Chern et al., 2012). The data imply that NPR1 is the core of a transcription complex on *PR* gene promoters. In addition to TGA factors, NPR1 interacts with the group of small NIM1-INTERACTING (NIMIN) proteins (Weigel et al., 2001). Like *NPR1*, *NIMIN* genes are dispersed in the whole plant kingdom (Chern et al., 2005; Zwicker et al., 2007). NIMIN proteins harbor nuclear localization signals, and thus target NPR1 in the nucleus (Weigel et al., 2001; Chern et al., 2005; Zwicker et al., 2007). However, their functional significance was not evident, when NIMINs were first identified.

Arabidopsis contains four *NIMIN* genes, *NIMIN1*, *NIMIN1b*, *NIMIN2*, and *NIMIN3* (Weigel et al., 2001). Of these, *NIMIN1* and *NIMIN2* have been studied in some detail. Both genes are strongly up-regulated by SA. In contrast, the two genes are not induced significantly in pathogen-infected necrotic tissue displaying ETI (Glocova et al., 2005). Hence, *NIMIN1* and *NIMIN2* seem to be specifically linked to the SA-dependent SAR response, rather than to ETI. Similarly, tobacco *NIMIN2*-type mRNAs accumulate in response to the SA signal molecule (Horvath et al., 1998; Zwicker et al., 2007). Although clearly structurally related, the *Arabidopsis* NIMIN proteins are distinct from each other. For example, they interact differentially with NPR1 (Weigel et al., 2001). NIMIN3 interacts with the At NPR1 N-terminal half, whereas NIMIN1, NIMIN1b, and NIMIN2 possess similar motifs by which they bind to the At NPR1 C-terminal third. In the C-terminus of Nt NPR1, the binding region of SA-induced NIMIN2-type proteins has been mapped from amino acids 494 to 510 (Maier et al., 2011). Notably, several *npr1* mutant alleles have been uncovered in the corresponding region of At NPR1, all of which affect responsiveness to BTH *in planta* (Canet et al., 2010). Furthermore, occurrence of the interaction domain for inducible NIMIN2-type proteins and the LENRV domain is coincident in NPR1 proteins and its paralogs from many species. Thus, these two domains appear to be intimately connected with the SA response.

The functional significance of NIMIN proteins for NPR1 activity has been addressed in overexpression experiments. Both *Arabidopsis* NIMIN1 and NEGATIVE REGULATOR OF DISEASE RESISTANCE (NRR), a NIMIN homolog from rice, are able to suppress induction of *PR* genes and to cause enhanced susceptibility to bacterial pathogens in transgenic plants (Chern et al., 2005, 2008; Weigel et al., 2005). From these data, it has been concluded that NIMIN proteins are repressors of NPR1. However, in tobacco, constitutive overexpression of Nt NIMIN2a produced only a delay in PR-1 protein accumulation, and it has been suggested that NIMIN proteins, although negatively affecting NPR1 activity, are, at bottom, positive regulators of NPR1-mediated *PR* gene induction (Zwicker et al., 2007). Apart from NIMIN1, the biological significance of other *Arabidopsis* NIMIN family members has not yet been addressed. Here, we provide evidence that the *Arabidopsis* NIMIN proteins affect NPR1 differentially at distinct stages of SAR, thus enabling the plant to strictly control defense gene activation in tissue distant from sites of pathogen entry undergoing ETI.

RESULTS

NIMIN3 IS NOT RESPONSIVE TO PLANT DEFENSE SIGNALS

Previously, we have shown that *NIMIN1* and *NIMIN2* are strongly induced by treatment of *Arabidopsis* plants with SA or Bion®, a commercial plant growth regulator containing the functional SA analog BTH, and that this induction is due to transcriptional gene activation (Weigel et al., 2001, 2005; Glocova et al., 2005). To further elucidate the functional relevance of *NIMIN* genes, we have now analyzed expression of *NIMIN3* in response to diverse signal molecules involved in plant defense reactions. Initially, transcript accumulation was monitored using reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. The primers used and the sizes of fragments generated by PCR from

plasmids carrying cDNAs for *NIMIN3* and various control genes are listed in **Table 1**. *NIMIN3* transcript levels were compared to expression of the *NIMIN1*, *NIMIN2*, and *PR-1* genes. Unlike *NIMIN1*, *NIMIN2*, and *PR-1*, expression of *NIMIN3* was neither induced by SA nor BTH (**Figure 1A**). Moreover, jasmonate (JA), another plant defense signal, had no effect on either of the *NIMIN* genes (data not shown). However, we were able to detect *NIMIN3* transcripts in several independent RNA preparations irrespective of whether they had been isolated from control or chemically induced plant tissue (**Figures 1A** and **2A**), suggesting that *NIMIN3* may be expressed constitutively at a low level. To address this question, we isolated 1.4 kb of the *NIMIN3* 5′-upstream region and fused it to the β-glucuronidase (*GUS*) reporter gene. The chimeric gene was transferred to the tobacco genome, and GUS enzyme activity was determined in seven independent primary transformants, all containing intact copies of the reporter gene construct (data not shown). As compared to transgenic tobacco plants carrying analogous $NIMIN1_{Pro}$*::GUS* or $NIMIN2_{Pro}$*::GUS* constructs (0.8 and 0 GUS units on an average, respectively; Glocova et al., 2005), untreated plants containing $NIMIN3_{Pro}$*::GUS* exhibited constitutive GUS enzyme activity (14.7 GUS units on an average; **Figure 1B**). Reporter gene expression from the $NIMIN3_{Pro}$*::GUS* construct in the tobacco genome was not enhanced significantly by treatment of plants with SA (0.3 and 1 mM; 17.6 GUS units on an average), BTH (0.34 mM), methyl JA (MeJA; 0.1 mM), or H_2O_2 (0.1 and 1 mM; data not shown). Likewise, gene expression from the *NIMIN3* promoter was not elevated by elicitation of HR or by exogenous application of the phytohormones 2,4-dichlorophenoxy acetic acid (2,4-D), gibberellic acid (GA), indole-3-acetic acid (IAA), or 1-naphthalene acetic acid (NAA; 0.01 and 0.1 mM each; data not shown). As determined by histochemical staining, *NIMIN3*-mediated reporter enzyme activity is mainly localized in leaf tissue (**Figure 1B**). Of note, *NIMIN3* gene expression is independent from an intact *NPR1* gene (**Figure 2A**).

SALICYLIC ACID-MEDIATED INDUCTION OF *NIMIN1* AND *NIMIN2* PROCEEDS THROUGH SEPARATE PATHWAYS

RNA analyses as depicted in **Figure 1A** had shown that *NIMIN1* was expressed only after induction, just as *PR-1*, while *NIMIN2* expression was occasionally observed prior to chemical treatment of plants. This finding was unexpected since the *NIMIN2* promoter exhibits clear chemical induction in transgenic tobacco plants (0 GUS units and 265.0 GUS units on an average for water and SA treatment, respectively, $n = 10$; **Figure 1B**; Glocova et al., 2005). It therefore seemed of interest to analyze regulation of the *NIMIN1* and *NIMIN2* genes in closer detail.

Initially, we used two *npr1* mutants, *npr1-1* and *npr1-2*, which are not able to support *PR-1* gene induction (Cao et al., 1994; Glazebrook et al., 1996). Surprisingly, *NIMIN1*, like *PR-1*, was inactive in absence of a functional *NPR1* gene (**Figure 2A**). Yet, *NIMIN2* expression was clearly detectable in both *npr1* mutants, although, in some experiments, *NIMIN2* transcript levels appeared to accumulate to lower overall levels in *npr1* than in wild-type plants (**Figure 2A** and data not shown). Our data are in conflict with another report. Blanco et al. (2009) have described that expression of both *NIMIN1* and *NIMIN2* is abolished in the *npr1-1* mutant. To support our results, we verified the identity of the *NIMIN2* RT-PCR products by digestion with restriction enzymes (data not shown). Hence, *NIMIN2* expression, unlike *NIMIN1* and *PR-1* expression, may be either independent or only partly dependent on *NPR1*. Furthermore, the kinetics of gene induction turned out to be different between *NIMIN1* and *NIMIN2*. Both genes are expressed transiently after SA application (**Figure 2B**). Yet, *NIMIN2* gene expression started immediately (0.5 h) after SA treatment, reached its maximum early (after 1 h) and was maintained at a high level for 24 h (**Figure 2B**). Thus, *NIMIN2* seems to be an immediate early SA responsive gene, as suggested previously for the tobacco *NIMIN2a* gene (Horvath et al., 1998). *NIMIN1* transcripts, on the other side, became most abundant only around 2 h after SA application (**Figure 2B**). This is clearly later than the onset of *NIMIN2* expression, yet earlier than the onset of *PR-1* induction. Notably, *NIMIN1* expression appeared even more transient than *NIMIN2* expression and was already shut down when *PR-1* transcripts began to accumulate. The time course of *NIMIN1* gene induction shown here is in accordance with previous results obtained by northern blotting (Weigel et al., 2005). Together, our data strongly suggest that SA-mediated

Table 1 | Primers and control plasmids used in RT-PCR analyses.

Gene	Control plasmid	Primer	Sequence	Fragment size (bp)
NIMIN1	pGBT9/NIMIN1	N1fwd	5′-CGGGATCCATATGTATCCTAAACAATTTAG	449
		N1bck	5′-AACCCGGGCTACTACAATGCAAGATTAAGATC	
NIMIN2	pGBT9/NIMIN2	N2fwd	5′-ACGCGTAGAAGAAGATAACGG	330
		N2bck	5′-CTAACGCTGTCTGGTTCCGGT	
NIMIN3	pGBT9/NIMIN3	N3fwd	5′-GGGGATCCATATGGACAGAGACAGAAAGAG	357
		N3bck	5′-TTCCCGGGCTACAGAGAAAGATTCAAGTC	
PR-1	pUC19/AtPR-1	PR1fwd	5′-GGGGATCCATATGAATTTTACTGGC	504
		PR1bck	5′-CTGAGCTCTTAGTATGGCTTCTCG	
Actin1	–	Act1	5′-CGATGAAGCTCAATCCAAACGA	302
		Act2	5′-CAGAGTCGAGCACAATACCG	

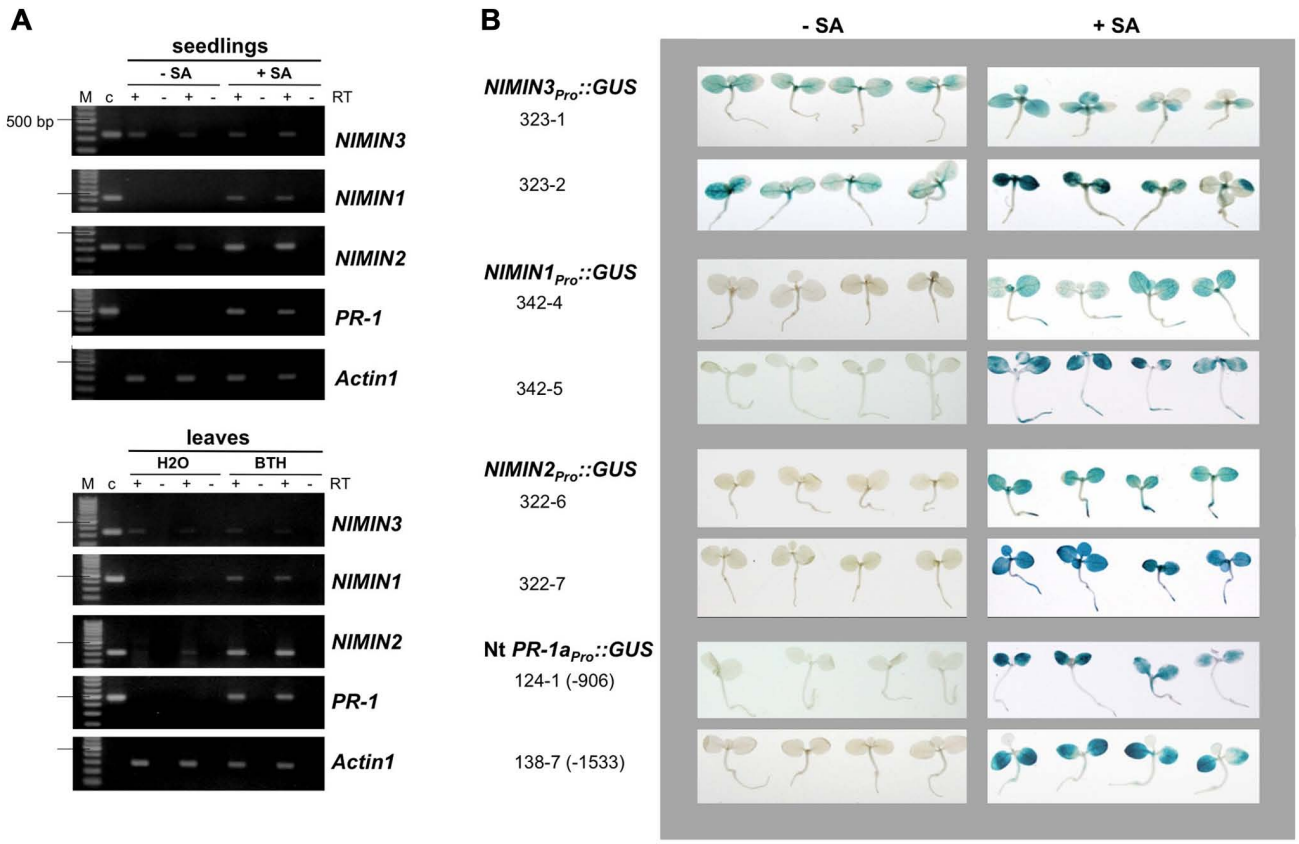


FIGURE 1 | *Arabidopsis NIMIN3* is expressed constitutively. (A) RT-PCR analyses of *NIMIN3* expression in *Arabidopsis* whole seedlings and leaf tissue. Expression of *NIMIN3* is compared to expression of *NIMIN1*, *NIMIN2*, and *PR-1*. RNA samples were isolated from 2-week-old whole seedlings grown either on MS medium or MS medium with addition of 0.3 mM SA and from leaves of 4-week-old plants 24 h after spraying with water or a suspension of Bion® containing 0.34 mM BTH. RT-PCR analyses were performed on DNase I-treated total RNA preparations in presence or absence of reverse transcriptase (RT) with primer combinations listed in **Table 1**. In lanes c, PCR products from 1 ng of plasmid DNAs carrying the respective cDNAs were loaded. The amplification of *Actin1* mRNA serves as an internal standard for different RNA samples used in the amplification reactions. **(B)** Expression of a $NIMIN3_{Pro}$*::GUS* reporter gene in transgenic tobacco seedlings. Expression from the *NIMIN3* promoter is compared to reporter gene expression from the *NIMIN1*, *NIMIN2*, and Nt *PR-1a* promoters. Tobacco seedlings (T1 generation) transformed with the indicated reporter genes were grown on MS medium with kanamycin or on selective medium supplemented with 0.3 mM SA. Two independent lines for each construct or, as in case of the Nt *PR-1a* promoter, two different constructs were analyzed. Seedlings were stained for GUS reporter enzyme activity when 4-weeks-old.

induction of the *NIMIN1* and *NIMIN2* genes proceeds through separate pathways.

The kinetics of gene induction were also monitored in tobacco seedlings containing $NIMIN_{Pro}$*::GUS* reporter gene constructs. Transgenic seeds were germinated on SA-containing medium. The germination of seeds occurred simultaneously for all lines analyzed, and the development of seedlings progressed similarly. GUS enzyme activities were first determined 7 days after sowing when small seedlings had emerged. With both $NIMIN2_{Pro}$*::GUS* and $NIMIN1_{Pro}$*::GUS*, we did not observe a clear induction profile (**Figure 2C**). GUS enzyme activity was already switched on to high levels early after germination. In contrast, *PR-1a* promoter activation and accumulation of the endogenous PR-1 proteins occurred with significant delay (**Figure 2C**). Thus, the kinetics of reporter gene activation from the *NIMIN1* and *NIMIN2* promoters in SA-treated tobacco seem to parallel the transcript accumulation patterns observed in *Arabidopsis*, i.e., *NIMIN* genes are induced by SA prior to *PR-1* genes. The data indicate that the molecular cues for early induction during the SAR response are contained within the 1 kb 5′-flanking regions of *NIMIN1* and *NIMIN2*, and that these cues are recognized in the heterologous species tobacco. Reporter gene expression from both the *NIMIN1* and *NIMIN2* promoters occurred in leaf and root tissue (**Figure 1B**). Likewise, green fluorescent protein (GFP) expression from the 0.8 kb *NIMIN1* promoter has been observed in roots, petioles, and leaves in transgenic *Arabidopsis* plants (Fonseca et al., 2010). This expression pattern distinguishes the SA-inducible *NIMIN1* and *NIMIN2* promoters from the *NIMIN3* promoter and the tobacco *PR-1a* promoter which are predominantly active in leaf tissue (**Figure 1B**).

NIMIN1 AND NIMIN3 SUPPRESS SALICYLIC ACID-INDUCED EXPRESSION FROM THE TOBACCO *PR-1a* PROMOTER

To unravel the functional significance of *NIMIN* gene expression at different times during the SAR response, we have developed an *in planta* assay for NIMIN activity. The gene coding for *GUS* under

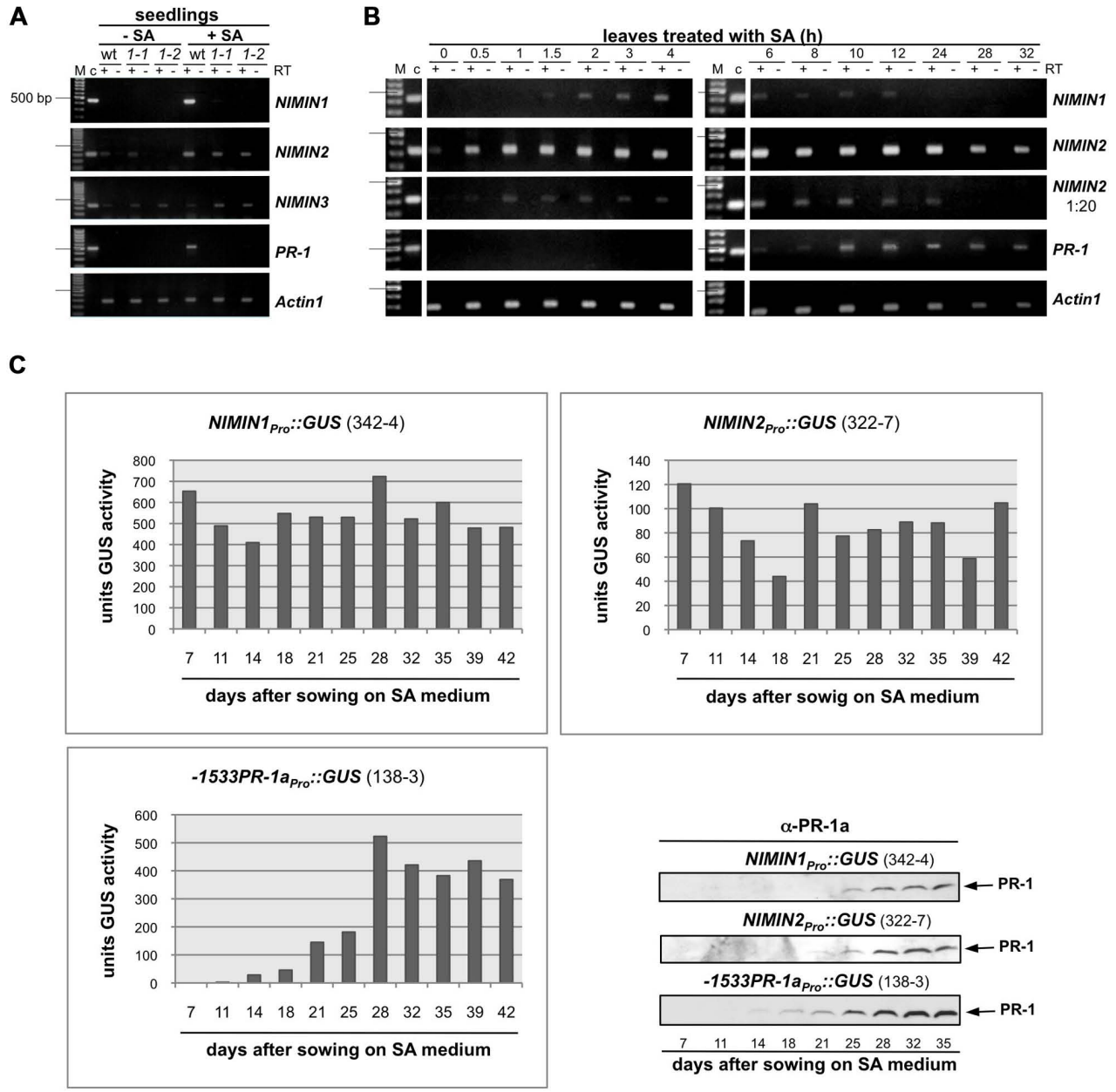


FIGURE 2 | Salicylic acid-induced *Arabidopsis NIMIN1* and *NIMIN2* are expressed differentially from each other and from *PR-1*. RNA samples were isolated from *Arabidopsis* seedlings or *Arabidopsis* leaves and analyzed as described in **Figure 1A**. Expression of *NIMIN1* and *NIMIN2* is compared to expression of *NIMIN3* and *PR-1*. **(A)** RT-PCR analyses of RNAs from wild-type (Col-0) and *npr1-1* and *npr1-2* mutant seedlings. *1-1, npr1-1; 1-2, npr1-2*. **(B)** RT-PCR analyses of RNAs from leaf tissue at different times after spraying plants with 1 mM SA. **(C)** Time course of SA-induced GUS reporter enzyme activities and PR-1 protein accumulation in tobacco seedlings transformed with $NIMIN1_{Pro}$*::GUS* or $NIMIN2_{Pro}$*::GUS*. Expression from the two *NIMIN* promoters is compared to reporter gene expression from the Nt *-1533PR-1a* promoter. For immunodetection of endogenous PR-1 proteins, equal amounts of protein were loaded in each lane of the SDS gels. Seedlings (T1 generation) were grown on selective medium with 0.3 mM SA. Similar results were obtained with independent lines of $NIMIN1_{Pro}$*::GUS*, $NIMIN2_{Pro}$*::GUS* and $-1533PR\text{-}1a_{Pro}$*::GUS*.

control of the tobacco *PR-1a* promoter (*-1533PR-1a*$_{Pro}$*::GUS*; Grüner and Pfitzner, 1994) was stably integrated in the genome of *Nicotiana benthamiana*. Several primary transformants were obtained all of which exhibited very strong and stringent induction of the reporter gene upon SA treatment of leaf tissue (data not shown). One typical line (3 GUS units uninduced and 1100 GUS units after SA treatment) was propagated, and T2 plants were used for infiltration experiments with an *Agrobacterium* strain carrying the gene for GFP (*mGFP4*) driven by the *Cauliflower mosaic virus* (CaMV) *35S RNA* promoter ($35S_{Pro}$*::mGFP4*). Infiltration of $35S_{Pro}$*::mGFP4* Agrobacteria yielded GUS enzyme activities only slightly above the background levels of non-infiltrated control

leaves, showing that agroinfiltration alone is not sufficient for efficient activation of the *PR-1a*$_{Pro}$*::GUS* reporter gene (**Figures 3A** and **4A**).

Next, we tested the influence of different NIMIN proteins on *PR-1a* gene induction after agroinfiltration of *N. benthamiana*. It has been shown previously that overexpression of *NIMIN1* suppresses SA-mediated *PR* gene induction and SAR in transgenic *Arabidopsis* plants (Weigel et al., 2005). However, the functional roles of NIMIN2 and NIMIN3 are not known. Initially, Agrobacteria adjusted to equal cell densities were infiltrated into leaves of

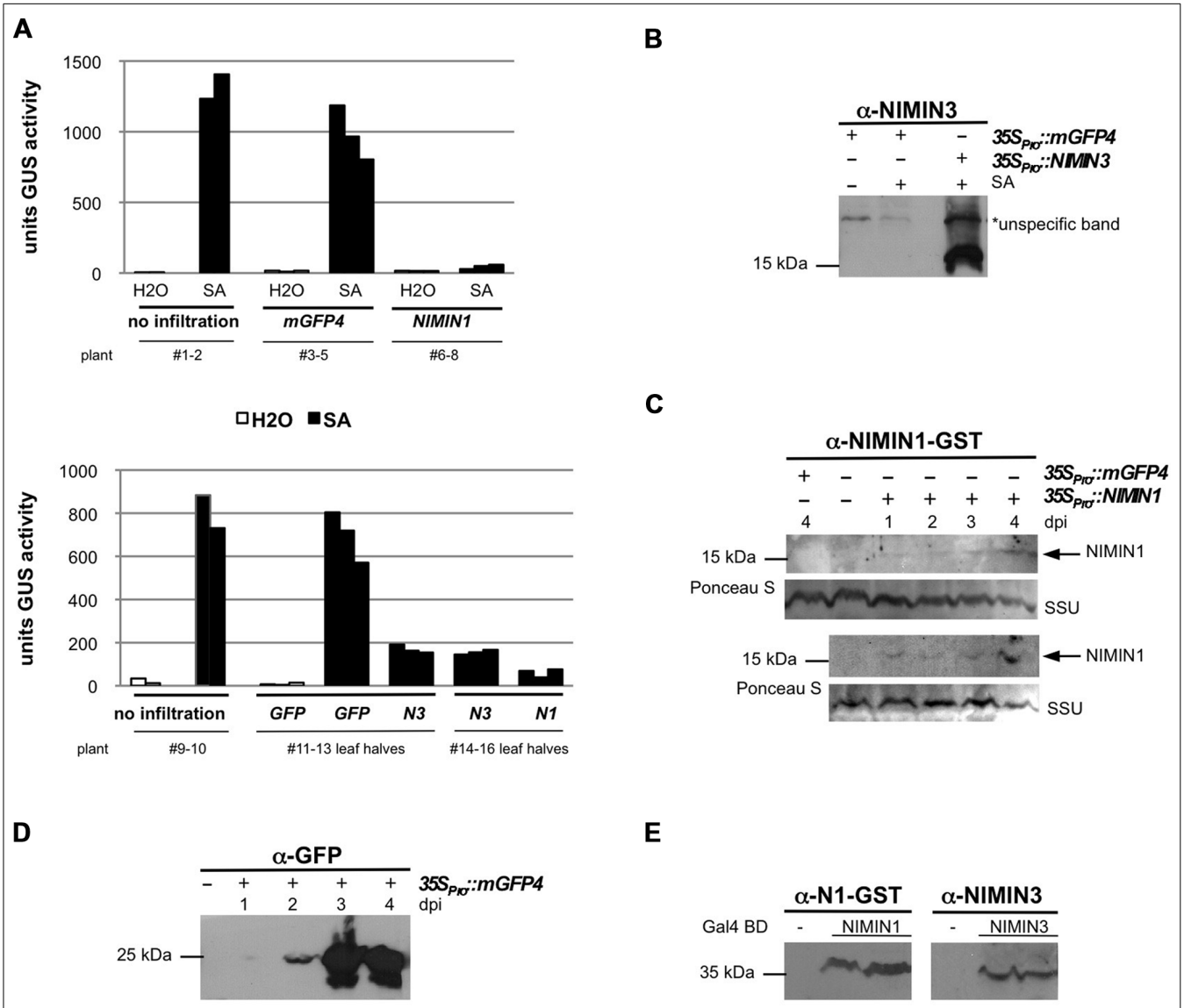


FIGURE 3 | ***Arabidopsis*** **NIMIN1 and NIMIN3 suppress salicylic acid-induced gene expression from the tobacco** ***PR-1a*** **promoter in** ***N. benthamiana***. **(A)** Effects of transient expression of *35S*$_{Pro}$*::NIMIN1* and *35S*$_{Pro}$*::NIMIN3* in an *N. benthamiana* reporter line with integrated *-1533PR-1a*$_{Pro}$*::GUS*. Three plants were infiltrated in parallel for each gene construct with *Agrobacterium* strains as indicated. For a better direct comparison, the two halves of the same leaf were infiltrated with Agrobacteria harboring *35S*$_{Pro}$*::NIMIN1* and *35S*$_{Pro}$*::NIMIN3,* respectively. Leaf disks excised from infiltrated leaf areas were floated on water or on 1 mM SA before determination of GUS enzyme activity. The three bars for each construct and treatment represent GUS activities from the three agroinfiltration experiments performed in parallel. Representative results are shown. N1, NIMIN1; N3, NIMIN3. **(B)** Immunodetection of NIMIN3 in extracts from agroinfiltrated and SA-floated leaf tissue. NIMIN3 accumulation was detected with a specific antiserum in an extract shown in **Figure 3A**. An unspecific band marked on the X-ray serves as loading control. Exposure of the X-ray film was for 1 min. **(C)** Immunodetection of NIMIN1 after agroinfiltration. Results from two independent time course experiments are shown. Leaf tissue was extracted after infiltration as indicated. Extracts were analyzed for protein accumulation with a specific antibody. As loading control, the region of the nitrocellulose filters with the small subunit of RuBisCO (SSU) stained with Ponceau S is shown. Exposure of the X-ray films was over night. dpi, days post-infiltration. **(D)** Immunodetection of green fluorescent protein (GFP) after agroinfiltration. Leaf tissue was extracted after infiltration as indicated. Exposure of the X-ray film was for 1 min. **(E)** Immunodetection of NIMIN1- and NIMIN3-Gal4 DNA binding domain (GBD) fusion proteins in extracts from transformed yeast. The NIMIN1 and NIMIN3 fusions were detected with the specific antisera used in **Figures 3B,C**.

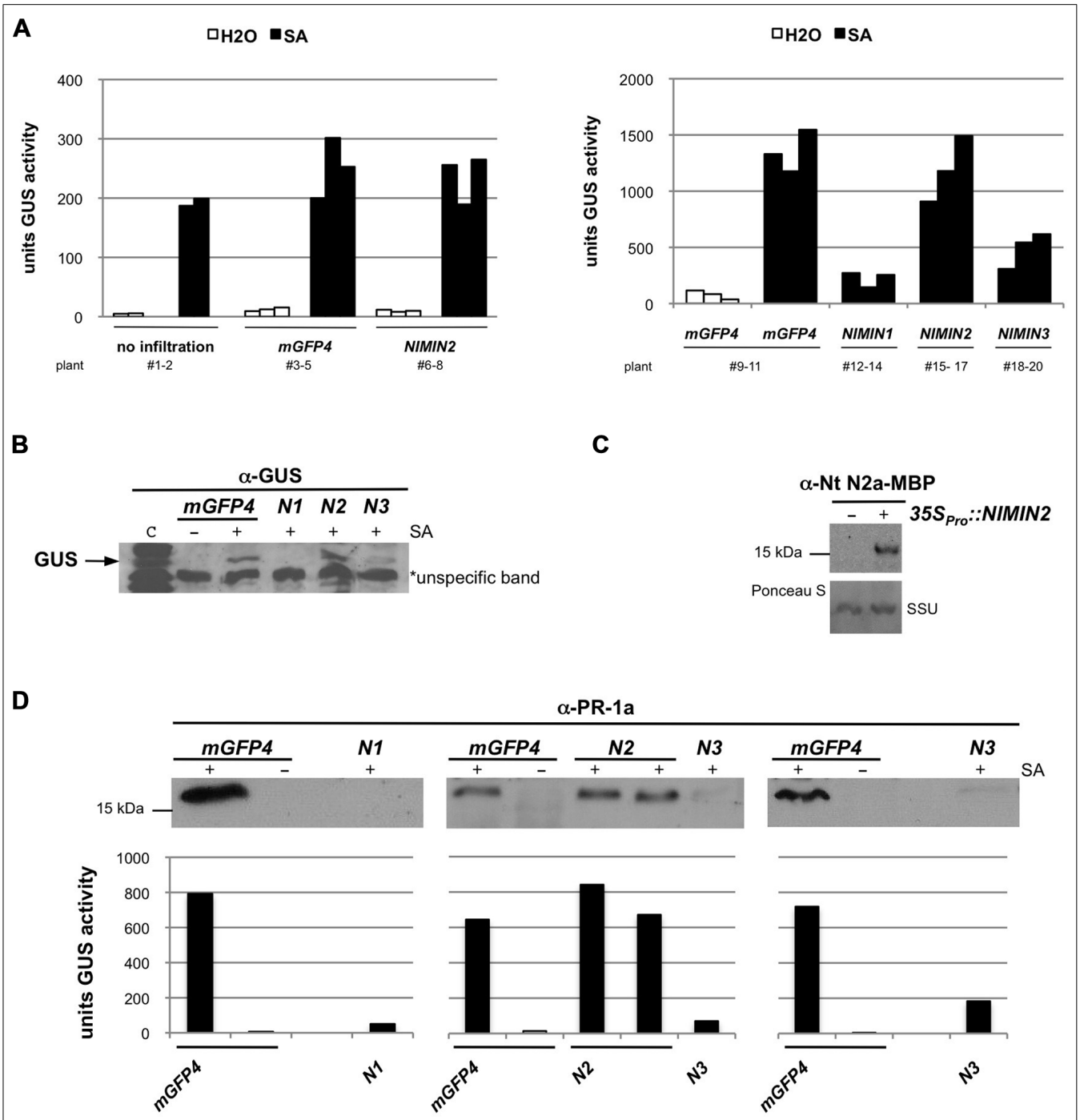


FIGURE 4 | ***Arabidopsis*** **NIMIN2 does not affect salicylic acid-induced gene expression from the tobacco** ***PR-1a*** **promoter in** ***N. benthamiana*****.** Transient expression assays and immunodetection were performed as described in **Figure 3**. N1, NIMIN1; N2, NIMIN2; N3, NIMIN3. **(A)** Effects of transient expression of *35S$_{Pro}$::NIMIN2* in the *N. benthamiana - 1533PR-1a$_{Pro}$::GUS* reporter line. The effects of NIMIN2 on the *PR-1a::GUS* reporter are compared to effects produced by NIMIN1 and NIMIN3. Representative results are shown. **(B)** Effects of transient expression of *35S$_{Pro}$::NIMIN1*, *35S$_{Pro}$::NIMIN2*, and *35S$_{Pro}$::NIMIN3* on accumulation of the GUS reporter protein in SA-treated leaf tissue. GUS accumulation was detected in extracts shown in **Figure 4A**. Lane c contains an extract from a tobacco plant stably transformed with *35S$_{Pro}$::GUS*. An unspecific band marked on the X-ray serves as loading control. **(C)** Immunodetection of NIMIN2 in agroinfiltrated tissue. NIMIN2 accumulation was detected with a specific antiserum in an extract shown in **Figure 4A**. **(D)** Effects of transient expression of *35S$_{Pro}$::NIMIN1*, *35S$_{Pro}$::NIMIN2*, and *35S$_{Pro}$::NIMIN3* on accumulation of the endogenous PR-1 protein in SA-treated *N. benthamiana* leaf tissue. GUS reporter enzyme activities of extracts analyzed for PR-1 protein accumulation are given below the immunodetections.

individual *N. benthamiana* plants with the *-1533PR-1a$_{Pro}$::GUS* reporter. In each experiment, three plants were infiltrated in parallel with the same *Agrobacterium* strain. After 4–5 days, disks were cut from leaf areas close to the infiltration sites. At this time, strong fluorescence was typically observed in tissue infiltrated with *35S$_{Pro}$::mGFP4* Agrobacteria, demonstrating efficient expression of the *GFP* reporter. GUS activity assays revealed that none of the NIMIN proteins is able to activate the *PR-1a$_{Pro}$::GUS* reporter gene on its own (**Figures 3A** and **4A** and data not shown). The excised leaf disks were then floated for 2 days on water or on a 1 mM SA solution. As controls, disks from non-agroinfiltrated leaves were incubated on water and SA. After floating, proteins were extracted from leaf tissue, and GUS reporter activity was determined. In other experiments, we have infiltrated the two halves of a single leaf with *Agrobacterium* strains harboring different constructs in order to allow an even more direct comparison between effects exerted by the respective NIMIN proteins. Consistent with what has been described for *NIMIN1* overexpression in transgenic *Arabidopsis* plants, agroinfiltration of *35S$_{Pro}$::NIMIN1* bacteria suppressed SA-mediated *PR-1a* promoter activation to nearly background levels as compared to GUS levels observed in *GFP* expressing leaf disks floated on water (**Figures 3A** and **4A**). Quite surprisingly, *NIMIN3* overexpression, too, clearly repressed *GUS* reporter gene induction from the Nt *PR-1a* promoter in *N. benthamiana* (**Figures 3A** and **4A**). Repression with NIMIN3 was, however, weaker than with NIMIN1 (**Figures 3A** and **4A**). The presence of NIMIN1 and NIMIN3 proteins in infiltrated *N. benthamiana* leaf tissue was monitored by immunodetection using specific antisera. NIMIN3 accumulated to high levels. The protein was readily detected in extracts from SA-floated leaf disks and also in extracts from agroinfiltrated tissue without SA induction (**Figure 3B** and data not shown). In contrast, we were not able to detect *NIMIN1* expression in extracts from SA-treated leaf tissue. We therefore performed time course experiments monitoring NIMIN1 accumulation in twofold concentrated extracts from 1 to 4 days after agroinfiltration. Whereas GFP accumulated to high levels at 3 and 4 days post-inoculation (dpi; **Figure 3D**), NIMIN1 protein was detected only faintly (**Figure 3C**). The inability to detect high amounts of NIMIN1 in agroinfiltrated plant tissue is, however, not due to a low sensitivity of the anti-NIMIN1 serum we used. Detection of NIMIN1 and NIMIN3-Gal4 DNA binding domain (GBD) fusion proteins, which are expressed to similar levels in yeast (Weigel et al., 2001), was similar for both NIMIN3 and NIMIN1 with the specific antisera (**Figure 3E**).

NIMIN2 DOES NOT SIGNIFICANTLY AFFECT SALICYLIC ACID-INDUCED EXPRESSION OF TOBACCO *PR-1* GENES

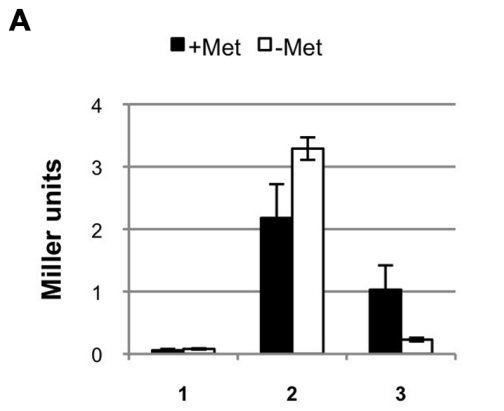
Likewise surprisingly, agroinfiltration of the *N. benthamiana* reporter line with *35S$_{Pro}$::NIMIN2* harboring bacteria did not repress SA-mediated induction of the *PR-1a$_{Pro}$::GUS* transgene (**Figures 4A,B**). Expression of *NIMIN2* in *N. benthamiana* leaf tissue was demonstrated by immunodetection using a specific antiserum directed against Nt NIMIN2a-maltose binding protein (MBP) which exhibits cross-reactivity with *Arabidopsis* NIMIN2 (**Figure 4C**). Thus, albeit similar to each other and possessing similar NPR1 interaction motifs, NIMIN2 and NIMIN1 seem to fulfill different, even opposing, functions in the SA signal transduction pathway.

We also tested whether transient expression of At *NIMIN* genes in *N. benthamiana* is able to suppress induction of endogenous *PR-1* genes. *N. benthamiana* (Nb) carries a gene for a basic PR-1 protein. The amino acid sequence for the basic PR-1 protein is co-linear with *N. tabacum* acidic PR-1 proteins except for a 19 amino acid-long extension at the C-terminus of Nb PR-1. In the co-linear region, the identity (similarity) between the basic Nb PR-1 protein and Nt PR-1a is 64% (87%). Consequently, using an antiserum raised against Nt PR-1a, we were able to detect a PR-1-related protein exhibiting a slightly higher molecular weight than the acidic Nt PR-1 proteins in extracts from *N. benthamiana* leaf disks floated on 1 mM SA (data not shown). SA induction of this protein was clearly suppressed in *N. benthamiana* tissue overexpressing *NIMIN1* or *NIMIN3*, but not in tissue overexpressing *NIMIN2* (**Figure 4D**).

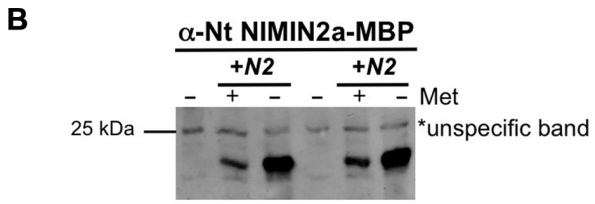
Arabidopsis NIMIN PROTEINS CANNOT BIND SIMULTANEOUSLY TO NPR1 IN YEAST

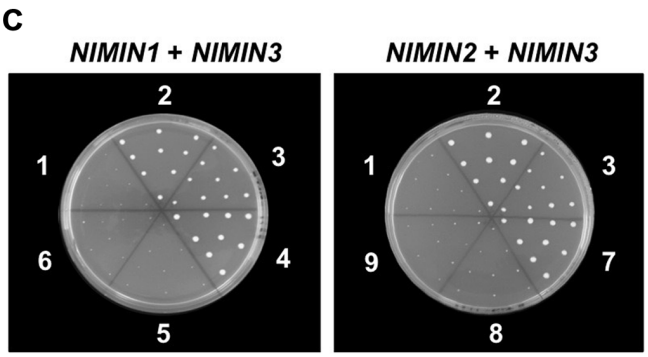
Differential regulation of *NIMIN* genes and differential effects of NIMIN proteins on *PR-1* induction strongly suggested that NIMINs serve unique functions at specific time points during the SAR response in *Arabidopsis*, an assumption fully consistent with our previous observation that NIMIN3 and NIMIN1/NIMIN2 bind to physically separate regions of At NPR1 (Weigel et al., 2001). Therefore, it was of interest to test whether NIMIN proteins are able to bind simultaneously to NPR1, or whether their binding excludes each other. To address this question, we made use of a yeast three-hybrid (Y3H) system. In this assay, interaction of two proteins can be monitored at different concentrations of a third protein whose expression level is controlled by methionine (Met) in the growth medium (Tirode et al., 1997). Previously, we have demonstrated that NIMIN proteins are able to interact with TGA transcription factors in presence of NPR1 (**Figures 5C** and **6B**; Weigel et al., 2001), showing that NIMINs and TGA factors possess independent binding sites on NPR1 which can be occupied at the same time. The same assay was used for monitoring binding of two different NIMIN proteins to NPR1. To this end, we used partial *NIMIN* cDNA clones which we had isolated in a yeast two-hybrid (Y2H) screen with the At NPR1 bait (Weigel et al., 2001).

Initially, we tested whether NIMIN1 and NIMIN2 can bind together to NPR1 in Y3H assays. Both proteins possess similar NPR1 interaction motifs by which they bind to the C-terminus of NPR1 (Weigel et al., 2001; **Figure 6A**). Truncated NIMIN1 or NIMIN2 including their NPR1 interaction motif were expressed as fusions with the Gal4 transcription activation domain (GAD), and full-length *NIMIN1* or *NIMIN2* were expressed from the *Met25* promoter, which is repressed in presence and de-repressed in absence of methionine. NPR1 was expressed as GBD fusion. The interactions of NIMIN1 or NIMIN2 with NPR1 were disrupted in presence of NIMIN2 or NIMIN1, respectively (**Figure 5A** and data not shown). Furthermore, complex formation between NPR1 and NIMIN1 was clearly dependent on the concentration of NIMIN2 (**Figures 5A,B**). Together, the data suggest that NIMIN1 and NIMIN2 may compete for the same binding site on NPR1.



GBD fusion	GAD fusion	Conditional (± Met)
1: NPR1	-	NIMIN2
2: NPR1	NIMIN1 35/142	-
3: NPR1	NIMIN1 35/142	NIMIN2





GBD fusion	GAD fusion	Additional (- Met)
1: NIMIN3	-	NPR1
2: NIMIN3	TGA2	NPR1
3: NPR1	NIMIN3 13/112	-
4: NPR1	NIMIN1 35/142	-
5: NIMIN3	NIMIN1 35/142	NPR1
6: NIMIN1	NIMIN3 13/112	NPR1
7: NPR1	NIMIN2 20/122	-
8: NIMIN3	NIMIN2 20/122	NPR1
9: NIMIN2	NIMIN3 13/112	NPR1

FIGURE 5 | *Arabidopsis* NIMIN1, NIMIN2, and NIMIN3 do not bind simultaneously to At NPR1 in yeast. (A) Yeast two-hybrid interaction of At NPR1-Gal4 DNA binding domain (GBD) and NIMIN1-Gal4 activation domain (GAD) fusion proteins in absence and presence of NIMIN2. *NIMIN2* was expressed from the *Met25* promoter which is repressed in presence and de-repressed in absence of methionine. **(B)** Immunodetection of NIMIN2 in yeast. Yeast cells analyzed for *lacZ* reporter gene expression in **Figure 5A** were probed for accumulation of NIMIN2 protein. N2, NIMIN2. **(C)** Yeast three-hybrid interaction of At NPR1 with NIMIN1 and NIMIN3 or with NIMIN2 and NIMIN3. NIMIN1, NIMIN2, and NIMIN3 were expressed as fusions with the GBD or GAD. Simultaneous interaction of At NPR1 with GAD-TGA2 and GBD-NIMIN3 serves as positive control for formation of a ternary protein complex.

Next, we asked whether NIMIN1 or NIMIN2 can bind to NPR1 in presence of NIMIN3 which interacts with NPR1 via a site distant from the NIMIN1/NIMIN2 binding site (Weigel et al., 2001). NIMIN1 and NIMIN3 or NIMIN2 and NIMIN3 were expressed as GBD or GAD fusions, while *NPR1* was expressed from the de-repressed *Met25* promoter. Surprisingly, the interaction between NIMIN1 and NPR1 and between NIMIN2 and NPR1 was disrupted in presence of NIMIN3 (**Figure 5C**). Hence, NIMIN3 binding to NPR1 seems to inhibit NIMIN1/NIMIN2 interaction, and simultaneous binding of NIMIN1, NIMIN2, and NIMIN3 to NPR1 may exclude each other.

NIMIN1 AND NIMIN2 INTERACT DIFFERENTIALLY WITH NPR1

In tobacco NPR1, binding of NIMIN2 proteins occurs in the region from amino acids 494 to 510 (Maier et al., 2011). The domain is highly conserved in NPR1 proteins from many plant species, including *Arabidopsis*, regarding both the sequence and its position within the amino acid chain (for At NPR1 94% identity, 100% similarity, from amino acids 496 to 512). To test whether both NIMIN1 and NIMIN2 bind to this region in At NPR1 and whether binding occurs in a similar fashion, we introduced mutations F507S and F508S into At NPR1. Nt NPR1 F505/506S is no longer able to interact with Nt NIMIN2a or Nt NIMIN2c (Maier et al., 2011). Similarly, mutation of F507/508S completely abolishes binding of NIMIN1 and NIMIN2 to At NPR1, but not binding of NIMIN3 (**Figure 6A**).

We then analyzed the relations of NPR1 with NIMIN1 and NIMIN2 in ternary protein complexes including TGA transcription factors. We have shown previously that SA administered to growth medium impairs formation of NPR1–NIMIN1 and NPR1–NIMIN2 complexes in Y2H assays, and that the sensitivity of loss of protein–protein interaction is very similar for both NIMIN1 and NIMIN2 ($IC_{50} \approx 20$ μM SA; Maier et al., 2011). Here, we monitored effects of SA on NPR1–NIMIN1 and NPR1–NIMIN2 interactions in presence of TGA2 or TGA6. Interaction of *Arabidopsis* NPR1 with TGA factors is not diminished with SA (**Figure 6B**; Maier et al., 2011). Ternary complexes comprising NIMIN1 were sensitive to SA as observed before for the

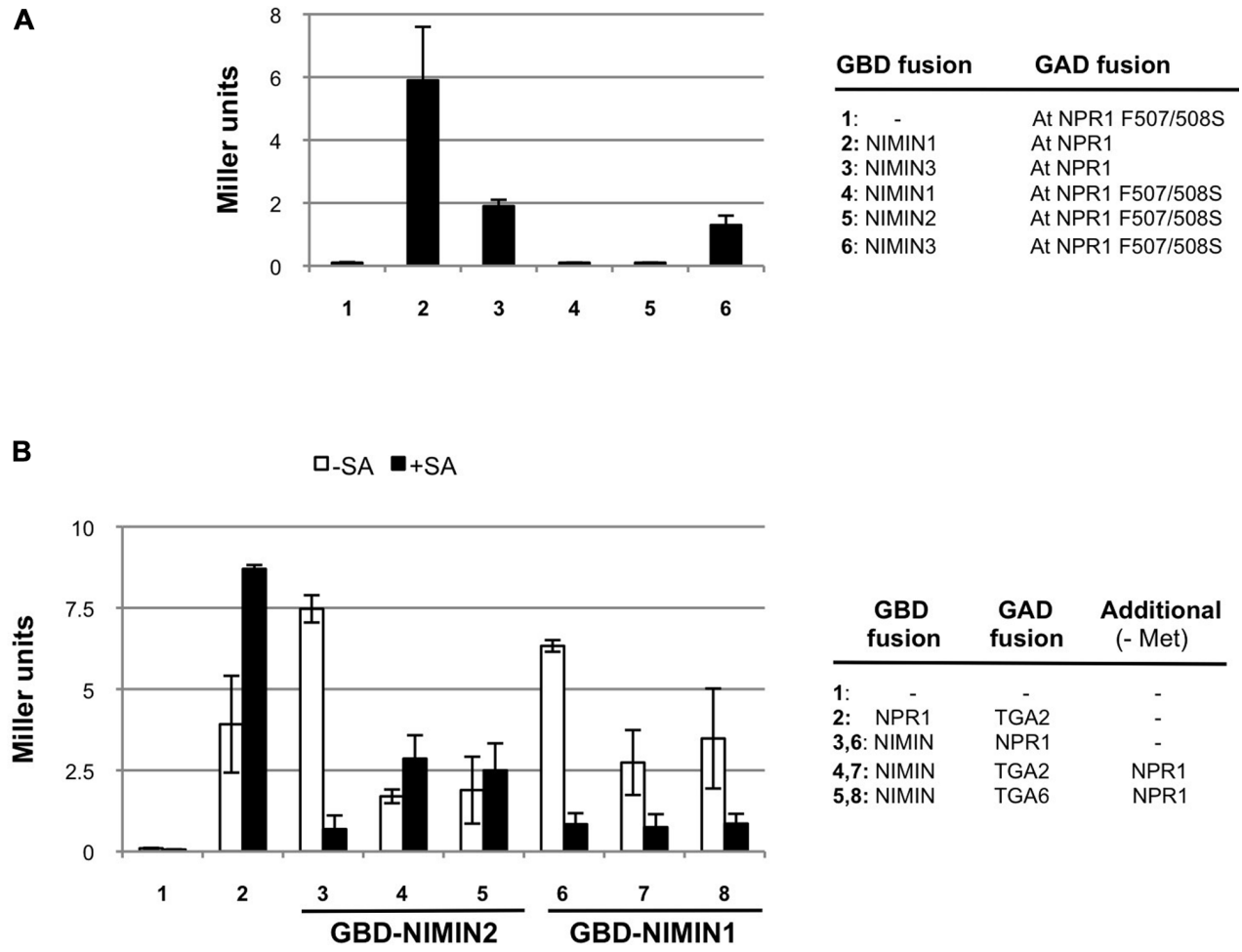


FIGURE 6 | *Arabidopsis* NIMIN1 and NIMIN2 interact differentially with At NPR1 in yeast. (A) Yeast two-hybrid interaction of NIMIN1, NIMIN2 or NIMIN3 expressed as GBD fusions with the mutant protein At NPR1 F507/508S expressed as GAD fusion. Interactions of GAD-At NPR1 with GBD-NIMIN1 and GBD-NIMIN3 serve as positive controls. **(B)** Effect of SA on formation of ternary protein complexes comprising GBD-NIMIN1 or GBD-NIMIN2, GAD-At TGA2 or GAD-At TGA6 and At NPR1. The binary interactions of At NPR1 with NIMIN1 or NIMIN2 or TGA2 serve as controls for effects of SA (concentration 0.3 mM) on the NPR1–NIMIN1/2 interaction.

NIMIN1–NPR1 binary interaction (**Figure 6B**). Quite surprisingly, however, ternary complexes comprising NIMIN2 proved to be stable in presence of SA (**Figure 6B**). Thus, although possessing similar NPR1 interaction motifs and binding to the same site in the C-terminus of NPR1, NIMIN1, and NIMIN2 can form complexes with NPR1 and TGA factors exhibiting differential sensitivity to SA, implying that these two NIMIN proteins interact differentially with NPR1 in transcription complexes on *PR* gene promoters.

DISCUSSION

NIM1-INTERACTING proteins have been identified through a Y2H screen with *Arabidopsis* NPR1 as bait. Although of rather small molecular weight, the proteins share several conserved regions with each other which are likely of functional relevance. Thus, all NIMIN proteins encompass an LxLxL/EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif at their C-terminus, and NIMIN1 and NIMIN2 possess a common motif for interaction with a domain in the C-terminus of *Arabidopsis* and tobacco NPR1. On the other hand, NIMIN1 and NIMIN3 have been reported to share a conserved PA/SFQPEDF signature (Weigel et al., 2001), suggesting that NIMIN1 and NIMIN3, albeit binding to different regions of NPR1, may exert similar activities. To understand the action of related, yet distinct, NIMIN proteins on NPR1, we have performed a comparative analysis of *Arabidopsis* NIMIN1, NIMIN2, and NIMIN3. We have studied the expression profiles of *NIMIN* genes, the effects of NIMIN proteins on SA induction of the SAR marker *PR-1* and their interaction with NPR1. Our results suggest that the *Arabidopsis* NIMIN proteins exert unique and complementary functions on NPR1 at different stages of the SAR response.

NIMIN3 REPRESSES *PR-1* IN UNCHALLENGED PLANTS

As opposed to *NIMIN1* and *NIMIN2*, which are clearly responsive to SA, *NIMIN3* is expressed constitutively at a low level in

Arabidopsis leaf tissue. In our current work, we have not found any indications for enhancement of *NIMIN3* expression by SA or other plant defense hormones. Most importantly, the *NIMIN3* promoter is weakly active in leaf tissue and does not respond to the SAR signal molecule SA. Hence, NIMIN3 is likely to function on a constitutive basis in unchallenged plants before the induction of SAR. This idea is consistent with our previous finding that NIMIN3 does not possess the interaction site by which NIMIN1 and NIMIN2 bind to the SA-sensitive NPR1 C-terminus (Weigel et al., 2001; Maier et al., 2011). When transiently overexpressed in the *N. benthamiana -1533PR-1a::GUS* reporter line created by us, NIMIN3, like NIMIN1, is able to suppress SA-induced activation of the reporter. Similarly, NIMIN3, like NIMIN1, also suppresses induced expression of an endogenous *PR-1* gene in *N. benthamiana*. Altogether, repression effects exerted by NIMIN3 in *N. benthamiana* seem moderate, when compared to effects observed with NIMIN1. On the other side, we did not expect suppression of *PR-1* gene induction to occur at all by NIMIN3 in *Nicotiana* species. First, a true NIMIN3 homolog has not been identified to date from tobacco or tomato. Furthermore, NPR1 family members from tobacco, Nt NPR1 and Nt NPR3, have not been found to interact with NIMIN3 in Y2H assays (Zwicker et al., 2007; Maier et al., 2011), whereas NIMIN3 clearly interacts with *Arabidopsis* NPR1 (Weigel et al., 2001). Thus, the biochemical basis of NIMIN3-mediated suppression of *PR-1* in *N. benthamiana* is not clear. However, we have noted previously that NIMIN3 and NIMIN1 share the conserved amino acid signature PA/SFQPEDF (from here on termed EDF motif; Weigel et al., 2001). This signature is also present in the rice (Os) NIMIN homolog NRR and some of its paralogs (consensus sequence WRP-F-W/MEDF; Chern et al., 2012). Mutations of NRR and its paralogs in this region have uncovered the motif as domain for strong interaction with rice NH1/NPR1 causing repression of transcription activity of Os NH1/NPR1 in a rice transient assay system. In contrast, the motif mediates only very weak interaction between NRR and *Arabidopsis* NPR1 (Chern et al., 2012). We have introduced mutations in the EDF motifs of NIMIN3 and NIMIN1 (E63A D64V in NIMIN3; E94A D95V in NIMIN1), and tested activities of the mutant proteins in Y2H assays with Gal4 AD-At NPR1 and in the *N. benthamiana* transient assay system. Unfortunately, the mutant proteins did not accumulate to detectable levels, neither in yeast nor in plant tissue, and therefore, the significance of the EDF domain for NIMIN3 and NIMIN1 could not be assessed (Masroor and Pfitzner, unpublished data). It is of interest, however, to note that binding of At NPR1 to NIMIN3 occurs within the 60 amino acid-long C-terminal half including the EDF motif (Weigel et al., 2001). Given the conservation of the amino acid sequence in NPR1 interactors from multiple plant species and the clear results in the rice system reported by Chern et al. (2012), we infer that the EDF signature is functional in *Arabidopsis* NIMINs, and that the domain is involved in regulation of *PR* genes via the NIMIN–NPR1 complex. The significance of the EDF domain for *PR* gene induction may, however, vary among different plant species. In this line, suppression of *PR-1* induction in *N. benthamiana* may be mediated via the EDF domain in NIMIN3 and NIMIN1, and suppression by NIMIN1 would be stronger because NIMIN1, unlike NIMIN3, can interact via a second domain with the NPR1 C-terminus. Of note, several cDNAs from *N. tabacum* and *N. benthamiana* coding for NIMIN proteins with the EDF motif (consensus WNL/PA/TF/L-T/PEDF) have been described in the databanks, underscoring our assumption that the EDF domain may have functional relevance also in tobacco. The mechanism by which the EDF domain in NIMIN proteins could suppress *PR-1* gene induction remains, however, elusive. Alternatively, suppression of *PR-1* induction in *N. benthamiana* by NIMIN3 and NIMIN1 may occur via the C-terminal LxLxL/EAR motif which has been implicated in recruiting the transcriptional co-repressor TOPLESS (*Arabidopsis* Interactome Mapping Consortium, 2011). In summary, our data would support the view that NIMIN3 can target the NPR1 complex in tobacco, and that NIMIN3 is a repressor of inadvertent *PR-1* gene expression in unchallenged *Arabidopsis* leaf tissue.

NIMIN2 DOES NOT AFFECT SALICYLIC ACID INDUCTION OF *PR-1*

We have noted previously that *NIMIN2* is responsive to SA (Weigel et al., 2001; Glocova et al., 2005). Here, using RT-PCR analyses, we show that *NIMIN2* mRNA accumulates very early after treatment of plants with SA, and, in several cases, *NIMIN2* mRNA was already detectable in plant tissue without exposure to chemicals at all. From our observations, we conclude that *NIMIN2* is more readily induced than *NIMIN1* or *PR-1*, consistent with the finding that *NIMIN2* expression, as opposed to *NIMIN1* and *PR-1* expression, is independent from an intact *NPR1* gene requiring activation by SA. Surprisingly, overexpression of *NIMIN2* in the *N. benthamiana -1533PR-1a::GUS* reporter line does not appear to have an effect on SA-induced *PR-1* gene expression. This finding is consistent with our previous observation showing that overexpression of a *NIMIN2* homolog, Nt *NIMIN2a*, in transgenic tobacco plants did not result in massive *PR-1* repression as reported in similar experiments for At *NIMIN1* and Os *NRR* overexpression (Chern et al., 2005, 2008; Weigel et al., 2005). Hence, NIMIN2 is likely to play a role at the very onset of SAR and is unlikely to be involved in repression of *PR-1* gene induction.

NIMIN1 CONTROLS EXPRESSION OF LATE SAR-INDUCED *PR-1*

NIMIN1 is an early SA-activated and *NPR1*-dependent gene which is induced after *NIMIN2*, but clearly before *PR-1*. *NIMIN1* is expressed only transiently, and the NIMIN1 protein does not appear to accumulate to high levels. These features are compatible with a role of NIMIN1 as regulator of late SAR genes, e.g., *PR-1*, preventing their premature activation. The repression effect exerted by NIMIN1 in the *N. benthamiana -1533PR-1a::GUS* reporter line is very strong. Above, we have argued that *PR-1* repression may be mediated via the EDF domain in NIMIN1, although we were not able to provide direct proof for this assumption. It is important to note, however, that NIMIN2 does not possess the EDF motif and does not repress *PR-1* gene induction in our system. Curiously, although not accumulating to substantial levels in agroinfiltrated *N. benthamiana* leaf tissue, NIMIN1 executes strong effects raising the question how NIMIN1 could suppress *PR-1* gene expression in near physical absence? Different scenarios seem conceivable. For example, NIMIN1 could be stable and exert its function only in direct association with NPR1. Any excess NIMIN1 protein would immediately

be degraded. In this scenario, NIMIN1 could act to prohibit contact of NPR1 to downstream transcription factors either by sterical hindrance or, in imitation to the action of a chaperone, by imposing a non-productive bent on NPR1. Together, our data support a view where NIMIN1 acts only later during the SAR response, after NIMIN2, keeping tight control over *PR-1* by promoting its repression. Notably, we were not able to detect simultaneous binding of NIMIN3, NIMIN2, or NIMIN1 to NPR1, and we found that NIMIN1 and NIMIN2 bind differentially to NPR1 in ternary protein complexes including TGA transcription factors.

WORKING MODEL FOR THE CONSECUTIVE ACTION OF *Arabidopsis* NIMIN PROTEINS IN THE COURSE OF SAR

Based on our findings, we propose sequential formation of different NIMIN–NPR1 complexes to promote defense gene activation at distinct stages of SAR (**Figure 7**). While NIMIN3 represses inadvertent *PR* gene activation in unchallenged plants, NIMIN2 is induced at low tissue levels of SA to relieve NIMIN3 repression by binding to the NPR1 C-terminus. This process may allow activation of early SA- and *NPR1*-dependent genes, e.g., *NIMIN1*. Interaction of NIMIN2 with the NPR1 C-terminus does not, however, appear to be sufficient to activate substantial expression of the late SAR gene *PR-1*. NIMIN2 action on NPR1 is transient and is followed by NIMIN1 replacing NIMIN2. NIMIN1 suppresses activation of *NPR1*-dependent SAR genes. NIMIN1 action on NPR1 seems even more transient than NIMIN2 action, and instability of NIMIN1 protein would be a crucial prerequisite for relief of *PR-1* gene repression. In this scenario, late SAR genes would be activated through direct action of SA on NPR1 (Maier et al., 2011; Fu et al., 2012; Wu et al., 2012) causing removal of repressing NIMIN1 from the NPR1 complex (Maier et al., 2011). In conclusion, consecutive action of NIMIN proteins with different biochemical capacities on the central SAR regulator NPR1 is needed to ensure sudden, strong and coordinate expression of defense genes to successfully combat invading pathogens. In this line, the NIMIN–NPR1 connection may constitute a molecular device to monitor ambient SA levels in diseased plants, enabling the plant to translate a steadily increasing gradient of the defense hormone SA into two clear decision steps, early and late SAR gene expression.

MATERIALS AND METHODS

DNA CONSTRUCTS

For transient gene expression assays, the coding regions from *NIMIN1*, *NIMIN2*, and *NIMIN3* were inserted as *Bam*HI/*Sac*I

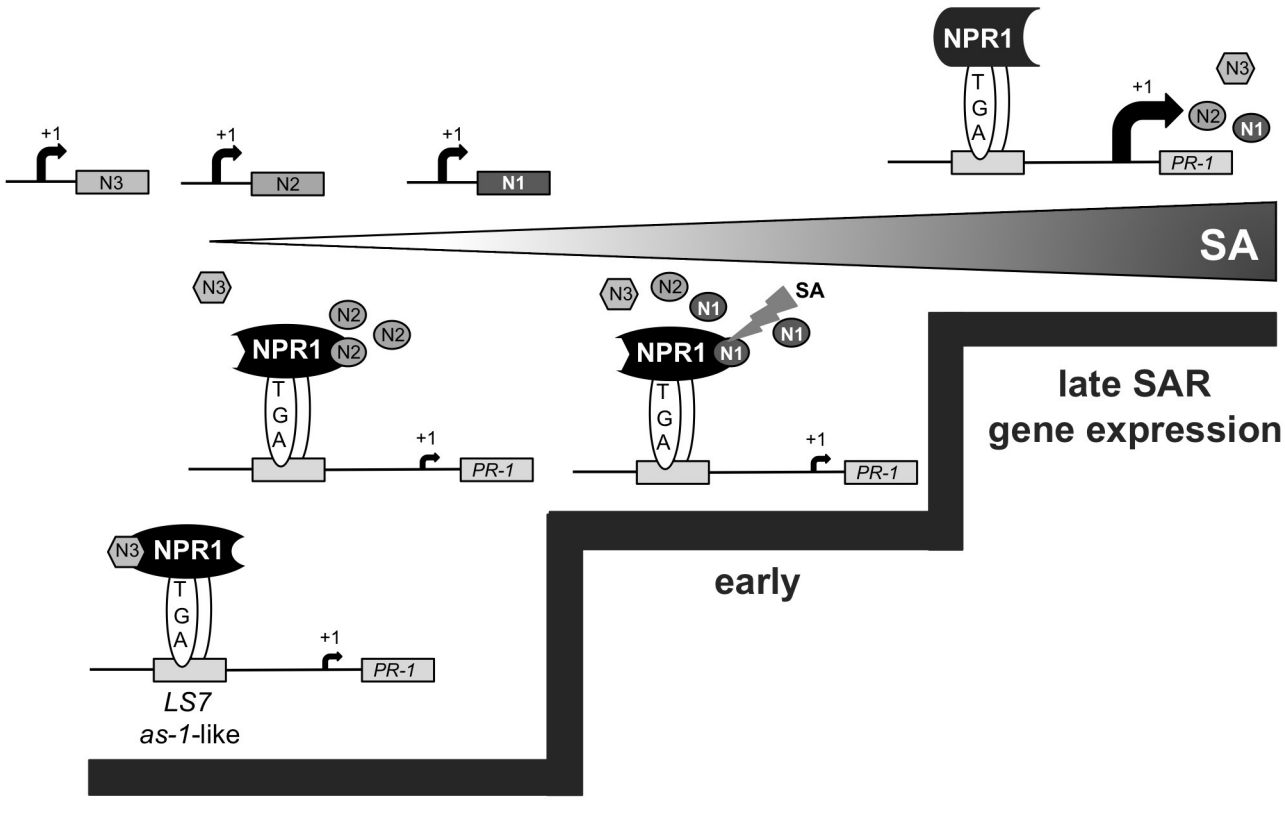


FIGURE 7 | Working model for the consecutive action of *Arabidopsis* NIMIN proteins in the course of SAR. The model implies sequential interaction between diverse NIMIN proteins and NPR1 to form regulatory complexes with differential biochemical capacities in the course of SAR. The model also suggests that sensing of ambient SA levels in diseased plants may occur through the various NIMIN–NPR1 complexes, enabling activation of *PR* genes at distinct threshold levels of SA (indicated by steps). In this scenario, the defense gene *PR-1* is induced late during SAR by direct action of SA on the NIMIN1–NPR1 regulatory complex.

fragments into pBin19/35S$_{Pro}$::GUS (Jefferson et al., 1987) from which the *GUS* reporter gene had been excised. The coding regions were amplified from the respective pGBT9 plasmids (Weigel et al., 2001) using C-terminal primers with the native stop codons and a *Sac*I restriction endonuclease site added 3′ to the stop codons.

The *NIMIN3$_{Pro}$::GUS* reporter gene was constructed in analogy to the *NIMIN1$_{Pro}$::GUS* and *NIMIN2$_{Pro}$::GUS* chimeric genes (Glocova et al., 2005). The *NIMIN3* promoter sequence was amplified from *Arabidopsis thaliana* (L.) Heynh. Col-0 genomic DNA using primers N3-P2 (5′-TTAAGCTTATACGGGACATA GTGCACAGCC) and N3-P1 (5′-AAGGATCCTGAACCGCTCTC TCTTCCTTCC). N3-P1 primes immediately upstream of the ATG translation start codon of *NIMIN3*. The resulting 1.4 kb fragment was ligated to *Hin*dIII/*Bam*HI cleaved pBin19/35S$_{Pro}$::GUS from which the *35S RNA* promoter had been removed.

To map the NIMIN1/NIMIN2 binding site in At NPR1, Phe-507 and Phe-508 were mutated to Ser using overlap extension PCR (Ho et al., 1989). The primers for mutagenesis were AtNPR1-14 (5′-CTCGGGAAACGAAGCAGCCCGCGCTGTTC) and AtNPR1-15 (5′-GAACAGCGCGGGCTGCTTCGTTTCCCGAG). The mutations were inserted in a C-terminal fragment of At *NPR1*. To this clone, the N-terminal At *NPR1* sequence was added as a 1.4 kb *Bam*HI/*Dra*III fragment, and the complete mutant sequence was ligated to *Bam*HI/*Sal*I cleaved pGBT9 and pGAD424.

All clones generated by PCR amplification were verified by DNA sequence analysis.

RNA ISOLATION AND RT-PCR ANALYSES

RNA isolation and RT-PCR analyses were performed as described by Zwicker et al. (2007). The primer combinations and control plasmids used for the different gene fragments are listed in **Table 1**. For the time course experiment shown in **Figure 2B**, RT-PCR assays were conducted to give approximately equal amounts of reaction products in order to enable direct comparison of *NIMIN1*, *NIMIN2*, and *PR-1* transcript accumulation at different time points after treatment of *Arabidopsis* with SA. To this end, RNAs were diluted 1:20 for RT-PCR amplification of *NIMIN2* transcripts.

GENERATION AND CULTIVATION OF TRANSGENIC PLANTS

Transformation of tobacco (*N. tabacum* L. cv. Samsun NN) by *Agrobacterium tumefaciens* was performed according to Grüner et al. (2003). Tobacco lines with *PR-1a$_{Pro}$::GUS*, *35S$_{Pro}$::GUS*, *NIMIN1$_{Pro}$::GUS*, and *NIMIN2$_{Pro}$::GUS* have been described earlier (Grüner et al., 2003; Glocova et al., 2005). For localization of GUS enzyme activity *in situ* (**Figure 1B**) and for determination of SA-induced GUS activity in time course experiments (**Figure 2C**), seeds from transgenic tobacco were sown on MS medium with 400 μg ml^{-1} kanamycin or on selective medium supplemented with 0.3 mM SA.

Agrobacterium-MEDIATED TRANSIENT GENE EXPRESSION IN *NICOTIANA BENTHAMIANA*

The *-1533PR-1a$_{Pro}$::GUS* gene construct (Grüner et al., 2003) was integrated via *Agrobacterium*-mediated transformation into the genome of *N. benthamiana* Domin. All primary transformants exhibited strong and stringent induction of the *GUS* reporter gene in response to SA. A line with an intermediate GUS enzyme activity was propagated by selfing, and plants of the T2 generation were used for agroinfiltration experiments.

The pBin19 gene constructs were transferred by triparental mating to *Agrobacterium tumefaciens* strain LBA4404. Recombinant *Agrobacterium* strains were grown at 30°C in minimal medium supplemented with 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} rifampicin to stationary phase. Cells were collected by centrifugation and resuspended in 10 mM $MgCl_2$ and 150 μM acetosyringone to give an optical density (OD_{600}) of 0.5 for all strains. Agrobacteria were incubated for 2–3 h at room temperature before agroinfiltration. To suppress post-transcriptional gene silencing, the bacterial suspensions were mixed with an equal volume of a strain carrying the p19 suppressor from *Tomato bushy stunt virus* (Voinnet et al., 2003). Four to six week-old greenhouse-grown *N. benthamiana* plants with integrated *-1533PR-1a$_{Pro}$::GUS* were agroinfiltrated in the abaxial air spaces. To allow for a direct comparison between effects produced by different *NIMIN* strains, leaves at the same position on the axis of different plants or the two halves of the same leaf were injected. In each experiment, three independent plants were infiltrated with the same *Agrobacterium* suspension, and plants infiltrated with a strain containing *35S$_{Pro}$::mGFP4* (Haseloff et al., 1997) were used to control gene expression levels in leaf tissue. Expression of *GFP* was monitored under UV light. GFP fluorescence remained always strictly confined to infiltrated leaf areas. Agroinfiltrated tissue was processed 4 or 5 days post-infiltration (dpi), when strong GFP fluorescence was observed. At this point of time, bacterial titers were similar in leaf tissue agroinfiltrated with strains *35S$_{Pro}$::mGFP4*, *35S$_{Pro}$::NIMIN1*, or *35S$_{Pro}$::NIMIN2* (Wöhrle and Pfitzner, unpublished data). Furthermore, co-overexpression of *mGFP4* and *NIMIN1* produced the same levels of GFP fluorescence and of GFP protein accumulation as overexpression of *mGFP4* alone (Masroor and Pfitzner, unpublished data).

GUS REPORTER GENE ASSAYS AND IMMUNODETECTION OF PROTEIN ACCUMULATION

Determination of GUS enzyme activity and histochemical localization of GUS activity *in situ* were performed as described previously (Weigel et al., 2001; Glocova et al., 2005). GUS activity is given in units (1 unit = 1 nmol 4-MU per hour per mg protein). For the time course experiment shown in **Figure 2C**, GUS enzyme activities were determined from pools of 10 seedlings for each data point. The same extracts were used for immunodetection of endogenous PR-1 proteins. Equal amounts of protein were loaded in each lane of the sodium dodecyl sulfate (SDS) gels.

To determine GUS enzyme activity after transient expression of *NIMIN* genes in *N. benthamiana*, two leaf disks each were punched out from non-infiltrated control or from agroinfiltrated leaf tissue at 4 or 5 dpi. Disks were floated for 2 days on water or on 1 mM SA and thereafter extracted with 150 μl GUS lysis buffer. The SA-induced reporter gene expression from the *PR-1a* promoter was compared in non-agroinfiltrated leaf tissue and in tissue infiltrated with *35S$_{Pro}$::mGFP4* and *35S$_{Pro}$::NIMIN* chimeric genes. The same extracts were used for immunodetection of protein accumulation.

Immunodetection of proteins separated by SDS gel electrophoresis was performed as described earlier (Zwicker et al., 2007). Specific antisera were raised in rabbits immunized with *E. coli* expressed and purified proteins NIMIN1-GST, Nt NIMIN2a-MBP, and NIMIN3 according to standard procedures. PR-1 protein accumulation in *N. benthamiana* was detected with a specific antiserum against Nt PR-1a. For detection of GFP and GUS proteins, rabbit polyclonal antisera were used as recommended by the manufacturers (Santa Cruz Biotechnology and Abcam, respectively). To analyze accumulation of NIMIN1 at different times after agroinfiltration (**Figure 3C**), four leaf disks were harvested directly from each infiltrated tissue and extracted with 150 µl GUS lysis buffer yielding twofold concentrated extracts. SA induction of the GUS reporter protein and of an endogenous *N. benthamiana* PR-1 protein was compared in tissue infiltrated with $35S_{Pro}$*::mGFP4* and $35S_{Pro}$*::NIMIN* chimeric genes. Equal extract volumes were loaded in each lane of an SDS gel. The loading of SDS gels for immunodetection of protein accumulation was checked by staining the nitrocellulose filters with Ponceau S (0.1% in 5% acetic acid). Alternatively, unspecific bands reacting with the antisera used are marked for demonstration of equal gel loading.

YEAST TWO-HYBRID AND THREE-HYBRID ASSAYS

Yeast two-hybrid and yeast three-hybrid analyses in absence and presence of SA were conducted as reported earlier (Weigel et al., 2001; Maier et al., 2011). *LacZ* reporter gene activities are given in Miller units. Most plasmids used in the protein–protein interaction assays have been described (Weigel et al., 2001). pGAD10/NIMIN1 35/142, pGAD10/NIMIN2 20/122, and pGAD10/NIMIN3 13/112 encode NIMIN proteins truncated at their N-terminus. The plasmids were isolated in a Y2H screen with the At NPR1 bait (Weigel et al., 2001).

ACKNOWLEDGMENTS

We would like to thank Sylvia Zwicker for communication of Y2H data, Xinnian Dong, Duke University, USA, and Jane Glazebrook, University of Minnesota, USA, for *npr1* mutant seeds, Jim Haseloff, University of Cambridge, England, for the generous gift of pBIN 35S-mGFP4, and Klaus Harter, Universität Tübingen, Germany, for providing the *Agrobacterium* strain with the p19 silencing suppressor. Thanks also to David Neeley for advice on the English language. Ashir Masroor was supported by a fellowship from Katholischer Akademischer Ausländer-Dienst (KAAD).

REFERENCES

Arabidopsis Interactome Mapping Consortium (2011). Evidence for network evolution in an *Arabidopsis* interactome map. *Science* 333, 601–607.

Blanco, F., Salinas, P., Cecchini, N. M., Jordana, X., Van Hummelen, P., Alvarez, M. E., et al. (2009). Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Mol. Biol.* 70, 79–102.

Canet, J. V., Dobón, A., Roig, A., and Tornero, P. (2010). Structure–function analysis of npr1 alleles in *Arabidopsis* reveals a role for its paralogs in the perception of salicylic acid. *Plant Cell Environ.* 33, 1911–1922.

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6, 1583–1592.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63.

Cao, H., Li, X., and Dong, X. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6531–6536.

Chern, M., Bai, W., Sze-To, W. H., Canlas, P. E., Bartley, L. E., and Ronald, P. C. (2012). A rice transient assay system identifies a novel domain in NRR required for interaction with NH1/OsNPR1 and inhibition of NH1-mediated transcriptional activation. *Plant Methods* 8, 6.

Chern, M., Canlas, P. E., Fitzgerald, H. A., and Ronald, P. C. (2005). Rice NRR, a negative regulator of disease resistance, interacts with *Arabidopsis* NPR1 and rice NH1. *Plant J.* 43, 623–635.

Chern, M., Canlas, P. E., and Ronald, P. C. (2008). Strong suppression of systemic acquired resistance in *Arabidopsis* by NRR is dependent on its ability to interact with NPR1 and its putative repression domain. *Mol. Plant* 1, 552–559.

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. (2000). The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12, 279–290.

Fonseca, J. P., Menossi, M., Thibaud-Nissen, F., and Town, C. D. (2010). Functional analysis of a TGA factor-binding site located in the promoter region controlling salicylic acid-induced NIMIN-1 expression in *Arabidopsis*. *Genet. Mol. Res.* 9, 167–175.

Friedrich, L., Lawton, K., Dietrich, R., Willits, M., Cade, R., and Ryals, J. (2001). NIM1 overexpression in *Arabidopsis* potentiates plant disease resistance and results in enhanced effectiveness of fungicides. *Mol. Plant Microbe Interact.* 14, 1114–1124.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut Rella, M., et al. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J.* 10, 61–70.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232.

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982.

Glocova, I., Thor, K., Roth, B., Babbick, M., Pfitzner, A. J. P., and Pfitzner, U. M. (2005). Salicylic acid (SA)-dependent gene activation can be uncoupled from cell death-mediated gene activation: the SA-inducible NIMIN-1 and NIMIN-2 promoters, unlike the PR-1a promoter, do not respond to cell death signals in tobacco. *Mol. Plant Pathol.* 6, 299–314.

Grüner, R., and Pfitzner, U. M. (1994). The upstream region of the gene for the pathogenesis-related protein 1a from tobacco responds to environmental as well as to developmental signals in transgenic plants. *Eur. J. Biochem.* 220, 247–255.

Grüner, R., Strompen, G., Pfitzner, A. J. P., and Pfitzner, U. M. (2003). Salicylic acid and the hypersensitive response initiate distinct signal transduction pathways in tobacco that converge on the as-1-like element of the PR-1a promoter. *Eur. J. Biochem.* 270, 4876–4886.

Haseloff, J., Siemering, K. R., Prasher, D. C., and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2122–2127.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51–59.

Horvath, D. M., Huang, D. J., and Chua, N.-H. (1998). Four classes of salicylate-induced tobacco genes. *Mol. Plant Microbe Interact.* 11, 895–905.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in *Arabidopsis*. *Plant J.* 16, 223–233.

Maier, F., Zwicker, S., Hückelhoven, A., Meissner, M., Funk, J., Pfitzner, A. J. P., et al. (2011). NON-EXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and

some NPR1-related proteins are sensitive to salicylic acid. *Mol. Plant Pathol.* 12, 73–91.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004.

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944.

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., and Després, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *Plant Cell* 18, 3670–3685.

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14, 340–358.

Ryals, J., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H. Y., et al. (1997). The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* 9, 425–439.

Shah, J., Tsui, F., and Klessig, D. F. (1997). Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. *Mol. Plant Microbe Interact.* 10, 69–78.

Strompen, G., Grüner, R., and Pfitzner, U. M. (1998). An as-1-like motif controls the level of expression of the gene for the pathogenesis-related protein 1a from tobacco. *Plant Mol. Biol.* 37, 871–883.

Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Egly, J. M. (1997). A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system. *J. Biol. Chem.* 272, 22995–22999.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., et al. (1992). Acquired resistance in *Arabidopsis. Plant Cell* 4, 645–656.

Uknes, S., Winter, A. M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., et al. (1993). Biological induction of systemic acquired resistance in *Arabidopsis. Mol. Plant Microbe Interact.* 6, 692–698.

van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162.

van Loon, L. C., and van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40, 199–211.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H., and Ryals, J. (1995). 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. *Mol. Plant Microbe Interact.* 8, 228–234.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., et al. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6, 959–965.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.* 33, 949–956.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., et al. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3, 1085–1094.

Weigel, R. R., Bäuscher, C., Pfitzner, A. J. P., and Pfitzner, U. M. (2001). NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from *Arabidopsis* that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. *Plant Mol. Biol.* 46, 143–160.

Weigel, R. R., Pfitzner, U. M., and Gatz, C. (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in *Arabidopsis. Plant Cell* 17, 1279–1291.

White, R. F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99, 410–412.

Wu, Y., Zhang, D., Chu, J. Y., Boyle, P., Wang, Y., Brindle, I. D., et al. (2012). The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Rep.* 1, 639–647.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528.

Zhou, J.-M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., et al. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Mol. Plant Microbe Interact.* 13, 191–202.

Zwicker, S., Mast, S., Stos, V., Pfitzner, A. J. P., and Pfitzner, U. M. (2007). Tobacco NIMIN2 proteins control PR gene induction through transient repression early in systemic acquired resistance. *Mol. Plant Pathol.* 8, 385–400.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 December 2012; accepted: 22 March 2013; published online: 12 April 2013.

*Citation: Hermann M, Maier F, Masroor A, Hirth S, Pfitzner AJP and Pfitzner UM (2013) The Arabidopsis NIMIN proteins affect NPR1 differentially. Front. Plant Sci. **4**:88. doi: 10.3389/fpls.2013.00088*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Hermann, Maier, Masroor, Hirth, Pfitzner and Pfitzner. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 06 May 2013
doi: 10.3389/fpls.2013.00116

Induced resistance in tomato by SAR activators during predisposing salinity stress

Matthew F. Pye[1], Fumiaki Hakuno[2], James D. MacDonald[1] and Richard M. Bostock[1]*

[1] *Department of Plant Pathology, University of California at Davis, Davis, CA, USA*
[2] *Research Center, Nihon Nohyaku Co., Ltd., Kawachi-Nagano, Osaka, Japan*

Edited by:
Corné M. Pieterse, Utrecht University, Netherlands

Reviewed by:
Zuhua He, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, China
Frederic Brunner, Eberhard Karls University Tübingen, Germany
Hideo Nakashita, RIKEN, Japan

***Correspondence:**
Richard M. Bostock, Department of Plant Pathology, University of California at Davis, One Shields Avenue, Davis, CA 95616, USA.
e-mail: rmbostock@ucdavis.edu

Plant activators are chemicals that induce disease resistance. The phytohormone salicylic acid (SA) is a crucial signal for systemic acquired resistance (SAR), and SA-mediated resistance is a target of several commercial plant activators, including Actigard (1,2,3-benzothiadiazole-7-thiocarboxylic acid-*S*-methyl-ester, BTH) and Tiadinil [*N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide, TDL]. BTH and TDL were examined for their impact on abscisic acid (ABA)-mediated, salt-induced disease predisposition in tomato seedlings. A brief episode of salt stress to roots significantly increased the severity of disease caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) and *Phytophthora capsici* relative to non-stressed plants. Root treatment with TDL induced resistance to *Pst* in leaves and provided protection in both non-stressed and salt-stressed seedlings in wild-type and highly susceptible NahG plants. Non-stressed and salt-stressed ABA-deficient *sitiens* mutants were highly resistant to *Pst*. Neither TDL nor BTH induced resistance to root infection by *Phytophthora capsici*, nor did they moderate the salt-induced increment in disease severity. Root treatment with these plant activators increased the levels of ABA in roots and shoots similar to levels observed in salt-stressed plants. The results indicate that SAR activators can protect tomato plants from bacterial speck disease under predisposing salt stress, and suggest that some SA-mediated defense responses function sufficiently in plants with elevated levels of ABA.

Keywords: Tiadinil, Actigard, induced susceptibility, phytohormones, *Pseudomonas syringae* pv. *tomato*, *Phytophthora capsici*, predisposition

INTRODUCTION

As sessile organisms, plants are presented with numerous biotic challenges such as herbivory and pathogen attack. Plants initiate responses to these challenges by harnessing tightly regulated phytohormone networks. Salicylic acid (SA) levels increase in plants following pathogen infection and SA is critical for the development of systemic acquired resistance (SAR; Métraux et al., 1990; Rasmussen et al., 1991). There are two enzymatic pathways for the generation of SA: one via phenylalanine ammonia lyase and the other via isochorismate synthase (ICS). In tomato (*Solanum lycopersicum*), *Arabidopsis* and *Nicotiana benthamiana*, most pathogen-induced SA appears to be synthesized via the ICS pathway (Wildermuth et al., 2001; Uppalapati et al., 2007; Catinot et al., 2008). Plants with compromised SA synthesis or signaling have greatly diminished defenses against pathogens, as is the case with SA-deficient transgenic plants expressing a bacterial salicylate hydroxylase (NahG; Gaffney et al., 1993) or ICS mutants like *sid2* (Wildermuth et al., 2001), and mutants in downstream targets of SA such as *npr1* (Mou et al., 2003). SAR induction by biotic agents coincides with increases in SA levels and a systemic transcriptional reprograming that primes the plant to respond rapidly to minimize the spread or severity of further infections (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Vlot et al., 2009). This transcriptional reprograming includes the expression of pathogenesis-related (PR) genes and deployment of peroxidases and other defense factors. In addition to induction by biotic agents, SAR responses are induced by exogenous application of SA to the foliage or roots (Ward et al., 1991).

Plant activators are chemicals that have no direct antimicrobial activity but induce disease resistance (Kessmann et al., 1994; Louws et al., 2001). A number of synthetic compounds have been developed that induce SAR by increasing SA accumulation (Iwai et al., 2007) and/or by acting on downstream targets of SA (Vernooij et al., 1995; Durrant and Dong, 2004). For example, the plant activator, probenazole, effective against bacterial, fungal, and oomycete diseases, stimulates SAR by increasing SA levels (Iwai et al., 2007). 1,2,3-Benzothiadiazole-7-thiocarboxylic acid-*S*-methyl-ester (BTH), sold under the trade name, Actigard, stimulates SAR in many plant species without inducing SA accumulation (Lawton et al., 1996). Tiadinil [TDL; *N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide] is a plant activator that was registered in Japan in 2003 under the trade name, V-GET. TDL was developed for disease management in rice where it is applied to nursery-grown seedlings for transplanting to production fields (Tsubata et al., 2006). TDL is very effective for control of rice blast disease caused by *Magnaporthe oryzae* (Yasuda et al., 2006) and appears to induce resistance in a manner similar to BTH by acting on downstream targets of SA (Lawton et al., 1996; Yasuda et al., 2004). The TDL metabolite,

4-methyl-1,2,3-thiadiazole-5-carboxylic acid, is responsible for the SAR activation (Yasuda et al., 2006).

Abiotic stress alters the susceptibility of plants to many pathogens (Cho et al., 2009). The effect of brief episodes of root stress such as salinity and water deficit at levels that commonly occur in agriculture is well documented in plant–oomycete interactions, wherein stress events predispose plants to levels of inoculum they would normally resist (DiLeo et al., 2010). The phytohormone abscisic acid (ABA) accumulates rapidly in roots and shoots as an adaptive response to these abiotic stresses, but also contributes to the increased disease proneness of the plants (Mohr and Cahill, 2003; Thaler and Bostock, 2004; Fan et al., 2009; DiLeo et al., 2010). Antagonism between SA and ABA is well documented in relation to plant defense responses to pathogens (Mohr and Cahill, 2007; Jiang et al., 2010). Previously, ABA was found to have an antagonistic effect on SAR which was induced by 1,2-benzisothiazol-3(2*H*)-one1,1-dioxide and BTH in *Arabidopsis* and tobacco (Yasuda et al., 2008; Kusajima et al., 2010). However, it is not known if plant activators that target SA signaling impact the ABA-mediated susceptibility to root pathogens that occurs following predisposing root stress in tomato.

Because of the potential for unwanted tradeoffs and signaling conflicts in plants exposed to different stresses, as can occur in the field, we investigated how predisposing root stress impacts chemically induced resistance in tomato. The objective of this study was to determine the effect of pretreatment of tomato seedlings with TDL and BTH on salt-induced predisposition to the foliar bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) and to the soilborne oomycete pathogen *Phytophthora capsici*. TDL is of particular interest in the context of soilborne pathogens such as *Phytophthora capsici* because it is often applied to plants as a root dip. We also determined the impact of SA, TDL and BTH on ABA accumulation during a predisposing episode of salt stress. The results show that TDL applied to roots strongly protects the leaves from disease caused by *Pst* in both non-stressed and salt-stressed plants. In contrast, neither TDL nor BTH protects roots from *Phytophthora capsici*. The protection induced by plant activators against *Pst* does not result from reduced ABA accumulation and, although overall disease is less in both non-stressed and salt-stressed plants by chemically induced SAR, plant activators do not reverse the salt-induced increment in disease severity.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Tomato plants (*Solanum lycopersicum*) of cultivars "New Yorker" or "Rheinlands Ruhm" and mutants within these backgrounds were used in experiments. "New Yorker" seeds were obtained from a commercial source (Totally Tomatoes, Randolph, WI, USA). The homozygous ABA-deficient mutant *sitiens* was compared with its isogenic, wild-type (WT) background, "Rheinlands Ruhm" (Tal and Nevo, 1973), and seeds for these were obtained from the C.M. Rick Tomato Genetics Resource Center at the University of California, Davis. NahG transgenic plants were generated in the "New Yorker" background, similar to the method used by Gaffney et al. (1993). The *nahG* construct containing the transgene salicylate hydroxylase under control of the CaMV 35S promoter in the binary vector pCIB200 was a gift of Syngenta Crop Protection, Inc.

Tomato plants were grown in a hydroponic format. Prior to use, tomato seeds were surface sterilized with the following protocol: 50% HCl (10 min) and rinsed with sterile deionized H_2O, 10% trisodium phosphate (15 min) and rinsed (3×) in sterile deionized H_2O, 70% ethanol (10 min), and rinsed (3×) with sterile deionized H_2O, and 50% commercial bleach (3% sodium hypochlorite; 20 min) followed by sterile deionized H_2O rinse (3×). Following surface-sterilization, seeds were placed on sterile germination paper in beakers containing sterile deionized H_2O, transferred after 1 week to trimmed 5 ml polypropylene pipette tips, secured with foam test tube plugs, and placed into aerated hydroponic containers filled with 4 L of aerated, 0.5× Hoagland's solution. Seedlings were grown for an additional 2 weeks in a growth chamber (150 μmol m^{-2} s^{-1}, 16 h photoperiod, 22°C, 70% RH) until at least two true leaves had developed on each plant.

SA TREATMENT, PLANT ACTIVATOR TREATMENT, SALT TREATMENTS, AND INOCULATION

Four-week-old hydroponically grown tomato plants were immersed in 50 ml of 0.5× Hoagland's solution containing 10 ppm (37 μM) TDL (Nihon Nohyaku Co., Ltd), 10 ppm (47 μM) BTH (Syngenta Crop Protection, Inc.), 10 ppm (62 μM) salicylic acid-sodium salt (SA; Sigma-Aldrich), or water for 7 days prior to salt stress and inoculation with a pepper isolate of *Phytophthora capsici* (from Yolo County, CA; also pathogenic on tomato) or *Pst*, (isolate B-64, gift of D. Cooksey). Pre-inoculation salt treatments consisted of exposing the roots to saline solution (0.2 M NaCl + 0.02 M $CaCl_2$) for 18 h. All seedlings collapsed within 10 min of exposure to saline solution and regained full turgor within 2 hr of salt removal. Shoots were dip inoculated with 2-day-old *Pst* cultures adjusted to 1×10^7 cfu ml^{-1} in 1 L of 10 mM $MgCl_2$ with 80 μl Silwet L77. Roots were inoculated with 2 ml of zoospore suspension to achieve a final concentration of 1×10^4 zoospores ml^{-1}.

Pst AND *Phytophthora capsici* DISEASE ANALYSES

Four days post-inoculation (dpi) *Pst*-infected leaflets were surface sterilized with 70% EtOH for 10 s, rinsed in sterile H_2O, and blotted dry. Samples were excised with a #3 hole punch (5 mm diameter) and ground in 200 μl 5 mM $MgCl_2$. A series of 10-fold dilutions were plated on King's B medium; colonies were counted after 2 days of growth at 28°C. The relationship of disease and *Phytophthora capsici* DNA content was determined by quantitative polymerase chain reaction (qPCR; DiLeo et al., 2010). To correct for variability across samples, a similar amount of hypocotyl and root tissue was extracted for each sample and the qPCR analyses were performed on DNA extracts adjusted for total DNA content as measured with a Nanodrop spectrophotometer model ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

ABA ANALYSES

To determine the effect of SA on ABA accumulation during salt stress, ABA levels were measured in WT plants pre-treated with SA, TDL, or BTH. Following salt stress treatment for 18 h, roots and shoots were collected and immediately frozen in liquid N_2.

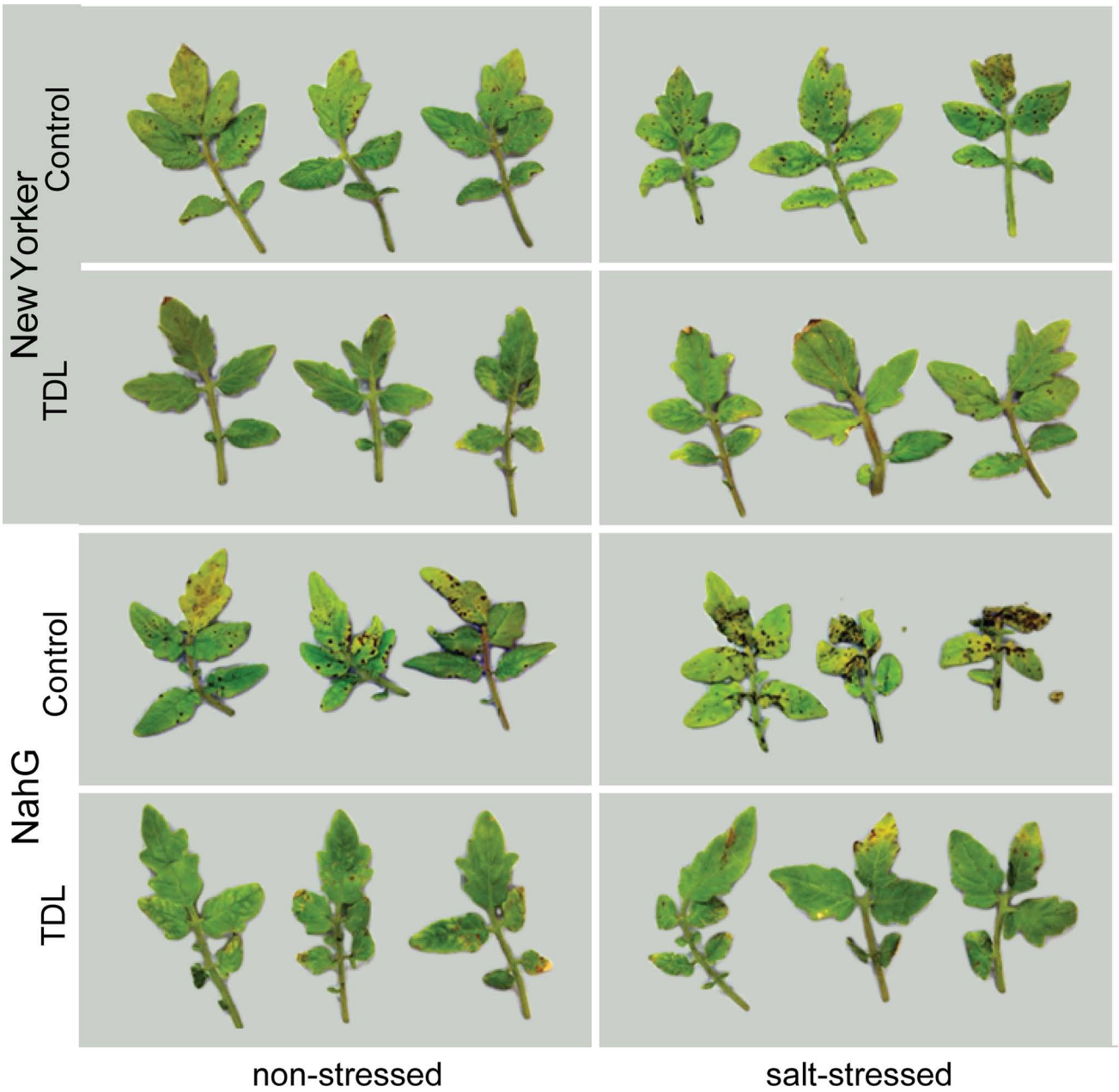


FIGURE 1 | *Pseudomonas syringae* pv. *tomato* colonization in WT background ("New Yorker") and NahG tomato leaves. Roots were pretreated with TDL for 7 days and then exposed for 18 h to salt stress (0.2 M NaCl/0.02 M $CaCl_2$). Shoots were dip-inoculated with a suspension of *Pst* adjusted to 10^7 cfu ml^{-1}. Symptoms photographed 4 dpi.

The tissues were lyophilized and placed at −20°C until extraction. The lyophilized tissue was ground in liquid N_2 to a fine powder with a mortar and pestle, 50–100 mg samples were collected, and each sample transferred to a microfuge tube. Cold 80% methanol (1.2 ml) containing butylated hydroxytoluene at 10 μg ml^{-1} was added to each tube, which was then vortexed. The extracts were placed on ice and agitated occasionally for 30 min. The tubes were centrifuged for 5 min at 10,000 × *g*, and the supernatants collected. The pellet was extracted with 0.5 ml of 80% methanol and centrifuged to collect the supernatant. This step was repeated, all three supernatants were combined, and the methanol concentration of the extract adjusted to 70%. The extracts were applied to pre-wetted Sep-pak C18 columns (Waters, Inc., Milford, MA, USA) and eluted with 5 ml of 70% methanol. The eluate (∼7.5 ml) containing ABA was concentrated to near dryness at 37°C under vacuum and the volume adjusted to 300 μl with deionized water. The samples were analyzed by competitive immunoassay with an ABA immunoassay kit according to the manufacturer's directions

(Agdia/Phytodetek, Elkhart, IN, USA). Results are expressed as nanomoles of (+)-ABA per gram dry weight of tissue. To determine the effect of the *nahG* transgene on ABA levels, roots and shoots from WT and NahG plants were processed using the same procedure as above.

SA ANALYSES

To determine the effect of the *nahG* transgene on SA accumulation following infection, SA was quantified in WT "New Yorker" and NahG backgrounds in non-inoculated plants and plants 3 dpi with *Pst*. Extraction of SA was carried out as previously described (Engelberth et al., 2004). Deuterated SA (C/D/N Isotopes, Inc., Quebec, Canada) was used as an internal standard. Methyl ester derivatives were analyzed by GC-MS in electronic ionization mode. Mass spectral analysis was done in selective ion monitoring mode. Fragment ions were SA-ME 152 and SA-D4-ME 156. Quantification calibration curves were generated with known quantities of pure SA.

STATISTICAL ANALYSES

Pseudomonas syringae pv. *tomato* disease assays in "New Yorker" and "Rheinlands Ruhm" backgrounds were performed three times, with three replicates for each treatment within each experiment. The *Phytophthora capsici* disease assay experiment was performed three times with five replicates for each treatment within each experiment. Experiments measuring ABA accumulation were performed five times. SA accumulation was measured in one experiment with three replicates for each treatment. Statistical analysis was performed on all data sets. Log transformation was used for data which pass the Shapiro–Wilk's test for normal distribution. The Tukey–Kramer test, Dunnett's test, Wilcoxon rank sums test or *T*-tests were used for means comparisons using JMP software (version 10.0; SAS Inc.) as indicated.

RESULTS

TDL PROTECTS TOMATO AGAINST THE BACTERIAL SPECK PATHOGEN *Pst* IN NON-STRESSED AND SALT-STRESSED SEEDLINGS

To determine if plant activators induce resistance to *Pst* under different stress regimes in our experimental format, roots of hydroponically grown seedlings of cv. "New Yorker" were treated with TDL and then either not salt-stressed or exposed to 0.2 M NaCl for 18 h prior to inoculation. In preliminary experiments, several concentrations of TDL were evaluated for phytotoxicity and for efficacy against bacterial speck disease with 10 ppm (37 μM) TDL selected as this concentration provided an optimal response. Concentrations higher than 10 ppm of TDL caused a slight bronzing of the roots and depressed growth of the seedlings, suggesting a mild phytotoxicity of the chemical in our experimental format at these higher levels. Inoculated salt-stressed seedlings had more severe disease symptoms (**Figure 1**) and a significantly higher titer of pathogen (**Figure 2**) than non-stressed, inoculated plants. Pretreatment with TDL at 10 ppm significantly reduced *Pst* colonization and symptom severity in "New Yorker" plants in both non-stressed and salt-treated seedlings (**Figure 2**). However, TDL did not prevent the proportional increase in *Pst* colonization observed in salt-stressed plants relative to the non-stressed controls.

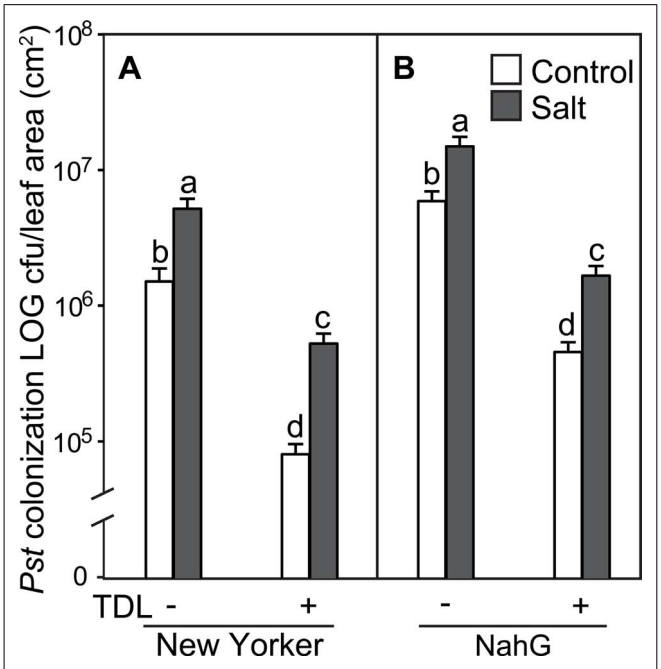


FIGURE 2 | *Pseudomonas syringae* pv. *tomato* colonization in (A) WT background ("New Yorker") and (B) NahG tomato leaves. Roots were pretreated with TDL for 7 days and then exposed for 18 h to salt stress (0.2 M NaCl/0.02 M $CaCl_2$). Shoots were dip-inoculated with a suspension of *Pst* adjusted to 10^7 cfu ml^{-1}. Colonization was evaluated 4 dpi. Bars represent the means ± SE from three expriments, $n = 15$. Letters above bars indicate significant differences between treatments at $\alpha = 0.05$ using the Tukey–Kramer test for mean separation.

Since TDL harnesses SA-mediated defenses, we treated SA-deficient NahG plants to see if TDL induces resistance under the different stress regimes in this highly susceptible background. As expected, NahG plants were more susceptible to *Pst* (**Figure 2**) and accumulated significantly less SA following *Pst* infection (data not shown) than the WT background "New Yorker." However, TDL provided strong protection in the NahG plants and mitigated the predisposing effect of salt-stress on bacterial speck disease.

TDL PROTECTS AGAINST *Pst* IN BOTH ABA-NORMAL AND ABA-DEFICIENT TOMATO SEEDLINGS

In a previous study we showed that ABA-deficient tomato mutants displayed a much reduced predisposition phenotype to salt stress (DiLeo et al., 2010). To determine if the protective effect of TDL is altered within an ABA-deficient tomato mutant, seedlings of WT (cv. "Rheinlands Ruhm") and an ABA-deficient mutant within this background, *sitiens*, were treated in the same format and stress regimes as above. TDL significantly reduced *Pst* symptoms (**Figure 3**) and colonization (**Figure 4**) in both non-stressed and salt-treated plants of "Rheinlands Ruhm." However, 3.6- and 5.4-fold increases in pathogen titer as a result of salt-stress were observed in both the control and TDL-treated plants, respectively, indicating that TDL did not prevent the proportional increase in *Pst* colonization in salt-stressed plants, similar to the results with "New Yorker" and NahG plants. In contrast, the *sitiens* mutant was not predisposed to *Pst* by salt stress and had significantly

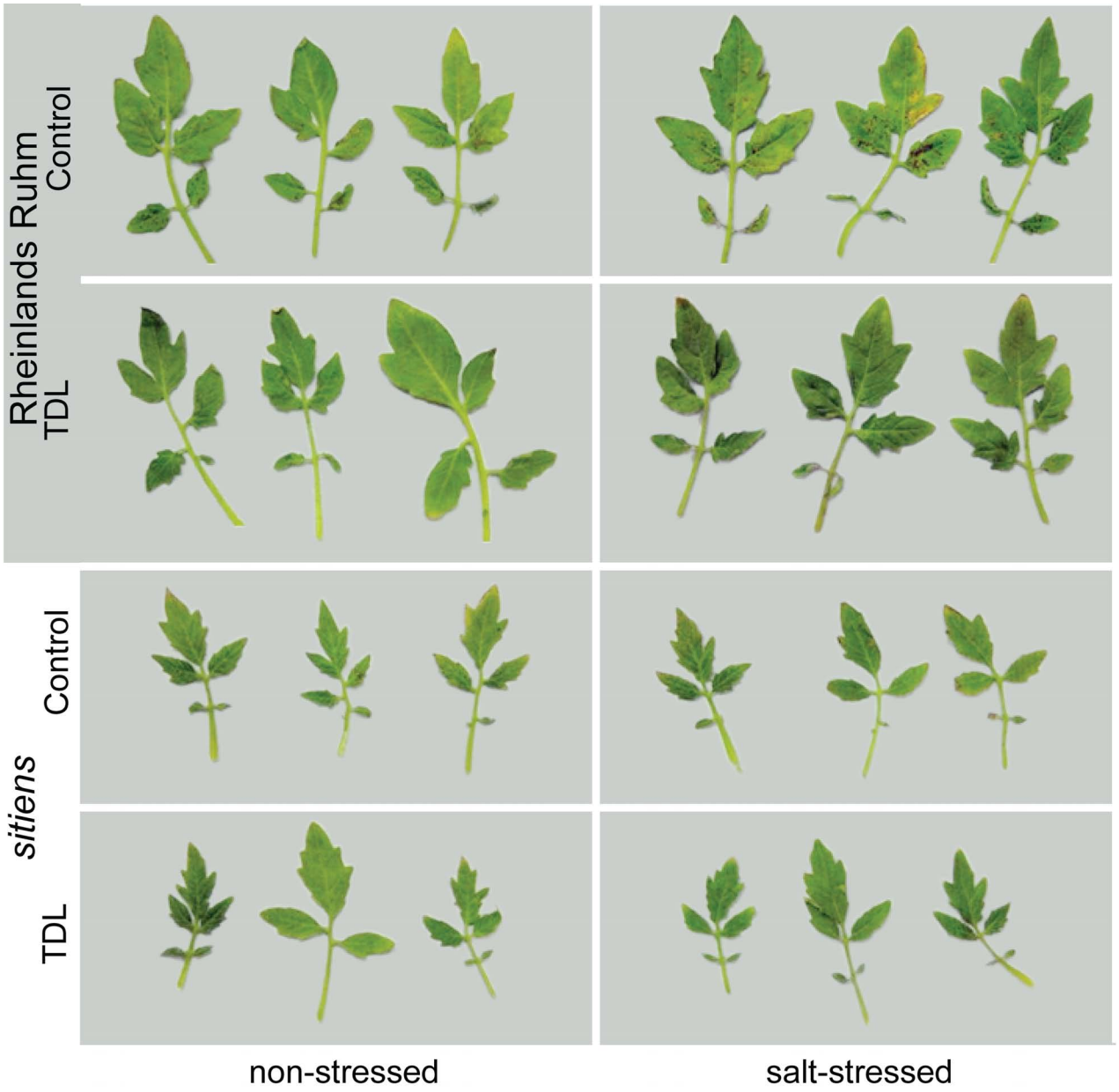


FIGURE 3 | *Pseudomonas syringae* pv. *tomato* colonization in background ("Rheinlands Ruhm") and *sitiens* tomato leaves. Roots were pretreated with TDL for 7 days and then exposed for 18 h to salt stress (0.2 M NaCl/0.02 M $CaCl_2$). Shoots were dip-inoculated with a suspension of *Pst* adjusted to 10^7 cfu ml^{-1}. Symptoms were photographed 4 dpi.

reduced symptoms (**Figure 3**) and colonization by the pathogen than the background "Rheinlands Ruhm" (**Figure 4**). Nonetheless, TDL pretreatment of *sitiens* provided further protection against *Pst* (**Figure 4**).

TDL AND BTH DO NOT REDUCE *Phytophthora capsici* DISEASE SEVERITY

To determine if plant activators protect tomato roots and crowns against the oomycete pathogen, *Phytophthora capsici*, and predisposing root stress, tomato seedlings were treated with TDL or BTH (10 ppm), not stressed or salt-stressed as above, and then inoculated. There was no protection provided by the plant activators against disease caused by *Phytophthora capsici* in either the control or salt-treated plants, as reflected in symptom severity (not shown) and pathogen colonization (**Figure 5**).

IMPACT OF SALINITY STRESS AND PLANT ACTIVATORS ON ROOT AND SHOOT ABA LEVELS

Because elevated levels of ABA in tomato can enhance susceptibility to *Pst* (Mohr and Cahill, 2007) and *Phytophthora capsici*

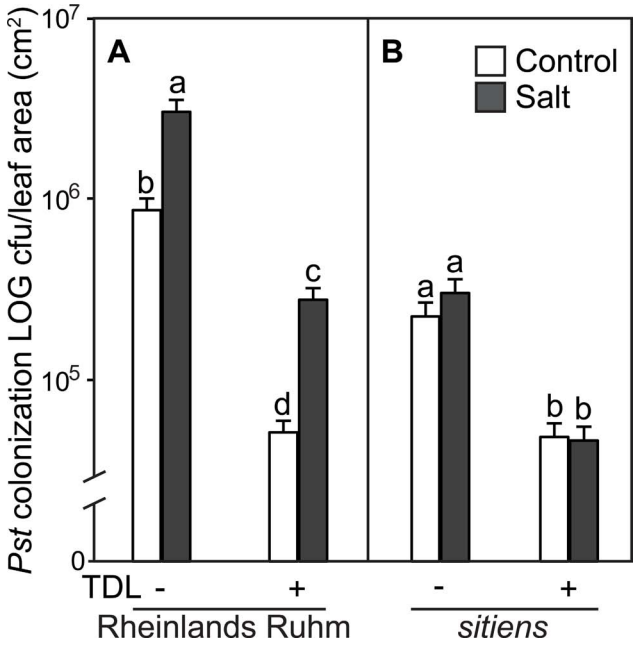


FIGURE 4 | *Pseudomonas syringae* pv. *tomato* colonization in (A) WT background ("Rheinlands Ruhm") and (B) *sitiens* tomato leaves. Roots were pretreated with TDL for 7 days and then exposed for 18 h to salt stress (0.2 M NaCl/0.02 M $CaCl_2$). Shoots were dip-inoculated with a suspension of *Pst* adjusted to 10^7 cfu ml^{-1}. Colonization was evaluated 4 dpi. Bars represent the means ± SE from three expriments, $n = 15$. Letters above bars indicate significant differences between treatments at $\alpha = 0.05$ using the Tukey–Kramer test for mean separation.

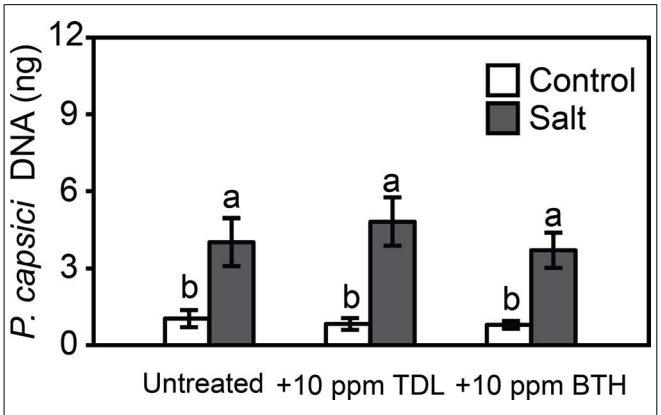


FIGURE 5 | *Phytophthora capsici* colonization 48 hpi on WT "New Yorker" non-stressed (control) or salt-stressed (0.2 M NaCl/0.02 M $CaCl_2$) roots for 18 h following pretreatment with TDL or BTH. Colonization estimated by quantitative polymerase chain reaction of pathogen DNA. Bars represent the means ± SE from three experiments ($n = 9$ for each treatment). Letters indicate significant differences between treatments by *T*-test ($\alpha = 0.05$).

(DiLeo et al., 2010), the effect of SA, TDL, and BTH on ABA levels was determined in roots and shoots. ABA concentrations in either shoots or roots at the time selected for inoculation in our treatment sequence were not altered by SA (**Figure 6**). However, a trend of increasing ABA accumulation was observed in TDL- and BTH-treated "New Yorker" plants relative to the corresponding control plants (**Figure 7**). Although the increase in ABA accumulation in the plants treated with these plant activators is not statistically significant at $P \leq 0.05$, it can be said that SA, TDL, and BTH do not reduce ABA content relative to untreated plants (**Figure 7**). In addition, salt stress did not further increase the levels of ABA in plants that had been pretreated with TDL or BTH, which were similar to the salt stressed controls.

DISCUSSION

In a previous study, we demonstrated the predisposing effect of salt stress and a role for ABA as a determinative factor in predisposition in the tomato–*Phytophthora capsici* interaction (DiLeo et al., 2010). The present study is the first report of salt-induced predisposition to the bacterial speck pathogen, *Pst*, in tomato. Furthermore, the results with the ABA-deficient *sitiens* mutant are consistent with the salt-induced susceptibility to *Pst* being mediated by ABA (**Figure 4**). These results conform to studies in *Arabidopsis* where ABA has been reported to promote susceptibility to *Pst* (de Torres-Zabala et al., 2007; Yasuda et al., 2008).

Because SA has been shown to protect tomato against salt stress, possibly by an ABA-dependent mechanism (Szepesi et al., 2009), plant activators that operate via the SA pathway were evaluated for effect on salt-induced predisposition. Protection of tomato against bacterial speck disease by BTH is well documented (Louws et al., 2001), and TDL has previously been shown to reduce the severity of bacterial and fungal infections without inducing SA accumulation (Yasuda et al., 2004, 2006). Here, TDL was shown to protect against *Pst* in both non-stressed and salt-stressed tomato plants. TDL pretreatment strongly reduced disease and colonization by *Pst* in both "New Yorker" and SA-deficient NahG plants. TDL, or more likely its biologically active metabolite, SV-03, presumably allows the NahG plants to mount an SAR response to *Pst* infection in the absence of SA accumulation (**Figure 2**). TDL provided protection in both non-stressed and salt-stressed plants, but did not reverse the predisposing effect of salt stress. An increase in *Pst* colonization was observed in the salt-stressed, TDL-pretreated plants of both genotypes, with comparable percentage increases relative to the corresponding non-stressed controls in "New Yorker" and NahG plants. This indicates that TDL does not reverse the salt-stress effect on disease, *per se*, and likely targets stress network signaling independently of an ABA-mediated process that conditions the salt-induced susceptibility observed in this system (**Figures 2** and **4**).

"Rheinlands Ruhm" also displayed salt-induced predisposition to *Pst*. Pretreatment with TDL significantly reduced *Pst* colonization in both "Rheinlands Ruhm" and *sitiens* (**Figure 4**). Similarly, TDL provided protection in both non-stressed and salt-stressed plants, but did not reverse the predisposing effect of salt stress in "Rheinlands Ruhm" plants. The salt-induced increment in colonization by the pathogen was comparable in both the untreated and TDL-treated plants (**Figure 4**). The ABA-deficient mutant, *sitiens*, is considerably less susceptible to *Pst* than its background "Rheinlands Ruhm," and does not exhibit salt-induced predisposition (**Figures 3** and **4**).

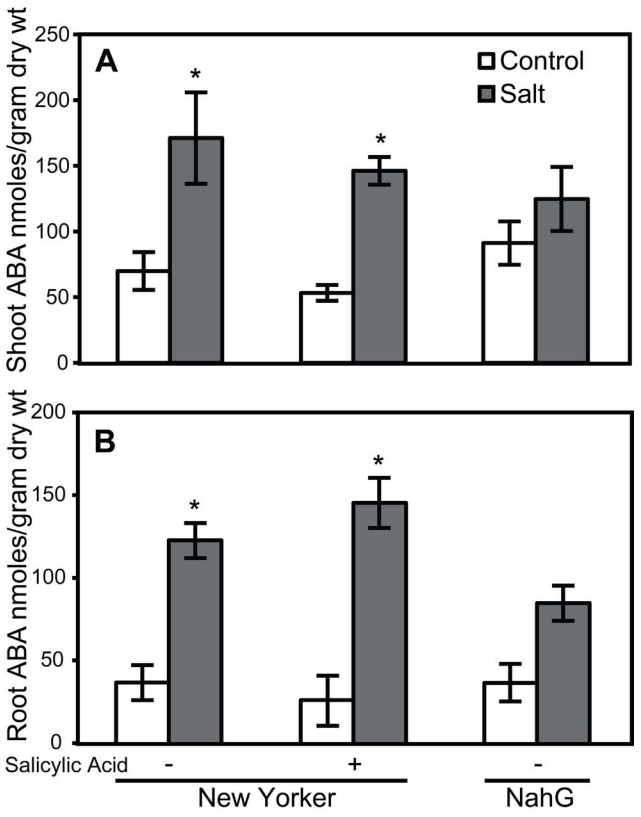


FIGURE 6 | ABA accumulation in shoots (A) and roots (B) of salt-stressed and non-stressed tomatoes with altered salicylic acid. ABA levels in the roots of "New Yorker" and NahG seedlings, non-stressed (control) and 18 h salt-stressed (0.2 M NaCl and 0.02 $CaCl_2$). + = seedling roots were treated with SA (62 μM) for 1 week prior to ABA measurement. Bars represent the means ± SE from five experiments ($n = 15$). Asterisks indicate significant differences over the "New Yorker" control by Dunnett's test $\alpha = 0.05$).

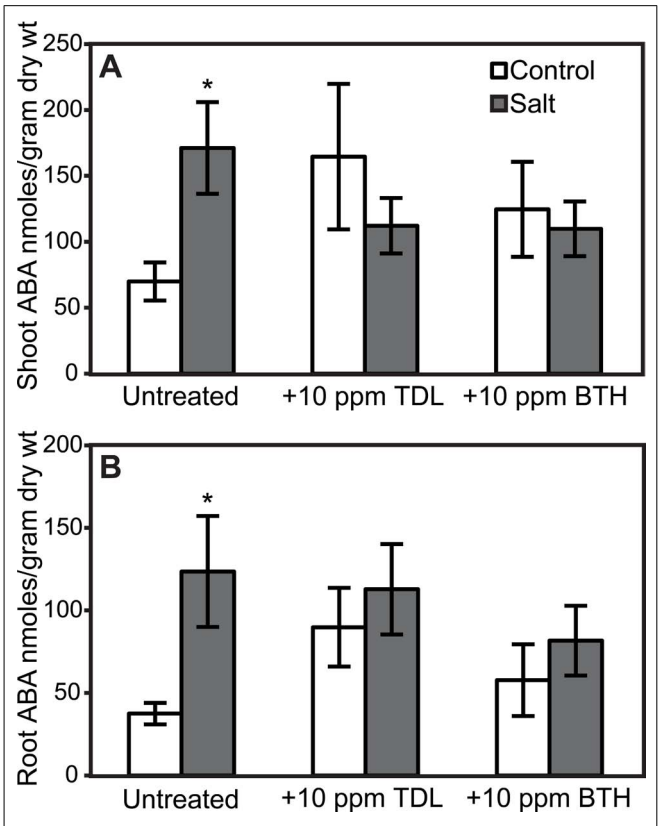


FIGURE 7 | ABA accumulation in shoots (A) and roots (B) of "New Yorker" plants non-stressed (control) or salt-stressed (0.2 M NaCl/0.02 M $CaCl_2$) for 18 h, with and without prior TDL or BTH treatment. Values are the means ± SE from three experiments ($n = 9$). Asterisks indicate a significant increase in shoot **(A)** ($\chi^2 = 8.65$, $P = 0.003$) and root **(B)** ($\chi^2 = 5.78$, $P = 0.016$) ABA in "New Yorker" salt over "New Yorker" control by Wilcoxon rank sums.

Protection by plant activators against foliar pathogens is well established (Louws et al., 2001; Yasuda et al., 2004). However, relatively few studies have examined these compounds against soilborne pathogens and so TDL and BTH were evaluated for protection against root infection by *Phytophthora capsici*. Neither TDL nor BTH induced resistance or impacted salt-induced predisposition to *Phytophthora capsici* (**Figure 5**). *Phytophthora capsici* is an aggressive root and crown pathogen with a hemibiotrophic parasitic habit (Lamour et al., 2012) that triggers both SA- and jasmonic acid-mediated responses during infection of tomato (unpublished data). The results suggest that SA responses in tomato play a less important role in defense against *Phytophthora capsici* than to *Pst*.

The impact of SA and plant activators on ABA accumulation was measured in tomato roots and shoots. SA treatment and SA-deficiency conferred by NahG did not significantly impact ABA levels (**Figure 6**). However, ABA accumulation in non-stressed TDL and BTH treatments trended higher than those observed in salt-stressed plants that did not receive a plant activator treatment (**Figure 7**). Protection by TDL against *Pst* is likely the result of a triggered SAR response and not the result of an antagonistic effect on ABA levels.

The efficacy of plant activators depends on the specific diseases targeted and the environmental context, which may present additional stressors to confound defense network signaling in the plant. A challenge for successful deployment of plant activators in the field is to manage the allocation, ecological and fitness costs that are associated with induced defenses (Heil, 2001; Heil and Baldwin, 2002; Heil and Bostock, 2002; Berger et al., 2007). These costs can be manifested by reduced growth and reproduction, vulnerability to other forms of attack, and potential interference with beneficial associations (Bostock, 2005). It would seem that the severity of these costs is conditioned in part by the milieu of abiotic stressors operative at any given time. Reactive oxygen species (ROS) contribute to the initiation of SAR (Alvarez et al., 1998), are induced by SA and BTH (Fitzgerald et al., 2004; van der Merwe and Dubery, 2006), and are essential co-substrates for induced defense responses such as lignin synthesis (Hammerschmidt and Kuc, 1982). ROS also are important in modulating abiotic stress networks, for example in ABA signaling and response (Cho et al., 2009). The potential compounding effect of ROS generated from multiple stressors presents a dilemma in that the plant

must reconcile these to adapt or else suffer the negative consequences of oxidative damage for failure to do so (Foyer and Noctor, 2009). Paradoxically, SA and BTH also are reported to protect plants against paraquat toxicity, which involves ROS generation for its herbicidal action (Silverman et al., 2005). How plants balance ROS's signaling roles and destructive effects within multiple stress contexts is unresolved and a critically important area of plant biology with relevance for optimizing induced resistance strategies in crop protection (Van Breusegem et al., 2008; Foyer and Noctor, 2009). Although our experiments were conducted under highly controlled conditions, the results with TDL are encouraging and show that chemically induced resistance to bacterial speck disease occurs in both salt-stressed and non-stressed plants and in plants severely compromised in SA accumulation. Future research with plant activators should consider their use within different abiotic stress contexts to fully assess outcomes in disease and pest protection.

ACKNOWLEDGMENTS

The authors acknowledge T. Savchenko for assistance with salicylic acid analyses, and K. Dehesh and G. Coaker for editorial suggestions. Research supported in part by a grant to Richard M. Bostock from the USDA Exotic/Invasive Pests and Diseases program and with funds from the University of California Agricultural Experiment Station.

REFERENCES

Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92, 773–784.

Berger, S., Sinha, A. K., and Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. *J. Exp. Bot.* 58, 4019–4026.

Bostock, R. M. (2005). Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* 43, 545–580.

Catinot, J., Buchala, A., Abou-Mansour, E., and Métraux, J.-P. (2008). Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett.* 582, 473–478.

Cho, D., Shin, D. J., Jeon, B. W., and Kwak, J. M. (2009). ROS-mediated ABA signaling. *J. Plant Biol.* 52, 102–113.

de Torres-Zabala, M., Truman, W., Bennett, M. H., Lafforgue, G., Mansfield, J. W., Rodriguez Egea, P., et al. (2007). *Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease. *EMBO J.* 26, 1434–1443.

DiLeo, M. V., Pye, M. F., Roubtsova, T. V., Duniway, J. M., Macdonald, J. D., Rizzo, D. M., et al. (2010). Abscisic acid in salt stress predisposition to phytophthora root and crown rot in tomato and *Chrysanthemum*. *Phytopathology* 100, 871–879.

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209.

Engelberth, J., Alborn, H. T., Schmelz, E. A., and Tumlinson, J. H. (2004). Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1781–1785.

Fan, J., Hill, L., Crooks, C., Doerner, P., and Lamb, C. (2009). Abscisic acid has a key role in modulating diverse plant–pathogen interactions. *Plant Physiol.* 150, 1750–1761.

Fitzgerald, H. A., Chern, M. S., Navarre, R., and Ronald, P. C. (2004). Overexpression of (At)NPR1 in rice leads to a BTH- and environment-induced lesion-mimic/cell death phenotype. *Mol. Plant Microbe Interact.* 17, 140–151.

Foyer, C. H., and Noctor, G. (2009). Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid. Redox Signal.* 11, 861–905.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., et al. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261, 754–756.

Hammerschmidt, R., and Kuc, J. (1982). Lignification as a mechanism for induced systemic resistance in cucumber. *Physiol. Plant Pathol.* 20, 61–71.

Heil, M. (2001). The ecological concept of costs of induced systemic resistance (ISR). *Eur. J. Plant Pathol.* 107, 137–146.

Heil, M., and Baldwin, I. T. (2002). Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends Plant Sci.* 7, 61–67.

Heil, M., and Bostock, R. M. (2002). Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Ann. Bot.* 89, 503–512.

Iwai, T., Seo, S., Mitsuhara, I., and Ohashi, Y. (2007). Probenazole-induced accumulation of salicylic acid confers resistance to *Magnaporthe grisea* in adult rice plants. *Plant Cell Physiol.* 48, 915–924.

Jiang, C.-J., Shimono, M., Sugano, S., Kojima, M., Yazawa, K., Yoshida, R., et al. (2010). Abscisic acid interacts antagonistically with salicylic acid signaling pathway in rice–*Magnaporthe grisea* interaction. *Mol. Plant Microbe Interact.* 23, 791–798.

Kessmann, H., Staub, T., Ligon, J., Oostendorp, M., and Ryals, J. (1994). Activation of systemic acquired disease resistance in plants. *Eur. J. Plant Pathol.* 100, 359–369.

Kusajima, M., Yasuda, M., Kawashima, A., Nojiri, H., Yamane, H., Nakajima, M., et al. (2010). Suppressive effect of abscisic acid on systemic acquired resistance in tobacco plants. *J. Gen. Plant Pathol.* 76, 161–167.

Lamour, K. H., Stam, R., Jupe, J., and Huitema, E. (2012). The oomycete broad-host-range pathogen *Phytophthora capsici*. *Mol. Plant Pathol.* 13, 329–337.

Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., et al. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., et al. (2001). Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Dis.* 85, 481–488.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250, 1002–1004.

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., et al. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250, 1004–1006.

Mohr, P. G., and Cahill, D. M. (2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. *Funct. Plant Biol.* 30, 461–469.

Mohr, P. G., and Cahill, D. M. (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato*. *Funct. Integr. Genomics* 7, 181–191.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944.

Rasmussen, J. B., Hammerschmidt, R., and Zook, M. N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv syringae. *Plant Physiol.* 97, 1342–1347.

Silverman, F. P., Petracek, P. D., Fledderman, C. M., Ju, Z. G., Heiman, D. F., and Warrior, P. (2005). Salicylate activity. 1. Protection of plants from paraquat injury. *J. Agric. Food Chem.* 53, 9764–9768.

Szepesi, Á., Csiszár, J., Gémes, K., Horváth, E., Horváth, F., Simon, M. L., et al. (2009). Salicylic acid improves acclimation to salt stress by stimulating abscisic aldehyde oxidase activity and abscisic acid accumulation, and increases Na+ content in leaves without toxicity symptoms in *Solanum lycopersicum* L. *J. Plant Physiol.* 166, 914–925.

Tal, M., and Nevo, Y. (1973). Abnormal stomatal behavior and root resistance, and hormonal imbalance in three wilty mutants of tomato. *Biochem. Genet.* 8, 291–300.

Thaler, J. S., and Bostock, R. M. (2004). Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. *Ecology* 85, 48–58.

Tsubata, K., Kuroda, K., Yamamoto, Y., and Yasokawa, N. (2006). Development of a novel plant activator for rice diseases, tiadinil. *J. Pestic. Sci.* 31, 161–162.

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Kunkel, B. N., Anand, A., Mysore, K. S., et al. (2007). The phytotoxin coronatine contributes to pathogen

fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato DC*3000. *Mol. Plant Microbe Interact.* 20, 955–965.

Van Breusegem, F., Bailey-Serres, J., and Mittler, R. (2008). Unraveling the tapestry of networks involving reactive oxygen species in plants. *Plant Physiol.* 147, 978–984.

van der Merwe, J. A., and Dubery, I. A. (2006). Benzothiadiazole inhibits mitochondrial NADH:ubiquinone oxidoreductase in tobacco. *J. Plant Physiol.* 163, 877–882.

Vernooij, B., Friedrich, L., Goy, P. A., Staub, T., Kessmann, H., and Ryals, J. (1995). 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. *Mol. Plant Microbe Interact.* 8, 228–234.

Vlot, A. C., Dempsey, D. M. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., et al. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3, 1085–1094.

Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414, 562–565.

Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., et al. (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid: mediated abiotic stress response in *Arabidopsis*. *Plant Cell* 20, 1678–1692.

Yasuda, M., Kusajima, M., Nakajima, M., Akutsu, K., Kudo, T., Yoshida, S., et al. (2006). Thiadiazole carboxylic acid moiety of tiadinil, SV-03, induces systemic acquired resistance in tobacco without salicylic acid accumulation. *J. Pestic. Sci.* 31, 329–334.

Yasuda, M., Nakashita, H., and Yoshida, S. (2004). Tiadinil, a novel class of activator of systemic acquired resistance, induces defense gene expression and disease resistance in tobacco. *J. Pestic. Sci.* 29, 46–49.

Conflict of Interest Statement: This research was supported in part with funds from Nihon-Nohyaku Co., Ltd., the manufacturer of Tiadinil. These funds were unrestricted to support research and expenses associated with Fumiaki Hakuno's sabbatical. We have no ongoing commercial or financial relationship with Nihon-Nohyaku Co., LTD. The purpose of this research was to provide an objective assessment of two commercial plant activators for their effects on disease within the context of predisposing abiotic stress.

Received: 05 March 2013; accepted: 12 April 2013; published online: 06 May 2013.

*Citation: Pye MF, Hakuno F, MacDonald JD and Bostock RM (2013) Induced resistance in tomato by SAR activators during predisposing salinity stress. Front. Plant Sci. **4**:116. doi: 10.3389/fpls.2013.00116*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Pye, Hakuno, MacDonald and Bostock. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 27 June 2013
doi: 10.3389/fpls.2013.00215

Integrating nitric oxide into salicylic acid and jasmonic acid/ethylene plant defense pathways

Luis A. J. Mur[1]*, Elena Prats[2], Sandra Pierre[1], Michael A. Hall[1] and Kim H. Hebelstrup[3]

[1] Molecular Plant Pathology Group, Institute of Environmental and Rural Science, Aberystwyth University, Aberystwyth, UK
[2] Institute for Sustainable Agriculture, Spanish National Research Council, Córdoba, Spain
[3] Section of Crop Genetics and Biotechnology, Department of Molecular Biology and Genetics, Aarhus University, Slagelse, Denmark

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Steven H. Spoel, University of Edinburgh, UK
Carmen Castresana, Centro Nacional de Biotecnologia, Spain

***Correspondence:**
Luis A. J. Mur, Molecular Plant Pathology Group, Institute of Environmental and Rural Science, Aberystwyth University, Edward Llwyd Building, Aberystwyth SY23 3DA, UK
e-mail: lum@aber.ac.uk

Plant defense against pests and pathogens is known to be conferred by either salicylic acid (SA) or jasmonic acid (JA)/ethylene (ET) pathways, depending on infection or herbivore-grazing strategy. It is well attested that SA and JA/ET pathways are mutually antagonistic allowing defense responses to be tailored to particular biotic stresses. Nitric oxide (NO) has emerged as a major signal influencing resistance mediated by both signaling pathways but no attempt has been made to integrate NO into established SA/JA/ET interactions. NO has been shown to act as an inducer or suppressor of signaling along each pathway. NO will initiate SA biosynthesis and nitrosylate key cysteines on TGA-class transcription factors to aid in the initiation of SA-dependent gene expression. Against this, *S*-nitrosylation of NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) will promote the NPR1 oligomerization within the cytoplasm to reduce TGA activation. In JA biosynthesis, NO will initiate the expression of JA biosynthetic enzymes, presumably to over-come any antagonistic effects of SA on JA-mediated transcription. NO will also initiate the expression of ET biosynthetic genes but a suppressive role is also observed in the *S*-nitrosylation and inhibition of *S*-adenosylmethionine transferases which provides methyl groups for ET production. Based on these data a model for NO action is proposed but we have also highlighted the need to understand when and how inductive and suppressive steps are used.

Keywords: nitric oxide, salicylic acid, jasmonic acid, ethylenes, pathogens, resistance mechanisms, signaling pathways

INTRODUCTION

Extensive characterization of plant interactions with pests and pathogens has allowed the major signaling networks governing biotic interactions to be elucidated (Davis, 1998; Preston, 2000; Quirino and Bent, 2003; Pieterse and Dicke, 2007). The hypersensitive response (HR) is effective mainly against (hemi)biotrophic pathogens and this form of defense is often associated with salicylic acid (SA; Mur et al., 2008a). SA acts via the induction of a plethora of defense genes, with the most-commonly described being acidic forms of pathogenesis-related protein (PR) genes such as *PR1* (Cao et al., 1994). The SA signaling pathway has now been extensively characterized (**Figure 1**). The translational activator NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1), localized in an oligomeric form in the cytoplasm (Fu et al., 2012) interact with the SA receptors NPR3 and NPR4 likely following redox changes at key cysteine residues that results in a monomeric NPR1 form which is translocated to the nucleus (Mou et al., 2003). Within the nucleus, NPR1 interacts with a range of TGA-class transcription factors which bind to TGACG motifs encoded within the promoters of SA-induced genes (Zhang et al., 1999).

Defenses against necrotrophic pathogens such as *Botrytis cinerea*, *Plectosphaerella cucumerina* (Berrocal-Lobo et al., 2002), and *Alternaria brassicicola* (Ton et al., 2002) have been linked to jasmonate and ethylene (hereafter referred to as JA and ET, respectively) signaling. Plant tolerance to insects is also strongly influenced by JA, so that there are similarities with resistance responses to necrotrophs (Howe and Jander, 2008). Both JA and ET signaling pathways have been exhaustively investigated and many good overviews are available (for example, Lin et al., 2009; Gfeller et al., 2010). Briefly, JA are lipoxygenase (LOX)-derived products of C18:3 acyl chains derived from phospholipids. JA is conjugated by JAR1 conjugatase to form (+)-7-iso-jasmonoyl-L-Ile (JA-Ile). JA-Ile interacts with the COI1 protein, a key part of a Skp-Cullin-F-box (SCF^{COI}) complex which targets JASMONATE ZIM DOMAIN (JAZ) proteins (Chini et al., 2007). This interaction lead to the destruction of the JAZ repressors via the proteasome relieving their suppressive effects on a wide range of transcriptional activators MYC2, MYC3, and MYC4 (Chini et al., 2007; Fernandez-Calvo et al., 2011). The ET receptors ETR1, ERS1, ETR2, ERS2, and EIN4 are kinases which act as signaling repressors until ET binding occurs (Hua and Meyerowitz, 1998). The negative regulation occurs through the activity of putative MAP3K, CTR1 (Hua and Meyerowitz, 1998) which phosphorylates EIN2. EIN2 is a central component in ET signaling that in the phosphorylated form is located in endoplasmic reticulum. It is likely that dephosphorylation results in EIN2 translocation to the nucleus (Ju et al., 2012). Within the nucleus transcriptional activation involves components such as EIN2 and EIN3 regulating the expression of key transcription factors *ORA59* and *ERF1* (AP2/EREBP;

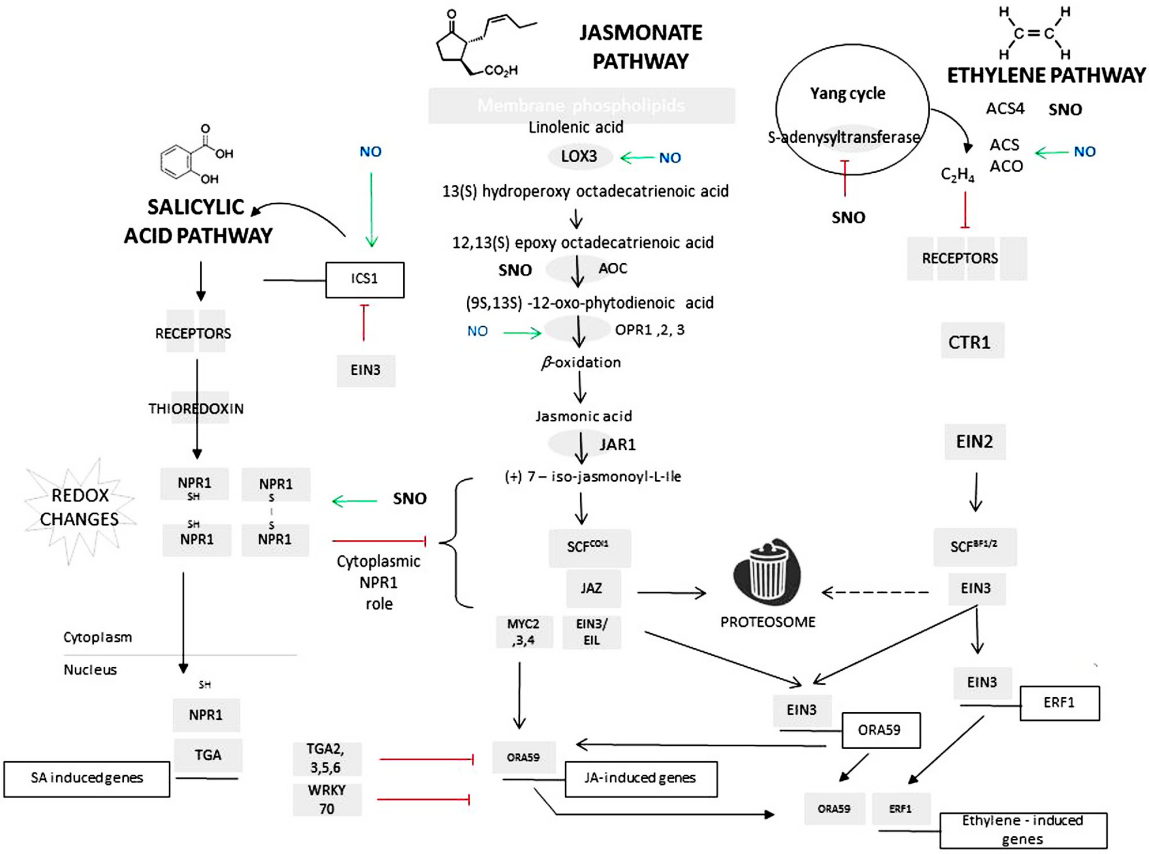


FIGURE 1 | The impact of nitric oxide on salicylic acid, jasmonate (and ethylene signaling cascades. Schematic versions of salicylic acid (SA), jasmonate (JA), and ethylene (ET) signaling cascade. Biosynthetic enzymes are represented as gray ovals and signaling components are gray rectangles. Abbreviations in the jasmonate biosynthetic pathway are as follows: LOX, lipoxygenase; AOC, allene oxide cyclase; OPR, oxo-phyto dienoate reductase; for the ethylene biosynthetic pathway: ACS, 1-aminocyclopropane-1-carboxylic acid synthase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase. Genes and their regulatory promoters are represented as open boxes. For details of signaling cascades, see main text. The known NO-regulated steps are indicated: green arrows indicating that NO has a promontory effects via induction of gene transcription; *S*-nitrosylative steps are indicated via a solid black bar and whether this promotes (green arrows) or inhibit a signaling (red bar) step. Note that for and ACO and ACS4, the effect of *S*-nitrosylation has yet to be determined.

Stepanova and Alonso, 2009). In the absence of ET, EIN3 is targeted by the SCF ligase, EIN3-binding F-box 1 and 2 (EBF1, EBF2) for destruction in the proteasome (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004). As JAZ repressors also interact with EIN3, this would appear to be a crucial mechanism governing the JA/ET synergistic interactions (Zhu et al., 2011).

Under natural conditions plants are exposed to attacks from a range of pathogens and pests with a variety of infection strategies. Cross-talk between SA and JA/ET pathways allows the plant to divert resources to the most appropriate defense mechanisms (Pieterse et al., 2012). Thus antagonistic relationships are most often reported but synergistic SA and JA/ET pathway interactions also occur (Mur et al., 2005, 2006, 2008b). SA can suppress JA effects through the suppression of the JA biosynthetic enzymes LOX2 (Spoel et al., 2003) and allene oxide synthase (AOS, Laudert and Weiler, 1998). However, there are many points downstream of JA biosynthesis that are targeted by SA (Leon-Reyes et al., 2010). SA–JA cross-talk components include the protein kinase MPK4 (Petersen et al., 2000) and in particular the interplay of transcriptional regulators appears to play large roles in SA–JA antagonism.

Many antagonism mechanisms centre on the role of NPR1; as SA-mediated suppression of JA-mediated expression was abolished in *npr1-1* mutants (Spoel et al., 2003; Bargmann et al., 2009; Leon-Reyes et al., 2009; Pieterse et al., 2009). This antagonistic mechanism partially reflects an additional as yet poorly defined cytoplasmic role for NPR1 (Pieterse and Van Loon, 2004) but this does not seem to involve interference with SCF^{COI1}-mediated targeting of JAZ proteins (Van der Does et al., 2013). However, the major SA–JA regulatory role for NPR1 appears to be nuclear-located. A transcription factor whose expression is partially NPR1-dependent is WRKY70 (Li et al., 2004). Overexpression of WRKY70 increased SA-dependent genes expression (*PR1*, *PR2*, and *PR5*) and suppressed JA-dependent defense gene transcription (*COR1* and *VSP1*); with anti-sense WRKY70 plant displaying opposite effects. Expression of WRKY70 is also partially induced by AtMyb44, which, interestingly, is induced via COI1 action suggesting a negative feedback step to modulate the

amplitude of the JA response (Shim et al., 2013). This would represent a previously unsuspected role for an SA/JA antagonistic mechanism.

TGA-class transcription factors also appear to play a role in SA–JA interactions (Ren et al., 2008). Whilst many TGA factors regulate SA responsive gene expression, TGA2, TGA5, and TGA6 also induce JA and ET defense genes and crucially, also regulate SA-mediated antagonism (Zander et al., 2010). This suppressive mechanism includes a redox-regulated step catalyzed by glutaredoxins (GRX). GRX catalyze reactions whereby the oxidation of glutathione is coupled with the reduction of cysteine residues to influence protein stability and/or activity. GRX interacts with TGA2 and over-expression of GRX480 countered the induction of the *ORA59* promoter by EIN3 (Zander et al., 2012). In a parallel study, GCC box *cis* elements such as those found in the promoter of the JA marker gene *PDF1.2* were revealed as key sites through which SA–JA antagonism is effected. Focusing on two GCC-binding transcription factors it was found that ORA59 but not ERF1 was the key transcriptional target for the SA antagonistic mechanism (Van der Does et al., 2013).

Many other hormones interact with SA–JA/ET (Robert-Seilaniantz et al., 2011) but no comprehensive attempt has been made to integrate nitric oxide (NO) – a major defense signal – into the canonical SA–JA/ET interaction network. However, using plants displaying modulated expression of non-symbiotic hemoglobins (Hb) which oxidizes NO, we have demonstrated that NO plays an important role in both networks (Mur et al., 2012). Similar conclusions were advanced by Chun et al. (2012) who expressed mammalian NO synthase (NOS) in tobacco and observed increased resistance to pathogens via elevated SA and JA/ET defense gene expression.

NITRIC OXIDE IN PLANT–PATHOGEN INTERACTIONS

Nitric oxide has emerged as a major player of plant resistance responses to biotrophic and hemibiotrophic pathogens influencing both basal defense and HR (Mur et al., 2005; Prats et al., 2005). Many studies on plant interactions with *Pseudomonas syringae* all indicate that NO is rapidly produced during the HR (Delledonne et al., 1998; Clarke et al., 2000; Mur et al., 2005) and perturbation of this NO generation has shown it clear contribution toward both cell death and other defense processes (Delledonne et al., 1998; Boccara et al., 2005; Mur et al., 2005; Prats et al., 2005).

Many groups with an interest in NO and plant defense are concentrating on thiol oxidation by NO, referred to as *S*-nitrosylation. *S*-nitrosylation comes about by the reaction of the oxidized form of NO, the nitrosonium ion NO^+, which can electrophilically attack thiolate to produce *S*-nitrosylated thiols. This reaction can generate large pools of *S*-nitrosoglutathione ($GSH + NO \rightarrow GSNO + H^+$). GSNO itself can act as a nitrosylating agent and thus could act as a biochemical "memory" so that the effects of NO could persist after its generation has ceased or act as a mobile signal through which NO effects can be propagated throughout a plant as a component in systemic acquired resistance (Espunya et al., 2012). The reduction of GSNO pools is caused by the action of GSNO reductase (GSNOR) and predictably in AtGSNOR1 mutants GSNO levels have been observed to increase (Feechan et al., 2005). These AtGSNOR1 mutants were observed to exhibit compromised resistance to pathogens whilst over-expression of GSNOR increased defense against virulent pathogens. Such data suggested that it can be deleterious to form a GSNO "store" when NO is being generated.

If the thiol group belongs to cysteine residues of proteins, this results is an *S*-nitrosoprotein which can impact on protein function. A fascinating example of this is the control of reactive oxygen species (ROS) generation and cell death through protein *S*-nitrosylation. *S*-nitrosylation of cysteine 890 residue flanking the flavin-binding domain of the NADPH oxidase which is a major source of ROS generation during HR, suppressed both ROS generation and cell death (Yun et al., 2011). In addition, during the HR, *S*-nitrosylation and inactivation of two plastid-located peroxiredoxins (Prx; Sakamoto et al., 2003; Romero-Puertas et al., 2007) has been demonstrated. Prx can detoxify the highly reactive peroxynitrite ion which forms following the co-generation of O_2^- and NO ($O_2^- + NO \rightarrow ONOO^-$) and will generate hydroxyl radicals ($ONOO^- + H^+ \rightarrow NO_2 + OH$). The toxicity of OH radicals is well-established and has been linked to PCD in animal systems so that through this Prx-mediated mechanism, NO acts with ROS to propagate cell death. This mechanism may appear to act in opposition to the apparently suppressive role of NO with NADPH oxidase but most likely reflected differential effects at different NO concentrations (Beligni and Lamattina, 1999; Garcia-Mata et al., 2003) and discrete roles at different stages in the development of the HR.

NITRIC OXIDE AND SALICYLIC ACID SIGNALING

Following one of its first descriptions in plants (Delledonne et al., 1998), NO was immediately associated with SA-mediated events (Durner et al., 1998). Generation of NO through infiltration of mammalian NOS into plant tissues initiated SA-dependent gene expression (Durner et al., 1998). A comprehensive bioinformatic analysis of NO responsive promoters in *Arabidopsis* found that *cis* elements linked to SA responsiveness [ocs element-like sequences (OCSEs) and W-boxes] were prominent (Palmieri et al., 2008). We recently used transgenic over-expression and silencing of endogenous plant Hb in *Arabidopsis* to modulate NO generation in response to the hemibiotrophic pathogen *Pseudomonas syringae*, which demonstrated increased levels of SA accumulation in response to enhanced levels of NO and both were decreased in Hb over-expressing plants. Such observations placed SA "downstream" of NO generation but other data demonstrated that SA can modulate NO production. Thus, exogenous application of SA reduced NO production from tomato root tips (Gemes et al., 2011) and from stomata to initiate stomatal closure (Hao et al., 2010; Sun et al., 2010).

Studies of the mechanisms through which NO interacts with SA signaling appear to be particularly advanced (**Figure 1**). As already stated, the oligomeric status of NPR1 is essential to its action and *S*-nitrosylation of cysteine-156 has been shown to facilitate its oligomerization (Tada et al., 2008). Chemical reduction of this *S*-nitrosylated cysteine residue by SA-activated thioredoxin will promote monomer formation (Tada et al., 2008). Thus NO could be seen to play a paradoxical role: on the one hand initiating SA to promote NPR1 monomer formation and translocation

from cytoplasm to nucleus, but on the other hand favoring oligomerization by initiating nitrosylation. These opposing roles are reinforced at other steps in SA signaling pathways. Thus, the positive effects of NO on SA pathway are further augmented by TGA1 *S*-nitrosylation that stabilizes the transcription factor and strengthens binding to cognate promoter sequences (Lindermayr et al., 2010). Against this are the effects of *S*-nitrosylation on SA-binding protein 3 (SABP3). SABP3 exhibits high affinity binding to SA and carbonic anhydrase activity. *S*-nitrosylation of SABP3 abolished SA binding and carbonic anhydrase activity (Wang et al., 2009). It may be assumed that this would have the same effect as silencing SABP3 gene expression which suppressed a HR elicited by *Pseudomonas syringae* pv. *tomato* (Slaymaker et al., 2002).

NITRIC OXIDE AND ETHYLENE/JASMONIC ACID SIGNALING

Nitric oxide has often been reported to have a suppressive effect on ET signaling. Leshem and Pinchasov (2000) used laser photoacoustic detection to measure both NO and ET in ripening avocados and strawberry and noted that, on ripening, NO levels were reduced as ET increased. A mechanistic understanding of this interaction was provided by (Lindermayr et al., 2006, 2010). The Yang (methylmethionine) cycle produces *S*-adenosylmethionine (AdoMet) which is the methyl donor linked to the production of a range of metabolites including ET and also polyamines (Roje, 2006). Lindermayr et al. (2006) reported the *S*-nitrosylation of a key cysteine (Cys-114) within the active site of a methionine adenosyltransferase (MAT1; At1g02500) following the application of the NO donor – GSNO. *S*-nitrosylation by GSNO suppressed MAT1 enzymatic activity and also ET production. *S*-nitrosylation has also been noted in the ET biosynthetic enzymes 1-aminocyclopropane-l-carboxylic (ACC) synthase 4, although this has not been linked to a loss in enzymatic activity (Abat and Deswal, 2009).

Against such observations are our results which show the simultaneous generation of both NO and ET during a bacterially-elicited HR in tobacco (Mur et al., 2008a, 2009, 2012). We also noted that infiltration of a NO^+ donor – sodium nitroprusside (SNP) – into tobacco leaves produced NO and also ET (Mur et al., 2005, 2008b). As SNP could induce ACC synthase expression (ACS), this seems to be one mechanism through which NO could boost ET production (Mur et al., 2008a,b). Similarly, the expression of mammalian NOS in transgenic tobacco increased ACC oxidase (the final enzyme in ET biosynthesis) and *ethylene-responsive element binding protein* (*EREBP*) expression (Chun et al., 2012).

Recently, we have also shown that NO positively contributes to elicit the production of jasmonates (Mur et al., 2012). Examining the transcriptional data provided by Palmieri et al. (2008) it can be seen that NO increases the expression of a range of JA biosynthetic genes. Thus, expression of LOX3 (At1g17420), 12-oxophytodienoate reductase 1, 2, and 3 (OPR1, 2, and 3; **Figure 1**), were induced by NO. Surprisingly, the expression of the intermediate JA biosynthetic enzyme – allene oxide cyclase (AOC) – is suppressed by NO (Palmieri et al., 2008) and AOC appears to be *S*-nitrosylated although an inhibitory effect has not been established (Romero-Puertas et al., 2008).

INTEGRATING NO INTO SA AND JA/ETHYLENE PATHWAYS: THE CHALLENGES

To highlight the roles of NO in each pathway, it is useful to consider two differing scenarios (**Figure 1**). Upon infection with a (hemi)biotrophic pathogen NO will contribute to the initiation of SA biosynthesis through the relief of EIN3 repression of the *isochorismate synthase 1* (*ICS1*) transcription (Chen et al., 2009). This relief possibly result from the suppression of ET biosynthesis through MAT1 nitrosylation so that EIN3 is degraded in the proteasome. SA will induce thioredoxins to reduce NPR1 protein to their monomeric form leading to their translocation to the nucleus. In the nucleus NPR1 will bind to TGA-class transcription factors. *S*-nitrosylation of TGA factors will increase affinity for their cognate promoters. Some TGA factors will bind to the ORA59 promoter to suppress both JA and ET-inducible genes. NPR1 will also contribute to the induction of WKRY70 to also suppress JA/ET expression. Another scenario is infection with a necrotrophic pathogen or attack by certain pests. JA biosynthesis occurs rapidly which is facilitated by NO-mediated induction of LOX3 and OPR1, 2, and 3. In addition, NO will induce ACS and ACO (1-aminocyclopropane-1-carboxylic acid oxidase) expression to increase ET biosynthesis.

This model is clearly too simplistic and poses a number of questions. When NO is generated, this should *S*-nitrosylate NPR1 to help maintain the oligomeric form which could suppress JA events (Pieterse and Van Loon, 2004; **Figure 1**). If so, how is JA biosynthesis achieved? Also, how can the induction of ACS by NO counter the effects of a MAT1 inhibition leading to a failure to provide methyl groups for ET production? Most importantly how is specificity conferred where SA and JA/ET pathways are simultaneous in play? One example of this is the *Pseudomonas syringae* pv. *phaseolicola* (*Psph*)-elicited HR in tobacco (Kenton et al., 1999; Mur et al., 2009).

As we have recently pointed out (Mur et al., 2013) this could reflect subtle spatial–temporal separation in around sites of infection or insect grazing. Hb gene expression is regulated in a cell-specific manner and therefore could represent a way in which spatial regulation of local NO levels can be modulated by the plant in reaction to pathogens (Hebelstrup et al., 2013). Subcellular separation in signaling events should also be considered (Mur et al., 2013). Additionally, NO concentration is a key determinant of what regulatory step is employed. Thus, during the *Psph*-elicited HR in tobacco, NO production is rapidly induced but does not peak until 10–24 h following challenge whereas both SA and ET production is initiated at ∼6 h following challenge (Mur et al., 2008b). During this phase it would be supposed that the positive effects of NO on SA and ET are paramount – as a low NO concentration effect – whilst JA production is suppressed via SA/JA antagonistic mechanisms possibly via *S*-nitrosylation of ACO. As NO production peaks it may be that the induction of JA-biosynthetic genes is initiated at high NO concentrations which could overcome any SA-antagonistic mechanism on this pathway. If the concentration-dependent mode for NO effects on SA/JA/ET signaling pathways is substantiated this suggest a major role for Hb. We have recently shown how reduced expression of Hb during the HR contributed to increased NO production (Mur et al., 2012) so this and NO generation mechanisms such as nitrate

reductase (Gupta et al., 2011) could be important arbiters of the interplay between defense signaling cascades.

One way to address such complex interactions is suggested by the recent work of Windram et al. (2012). These authors sampled at 2 h intervals for 48 h to extensively describe transcriptomic changes occurring following attack of *Arabidopsis* by *B. cinerea* and then used Systems Biology modeling approaches to characterize key signaling hubs. Marrying this approach with careful measurements of signal generation patterns, would undoubtedly improve our understanding of the signaling interactions during plant defense and the place of NO in this network.

REFERENCES

Abat, J. K., and Deswal, R. (2009). Differential modulation of *S*-nitrosoproteome of *Brassica juncea* by low temperature: change in *S*-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity. *Proteomics* 9, 4368–4380. doi: 10.1002/pmic.200800985

Bargmann, B. O., Laxalt, A. M., ter Riet, B., Testerink, C., Merquiol, E., Mosblech, A., et al. (2009). Reassessing the role of phospholipase D in the *Arabidopsis* wounding response. *Plant Cell Environ.* 32, 837–850. doi: 10.1111/j.1365-3040.2009.01962.x

Beligni, M. V., and Lamattina, L. (1999). Is nitric oxide toxic or protective? *Trends in Plant Sci.* 4, 299–300. doi: 10.1016/S1360-1385(99)01451-X

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. *Plant J.* 29, 23–32. doi: 10.1046/j.1365-313x.2002.01191.x

Boccara, M., Mills, C. E., Zeier, J., Anzi, C., Lamb, C., Poole, R. K., et al. (2005). Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. *Plant J.* 43, 226–237. doi: 10.1111/j.1365-313X.2005.02443.x

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. N. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired-resistance. *Plant Cell* 6, 1583–1592.

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., et al. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in *Arabidopsis*. *Plant Cell* 21, 2527–2540. doi: 10.1105/tpc.108.065193

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J. M., Lorenzo, O., et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448, 666–671. doi: 10.1038/nature06006

Chun, H. J., Park, H. C., Koo, S. C., Lee, J. H., Park, C. Y., Choi, M. S., et al. (2012). Constitutive expression of mammalian nitric oxide synthase in tobacco plants triggers disease resistance to pathogens. *Mol. Cells* 34, 463–471. doi: 10.1007/s10059-012-0213-0

Clarke, A., Desikan, R., Hurst, R. D., Hancock, J. T., and Neill, S. J. (2000). NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J.* 24, 667–677. doi: 10.1046/j.1365-313x.2000.00911.x

Davis, K. R. (1998). *Arabidopsis thaliana*. A robust model system for studying plant–pathogen interactions. *Subcell. Biochem.* 29, 253–285.

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* 394, 585–588. doi: 10.1038/29087

Durner, J., Wendehenne, D., and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10328–10333. doi: 10.1073/pnas.95.17.10328

Espunya, M. C., De Michele, R., Gomez-Cadenas, A., and Martinez, M. C. (2012). *S*-nitrosoglutathione is a component of wound- and salicylic acid-induced systemic responses in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 3219–3227. doi: 10.1093/jxb/ers043

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Fernandez-Calvo, P., Chini, A., Fernandez-Barbero, G., Chico, J. M., Gimenez-Ibanez, S., Geerinck, J., et al. (2011). The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 Are Targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* 23, 701–715. doi: 10.1105/tpc.110.080788

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162

Gagne, J. M., Smalle, J., Gingerich, D. J., Walker, J. M., Yoo, S. D., Yanagisawa, S., et al. (2004). *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 6803–6808. doi: 10.1073/pnas.0401698101

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L., and Blatt, M. R. (2003). Nitric oxide regulates K+ and Cl− channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proc. Natl. Acad. Sci. U.S.A.* 100, 11116–11121. doi: 10.1073/pnas.1434381100

Gemes, K., Poor, P., Horvath, E., Kolbert, Z., Szopko, D., Szepesi, A., et al. (2011). Cross-talk between salicylic acid and NaCl-generated reactive oxygen species and nitric oxide in tomato during acclimation to high salinity. *Physiol. Plant.* 142, 179–192. doi: 10.1111/j.1399-3054.2011.01461.x

Gfeller, A., Liechti, R., and Farmer, E. E. (2010). *Arabidopsis* jasmonate signaling pathway. *Sci. Signal.* 3, cm4. doi: 10.1126/scisignal.3109cm4

Guo, H., and Ecker, J. R. (2003). Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* 115, 667–677. doi: 10.1016/S0092-8674(03)00969-3

Gupta, K. J., Fernie, A. R., Kaiser, W. M., and Van Dongen, J. T. (2011). On the origins of nitric oxide. *Trends Plant Sci.* 16, 160–168. doi: 10.1016/j.tplants.2010.11.007

Hao, F. S., Zhao, S. L., Dong, H., Zhang, H., Sun, L. R., and Miao, C. (2010). Nia1 and Nia2 are involved in exogenous salicylic acid-induced nitric oxide generation and stomata! closure in *Arabidopsis*. *J. Integr. Plant Biol.* 52, 298–307. doi: 10.1111/j.1744-7909.2010.00920.x

Hebelstrup, K. H., Shah, J. K., and Igamberdiev, A. U. (2013). The role of nitric oxide and hemoglobin in plant development and morphogenesis. *Physiol. Plant.* [Epub ahead of print]. doi: 10.1111/ppl.12062

Howe, G. A., and Jander, G. (2008). Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* 59, 41–66. doi: 10.1146/annurev.arplant.59.032607.092825

Hua, J., and Meyerowitz, E. M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* 94, 261–271. doi: 10.1016/S0092-8674(00)81425-7

Ju, C., Yoon, G. M., Shemansky, J. M., Lin, D. Y., Ying, Z. I., Chang, J., et al. (2012). CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19486–19491. doi: 10.1073/pnas.1214848109

Kenton, P., Mur, L. A. J., Atzorn, R., Wasternack, C., and Draper, J. (1999). (−)-Jasmonic acid accumulation in tobacco hypersensitive response lesions. *Mol. Plant Microbe Interact.* 12, 74–78. doi: 10.1094/MPMI.1999.12.1.74

Laudert, D., and Weiler, E. W. (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. *Plant J.* 15, 675–684. doi: 10.1046/j.1365-313x.1998.00245.x

Leon-Reyes, A., Du, Y., Koornneef, A., Proietti, S., Körbes, A. P., Memelink, J., et al. (2010). Ethylene signaling renders the jasmonate response of Arabidopsis insensitive to future suppression by salicylic Acid. *Mol. Plant Microbe Interact.* 23, 187–197.

Leon-Reyes, A., Spoel, S. H., De Lange, E. S., Abe, H., Kobayashi, M., Tsuda, S., et al. (2009). Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. *Plant Physiol.* 149, 1797–1809. doi: 10.1104/pp.108.133926

Leshem, Y. Y., and Pinchasov, Y. (2000). Non-invasive photoacoustic spectroscopic determination of relative endogenous nitric oxide and ethylene content stoichiometry during the ripening of strawberries *Fragaria anannasa* (Duch.) and avocados *Persea americana* (Mill.). *J.*

Exp. Bot. 51, 1471–1473. doi: 10.1093/jexbot/51.349.1471

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331. doi: 10.1105/tpc.016980

Lin, Z. F., Zhong, S. L., and Grierson, D. (2009). Recent advances in ethylene research. *J. Exp. Bot.* 60, 3311–3336. doi: 10.1093/jxb/erp204

Lindermayr, C., Saalbach, G., Bahnweg, G., and Durner, J. (2006). Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein *S*-nitrosylation. *J. Biol. Chem.* 281, 4285–4291. doi: 10.1074/jbc.M511635200

Lindermayr, C., Sell, S., Muller, B., Leister, D., and Durnera, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Mou, Z., Fan, W. H., and Dong, X. N. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944. doi: 10.1016/S0092-8674(03)00429-X

Mur, L. A., Lloyd, A. J., Cristescu, S. M., Harren, F. J., Hall, M. A., and Smith, A. R. (2009). Biphasic ethylene production during the hypersensitive response in *Arabidopsis*: a window into defense priming mechanisms? *Plant Signal. Behav.* 4, 610–613. doi: 10.4161/psb.4.7.8904

Mur, L. A., Mandon, J., Persijn, S., Cristescu, S. M., Moshkov, I. E., Novikova, G. V., et al. (2013). Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* 5, pls052. doi: 10.1093/aobpla/pls052

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* 140, 249–262. doi: 10.1104/pp.105.072348

Mur, L. A. J., Kenton, P., Lloyd, A. J., Ougham, H., and Prats, E. (2008a). The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* 59, 501–520. doi: 10.1093/jxb/erm239

Mur, L. A. J., Laarhoven, L. J. J., Harren, F. J. M., Hall, M. A., and Smith, A. R. (2008b). Nitric oxide interacts with salicylate to regulate biphasic ethylene production during the hypersensitive response. *Plant Physiol.* 148, 1537–1546. doi: 10.1104/pp. 108.124404

Mur, L. A. J., Santosa, I. E., Laarhoven, L. J. J., Holton, N. J., Harren, F. J. M., and Smith, A. R. (2005). Laser photoacoustic detection allows in planta detection of nitric oxide in tobacco following challenge with avirulent and virulent *Pseudomonas syringae* pathovars. *Plant Physiol.* 138, 1247–1258. doi: 10.1104/pp.104.055772

Mur, L. A. J., Sivakumaran, A., Mandon, J., Cristescu, S. M., Harren, F. J. M., and Hebelstrup, K. H. (2012). Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. *J. Exp. Bot.* 63, 4375–4387. doi: 10.1093/jxb/ers116

Palmieri, M. C., Sell, S., Huang, X., Scherf, M., Werner, T., Durner, J., et al. (2008). Nitric oxide-responsive genes and promoters in *Arabidopsis thaliana:* a bioinformatics approach. *J. Exp. Bot.* 59, 177–186. doi: 10.1093/jxb/erm345

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., et al. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. *Cell* 103, 1111–1120.

Pieterse, C. M., and Van Loon, L. (2004). NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant Biol.* 7, 456–464. doi: 10.1016/j.pbi.2004.05.006

Pieterse, C. M. J., and Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends Plant Sci.* 12, 564–569. doi: 10.1016/j.tplants.2007.09.004

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., et al. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two *Arabidopsis* F box proteins: EBF1 and EBF2. *Cell* 115, 679–689. doi: 10.1016/S0092-8674(03)00968-1

Prats, E., Mur, L. A., Sanderson, R., and Carver, T. L. (2005). Nitric oxide contributes both to papilla-based resistance and the hypersensitive response in barley attacked by *Blumeria graminis* f. sp. *hordei*. *Mol. Plant Pathol.* 6, 65–78. doi: 10.1111/j.1364-3703.2004.00266.x

Preston, G. M. (2000). *Pseudomonas syringae* pv. *tomato*: the right pathogen, of the right plant, at the right time. *Mol. Plant Pathol.* 1, 263–275. doi: 10.1046/j.1364-3703.2000.00036.x

Quirino, B. F., and Bent, A. F. (2003). Deciphering host resistance and pathogen virulence: the *Arabidopsis/Pseudomonas* interaction as a model. *Mol. Plant Pathol.* 4, 517–530. doi: 10.1046/j.1364-3703.2003.00198.x

Ren, C. M., Zhu, Q., Gao, B. D., Ke, S. Y., Yu, W. C., Xie, D. X., et al. (2008). Transcription factor WRKY70 displays important but no indispensable roles in jasmonate and salicylic acid signaling. *J. Integr. Plant Biol.* 50, 630–637. doi: 10.1111/j.1744-7909.2008.00653.x

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Roje, S. (2006). *S*-adenosyl-L-methionine: beyond the universal methyl group donor. *Phytochemistry* 67, 1686–1698. doi: 10.1016/j.phytochem.2006.04.019

Romero-Puertas, M. C., Campostrini, N., Matte, A., Righetti, P. G., Perazzolli, M., Zolla, L., et al. (2008). Proteomic analysis of *S*-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* 8, 1459–1469. doi: 10.1002/pmic.200700536

Romero-Puertas, M. C., Laxa, M., Matte, A., Zaninotto, F., Finkemeier, I., Jones, A. M. E., et al. (2007). *S*-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. *Plant Cell* 19, 4120–4130. doi: 10.1105/tpc.107.055061

Sakamoto, A., Tsukamoto, S., Yamamoto, H., Ueda-Hashimoto, M., Takahashi, M., Suzuki, H., et al. (2003). Functional complementation in yeast reveals a protective role of chloroplast 2-Cys peroxiredoxin against reactive nitrogen species. *Plant J.* 33, 841–851. doi: 10.1046/j.1365-313X.2003.01669.x

Shim, J. S., Jung, C., Lee, S., Min, K., Lee, Y. W., Choi, Y., et al. (2013). AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J.* 73, 483–495. doi: 10.1111/tpj.12051

Slaymaker, D. H., Navarre, D. A., Clark, D., Del Pozo, O., Martin, G. B., and Klessig, D. F. (2002). The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11640–11645. doi: 10.1073/pnas.182427699

Spoel, S. H., Koornneef, A., Claessens, S. M., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159 PMCid:150028

Stepanova, A. N., and Alonso, J. M. (2009). Ethylene signaling and response: where different regulatory modules meet. *Curr. Opin. Plant Biol.* 12, 548–555. doi: 10.1016/j.pbi.2009.07.009

Sun, L. R., Hao, F. S., Lu, B. S., and Ma, L. Y. (2010). AtNOA1 modulates nitric oxide accumulation and stomatal closure induced by salicylic acid in *Arabidopsis*. *Plant Signal. Behav.* 5, 1022–1024. doi: 10.4161/psb.5.8.12293

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Ton, J., Van Pelt, J. A., Van Loon, L. C., and Pieterse, C. M. J. (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.* 15, 27–34. doi: 10.1094/MPMI.2002.15.1.27

Van der Does, D., Leon-Reyes, A., Koornneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744–761. doi: 10.1105/tpc.112.108548

Wang, Y. Q., Feechan, A., Yun, B. W., Shafiei, R., Hofmann, A., Taylor, P., et al. (2009). *S*-nitrosylation of AtSABP3 antagonizes the expression of plant immunity. *J. Biol. Chem.* 284, 2131–2137. doi: 10.1074/jbc.M806782200

Windram, O., Madhou, P., Mchattie, S., Hill, C., Hickman, R., Cooke, E., et al. (2012). *Arabidopsis* defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell* 24, 3530–3557. doi: 10.1105/tpc.112.102046

Yun B. W., Feechan A., Yin M., Saidi N. B., Le Bihan T., Yu M., et al. (2011) S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. *Nature* 478, 264–268. doi: 10.1038/nature10427

Zander, M., Chen, S. X., Imkampe, J., Thurow, C., and Gatz, C. (2012). Repression of the *Arabidopsis thaliana* jasmonic acid/ethylene-induced defense pathway by TGA-interacting glutaredoxins depends on their c-terminal ALWL motif. *Mol. Plant* 5, 831–840. doi: 10.1093/mp/ssr113

Zander, M., La Camera, S., Lamotte, O., Metraux, J. P., and Gatz, C. (2010). *Arabidopsis thaliana* class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210. doi: 10.1111/j.1365-313X.2009.04044.x

Zhang, Y. L., Fan, W. H., Kinkema, M., Li, X., and Dong, X. N. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528. doi: 10.1073/pnas.96.11.6523

Zhu, Z. Q., An, F. Y., Feng, Y., Li, P. P., Xue, L., Mu, A., et al. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12539–12544. doi: 10.1073/pnas.1103959108

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 April 2013; accepted: 05 June 2013; published online: 27 June 2013.

Citation: Mur LAJ, Prats E, Pierre S, Hall MA and Hebelstrup KH (2013) Integrating nitric oxide into salicylic acid and jasmonic acid/ethylene plant defense pathways. Front. Plant Sci. ***4****:215. doi: 10.3389/fpls.2013.00215*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Mur, Prats, Pierre, Hall and Hebelstrup. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 16 May 2013
doi: 10.3389/fpls.2013.00127

A component of the Sec61 ER protein transporting pore is required for plant susceptibility to powdery mildew

Wen-Jing Zhang†, Susanne Hanisch†, Mark Kwaaitaal, Carsten Pedersen and Hans Thordal-Christensen*

Section for Plant and Soil Science, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Frederiksberg, Denmark

Edited by:
Corné M. Pieterse, Utrecht University, Netherlands

Reviewed by:
Ralph Huckelhoven, Technische Universitaet Muenchen, Germany
Thomas Liebrand, Wageningen University, Netherlands

***Correspondence:**
Hans Thordal-Christensen, Section for Plant and Soil Science, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark.
e-mail: htc@life.ku.dk

†*Wen-Jing Zhang and Susanne Hanisch have contributed equally to this work.*

Biotrophic pathogens, like the powdery mildew fungi, require living plant cells for their growth and reproduction. During infection, a specialized structure called the haustorium is formed by the fungus. The haustorium is surrounded by a plant cell-derived extrahaustorial membrane (EHM). Over the EHM, the fungus obtains nutrients from and secretes effector proteins into the plant cell. In the plant cell these effectors interfere with cellular processes such as pathogen defense and membrane trafficking. However, the mechanisms behind effector delivery are largely unknown. This paper provides a model for and new insights into a putative transfer mechanism of effectors into the plant cell. We show that silencing of the barley Sec61βa transcript results in decreased susceptibility to the powdery mildew fungus. *Hv*Sec61βa is a component of both the endoplasmic reticulum (ER) translocon and retrotranslocon pores, the latter being part of the ER-associated protein degradation machinery. We provide support for a model suggesting that the retrotranslocon function of *Hv*Sec61βa is required for successful powdery mildew fungal infection. *Hv*Sec61βa-GFP and a luminal ER marker were co-localized to the ER, which was found to be in close proximity to the EHM around the haustorial body, but not the haustorial fingers. This differential EHM proximity suggests that the ER, including *Hv*Sec61βa, may be actively recruited by the haustorium, potentially to provide efficient effector transfer to the cytosol. Effector transport across this EHM-ER interface may occur by a vesicle-mediated process, while the Sec61 retrotranslocon pore potentially provides an escape route for these proteins to reach the cytosol.

Keywords: powdery mildew, haustorium, extrahaustorial membrane (EHM), endoplasmic reticulum-associated degradation (ERAD), Sec61 complex, susceptibility factor

INTRODUCTION

Many filamentous plant pathogenic fungi and oomycetes rely on placing a feeding structure, a so-called haustorium inside host cells in order to exploit host resources and to transfer effector proteins to the host cytosol. By unknown mechanisms, these pathogens trigger the host cells to generate an extrahaustorial membrane (EHM), which allows the host cells to stay alive despite the severe haustorial invasions (Gan et al., 2012). In between the haustorium and the EHM, a sealed compartment, called the extrahaustorial matrix (EHMx) is present. Many of these pathogens, such as powdery mildew fungi, have genetically lost certain general life-sustaining processes during their evolution (Spanu et al., 2010). This prevents them from living on dead biological material, making them strict biotrophs. In the meantime, they secrete hundreds of effectors from the haustoria, mediated by signal peptides (SPs) and default secretion. Many of these effectors are transferred to the host cytosol, where they play important roles in pathogenicity by assisting in nutrient acquisition, suppression of defense and reprogramming cellular processes (Bozkurt et al., 2012; Pedersen et al., 2012; Saunders et al., 2012). An inherent problem, which is poorly understood, concerns how the effectors escape the EHM-delimited haustorial compartment to access the plant cytosol. This requires a mechanism to cross membranes, such as a protein transmitting pore. Essentially, the only currently established element of this process is the RxLR-dEER motif, located a few amino acids downstream of the SP cleavage site in many oomycete effectors. By an unknown process, this motif guides the effectors to be transported across membranes and allows them to enter the host cytosol (Whisson et al., 2007).

The endoplasmic reticulum (ER) is a major organelle in eukaryotic cells, which forms an extended network, functioning in, e.g., protein processing and sorting. Voegele et al. (2009) have previously suggested that the ER plays a role in transfer of effector to the plant cytosol. In the ER, proper folding and modification of proteins is assisted and validated by the ER quality control (ER-QC) machinery. If proteins finally fail the quality check, they are recognized by the ER-associated protein degradation (ERAD) machinery and retrotranslocated into the cytosol to be degraded by proteasomes (Nakatsukasa and Brodsky, 2008). Effectors may exploit this retrotranslocon pore in order to get access to the plant cytosol. Different multicomponent retrotranslocon pores have been described in yeast and mammals, in which, e.g., Derlin, Hrd, and Sec61 proteins are major elements (Kawaguchi and Ng, 2007; Nakatsukasa and Brodsky, 2008). The ERAD substrates are ubiquitinated during the retrotranslocation process by retrotranslocon-associated ubiquitin ligases, and this targets them for proteasomal degradation as soon as they reach the cytosol (Carvalho et al., 2006, 2010).

The Sec61 pore can translocate proteins bi-directionally, and it is primarily known as the translocon pore, mediating the process of SP-dependent protein translocation into the ER. The Sec61 pore is a doughnut-shaped heterotrimeric complex, consisting of the subunits, Sec61α, Sec61β, and Sec61γ. SP and Sec61-dependent translocation into the ER can occur either co- or post-translationally (Zimmermann et al., 2011). The ERAD pathway has in several cases been shown to be recruited by opportunistic pathogens for transfer of polypeptides into the host cell cytosol. For example, cholera toxin, shiga toxin, and *Pseudomonas aeruginosa* exotoxin enter the cytosol through retrotranslocon pores, but escape from ubiquitination and proteasome-mediated degradation (Rodighiero et al., 2002; Blanke, 2006). Retrotranslocation of cholera toxin occurs through the Sec61 retrotranslocon pore, and depletion of the Sec61 complex prevented the retrotranslocation of this toxin into the cytosol (Schmitz et al., 2000; Teter et al., 2002).

Here we aimed at studying the role of the Sec61 pore in plant susceptibility to the powdery mildew fungus. Barley (*Hordeum vulgare*) has two Sec61α, two Sec61β, and one Sec61γ protein[1] (Deng et al., 2007; Mayer et al., 2012), and to unravel the role of the pore, we made use of the fact that the Sec61β component is essential for retrotranslocon activity for various substrates, but less important for translocon activity under non-stressed conditions (Finke et al., 1996; Van den Berg et al., 2004; Liao and Carpenter, 2007; Kelkar and Dobberstein, 2009; Zhao and Jantti, 2009; Wang et al., 2010; Guerriero and Brodsky, 2012). We show that silencing of *HvSec61βa* reduced the susceptibility of barley epidermal cells to the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*). In addition, the *Hv*Sec61βa-GFP-labeled ER network is differentially associated to the body, and not the fingers of the powdery mildew fungal haustorium. To explain the role of the Sec61βa in pathogenicity, we propose a model in which the fungus actively recruits the ER in order to exploit the Sec61 pore for pathogenicity.

MATERIALS AND METHODS

PLANTS AND FUNGI

Barley (*Hordeum vulgare*) cv. Golden Promise plants were used for transient transformation and subsequent studies with and without powdery mildew fungal inoculation. The barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*), isolate DH14, was maintained on susceptible barley, cv. Golden Promise, grown at 20°C, 16 h light (150 μE/sm^2)/8 h dark, by weekly inoculum transfer. These growth conditions were used throughout the studies.

CLONING

To generate a gene-specific RNA interference (RNAi) construct to silence *HvSec61βa* (AK252927.1), its coding sequence was PCR-amplified using the primer pair Sec61βa_F1 (CACCATGGTGGCTAATGGTGACG) and Sec61βa_R1 (GGGGTGCGGTACAGCTTTC) on cDNA generated from mRNA isolated from 7-day-old barley leaf material. The PCR product was TOPO-cloned into the pENTR/D-TOPO vector (Invitrogen). Positive clones were validated by sequencing. Using Gateway LR cloning, according to the manufacturer's instructions (Invitrogen), the insert was transferred to the 35S-promoter driven destination vector, pIPKTA30N (Douchkov et al., 2005), to generate the final RNAi construct. To generate the Sec61βa-GFP construct for localization, the full-length Sec61βa coding sequence, without stop-codon, was amplified with the primer pair HvSec61βa_KZK_GWY_FW (GGGGACAAGTTTGTACAAAAAAGCAGGCACCATGGTGGCTAATGGTGACGCCCCT) and HvSec61βa_ns_GWY_Rv (GGGGACCACTTTGTACAAGAAAGCTGGGTTATTAGGGGTGCGGTACAGCTTGCC) on the pENTR clone described above, and using a BP clonase reaction it was cloned into the pDONR201 vector (Invitrogen). Positive clones were confirmed by sequencing. Using a Gateway LR clonase reaction according to the manufacturer's instructions (Invitrogen), the insert was transferred to the 35S-promoter-driven destination vector, P2GWF7 (Curtis and Grossniklaus, 2003). All final clones were verified by restriction enzyme digestion.

PARTICLE BOMBARDMENT

Transformation of gene constructs into epidermal cells of 7-day-old barley leaves was conducted by particle bombardment, essentially as described by Douchkov et al. (2005). For transient induced gene silencing (TIGS) studies, the constructs were co-transformed with a β-glucuronidase (GUS) reporter construct, followed by inoculation with *Bgh* 2 days later (inoculation density around 200 conidia per mm^2). Three days after inoculation, the leaves were GUS-stained, and the relative susceptibility index was calculated by dividing the number of GUS-stained epidermal cells containing a haustorium by the total number of GUS-stained cells. The data were normalized to the empty vector (pIPKTA30N) control. The experiments were repeated at least three times. A cell viability test was performed by co-transformation of the *HvSec61βa* RNAi construct or the empty vector control, pIPKTA30N, with the anthocyanin biosynthesis-activating construct, pBC17 (Schweizer et al., 2000). Two days after transformation, the leaves were inoculated with *Bgh* at a density of 200 conidia per mm^2, and after another 3 days, the anthocyanin-stained cells were counted. Constructs for marker proteins, fused with fluorescent proteins, were transformed and inoculated with *Bgh* 1 day later, and examined by confocal microscopy 2 days after transformation.

CONFOCAL MICROSCOPY

A Leica SP5-X confocal laser scanning microscope, mounted with a 63 × 1.2 numerical aperture water-immersion objective, was used. For fluorescent protein detection and localization, GFP was excited at 488 nm, and the fluorescence emission was detected between 518 and 540 nm. mCherry fluorescence was excited at 543 nm and fluorescence emission was detected between 590 and 640 nm. 3D projections were created using the Image Surfer 1.2 software[2] (Feng et al., 2007).

[1] http://webblast.ipk-gatersleben.de/barley/viroblast.php

[2] www.imagesurfer.org

RESULTS

*Hv*Sec61βa IS A POTENTIAL SUSCEPTIBILITY FACTOR FOR THE BARLEY POWDERY MILDEW FUNGUS

In barley two *Sec61*β genes have been identified, which are named *HvSec61*β*a* and *HvSec61*β*b*. Interestingly, the *HvSec61*β*a* transcript accumulates in leaves after attack by *Bgh*[3] (Dash et al., 2012). Therefore, we selected to analyze the role of *HvSec61*β*a* in the barley/*Bgh* interaction, and performed single cell TIGS of this gene. A 35S-promoter-driven RNAi construct, covering the full-length coding region of this gene, was generated and transiently transformed together with a GUS reporter-gene construct into barley epidermal cells (Douchkov et al., 2005). After 2 days, the leaves were inoculated with *Bgh* and transformed cells were stained for GUS activity 3 days thereafter. Infection success of *Bgh* was evaluated microscopically by scoring the total number of GUS-stained cells and the number of GUS-stained cells containing one or more haustoria. Subsequently, the data were normalized to the empty vector control. The RNAi construct of *HvSec61*β*a* resulted in more than 40% reduction in susceptibility to *Bgh* (**Figure 1A**). As a positive control, the relative susceptibility of cells transformed with an *Mlo*-RNAi construct was included (Douchkov et al., 2005). These cells were 70% less susceptible than the control cells. In order to confirm that the RNAi construct in fact results in silencing of *HvSec61*β*a*, we co-transformed barley epidermal cells with the RNAi construct of *HvSec61*β*a* and a 35S promoter-driven *Hv*Sec61βa-GFP fusion construct. Five days after transformation together with a reference construct for cytosolic mCherry expression, confocal imaging revealed that the RNAi construct prevented appearance of GFP signal, while it did not affect the signal from mCherry in the same cell (**Figure 1B**). The reduced *Hv*Sec61βa-GFP signal indicated that the *HvSec61*β*a* RNAi silencing construct indeed induced degradation of *HvSec61*β*a* encoding mRNA and likely as well impaired endogenous *HvSec61*β*a* transcript and protein accumulation. Thus, the observed increased resistance of *HvSec61*β*a*-silenced cells indicates a potential role of *Hv*Sec61βa as a susceptibility factor for efficient *Bgh* infection.

In order to analyze whether the reduced susceptibility could be due to reduced viability of the cells in which *HvSec61*β*a* was silenced, a second experiment was performed. Co-transformation was performed with an anthocyanin biosynthesis gene activation construct, pBC17, causing the transformed cells to accumulate the red anthocyanin pigment as long as they stay alive (Schweizer et al., 2000). Two days after transformation, the leaves were inoculated with a high density of *Bgh* conidia (≈200 per mm^2). Similar numbers of anthocyanin accumulating cells were detected in *HvSec61*β*a*-silenced and non-silenced cells after *Bgh* infection (**Figure 1C**). Therefore, this result confirmed that the *HvSec61*β*a* RNAi construct did not affect the viability of the barley cells after inoculation.

*Hv*Sec61β LOCALIZATION IN UNINFECTED AND INFECTED BARLEY CELLS

Next we aimed to subcellularly localize *Hv*Sec61βa to search for clues for the powdery mildew-related function of this protein. Sec61β is a small ∼8 kDa protein with a single transmembrane domain, and GFP-tagging has previously been used for its localization (Rolls et al., 1999; Voeltz et al., 2006). Therefore, we co-expressed our 35S promoter-driven *Hv*Sec61βa-GFP fusion construct together with a 35S promoter-driven SP-mCherry-HDEL construct (Nelson et al., 2007) in infected and uninfected barley epidermal cells. The SP targets mCherry to the ER and the ER retrieval motif HDEL (His-Asp-Glu-Leu) at the C-terminus retains it in the lumen of the ER (Gomord et al., 1997). Confocal images of epidermal single cells expressing *Hv*Sec61βa-GFP and SP-mCherry-HDEL were recorded 48 h after particle bombardment (**Figure 2**). Intense GFP signal was observed in the ER cortical network throughout the cells expressing GFP-tagged

[3]http://www.plexdb.org/plex.php?database=Barley

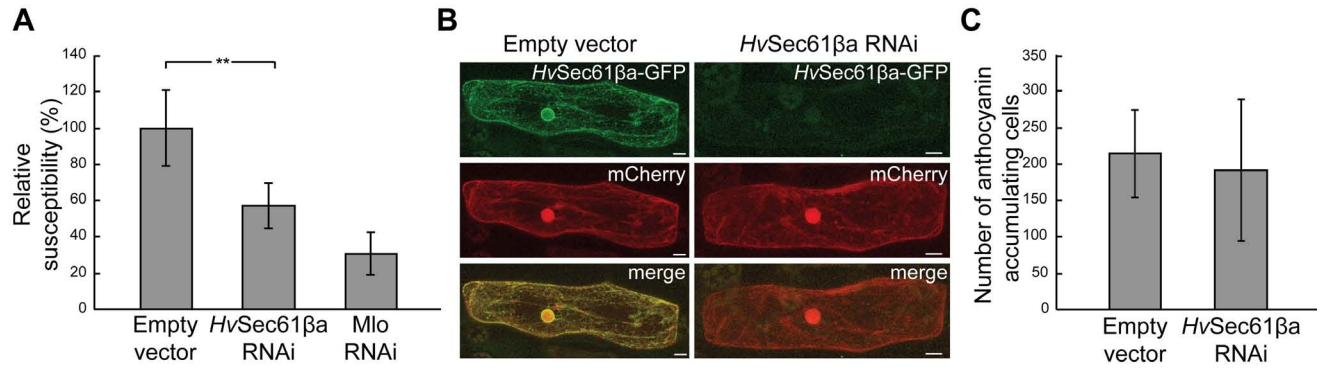


FIGURE 1 | Silencing of *Hv*Sec61βa reduces susceptibility to *Bgh* without affecting plant cell viability. (A) Susceptibility after co-transformation of empty vector control, *HvSec61*β*a*-RNAi and *Mlo*-RNAi constructs with a GUS-reporter construct into barley epidermal cells, followed by inoculation with *Bgh* 3 days later. The relative susceptibility was calculated as described in Materials and Methods. Mlo-RNAi was used as a positive control. Error bars represent standard deviation (SD). **, $P < 0.01$ (Student's *t*-test). **(B)** *HvSec61*β*a*-RNAi reduced the GFP signal originating from *Hv*Sec61βa-GFP, but not the fluorescence signal from cytosolic mCherry 5 days after transformation. Micrographs show maximum intensity projections. **(C)** Number of pBC17-transformed cells accumulating anthocyanin, reflecting cell viability, after co-bombardment with either an empty vector control or the *HvSec61*β*a*-RNAi construct on similarly sized pieces of leaf. Two days after bombardment, leaves were inoculated with *Bgh*, and the number of anthocyanin-accumulating cells was scored 3 days later ($n = 4$).

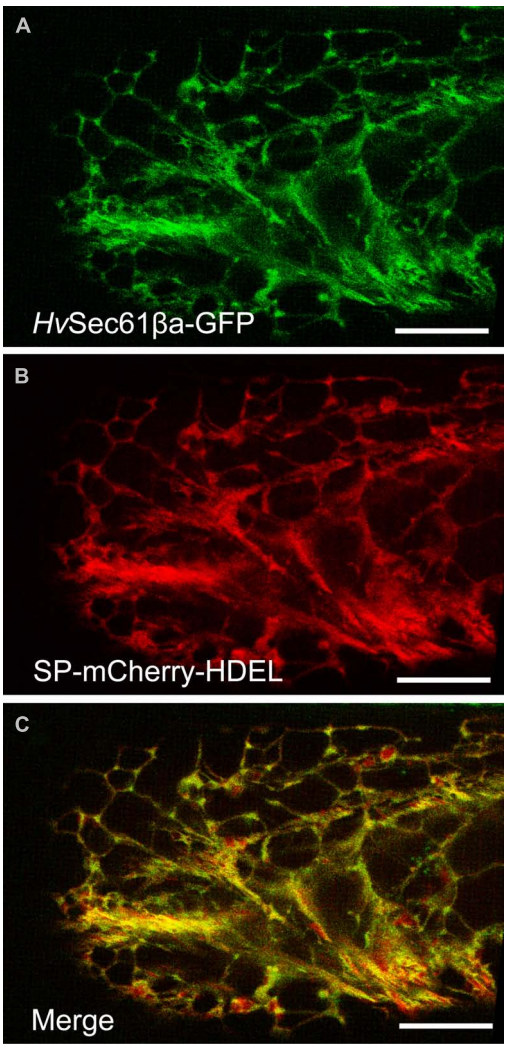


FIGURE 2 | *Hv*Sec61βa co-localizes with an ER luminal marker. (A) Maximum intensity projection of a z-series of confocal images of a barley epidermal cell expressing *Hv*Sec61βa-GFP reveals the ER localization of *Hv*Sec61βa-GFP with the typical distribution within the reticular ER network. **(B)** In the same epidermal cell, the 35S promoter-driven SP-mCherry-HDEL construct is expressed and labels the ER. **(C)** The merged image shows that the *Hv*Sec61βa-GFP and SP-mCherry-HDEL signals largely overlap. Scale bar, 20 μm.

*Hv*Sec61βa (**Figure 2A**). In addition, the *Hv*Sec61βa-GFP signal largely colocalized with mCherry signal from the luminal ER marker (**Figures 2B,C**). The colocalisation is near perfect in the tubular parts of the ER, while the cisternal parts have relatively more mCherry signal. This likely reflects that *Hv*Sec61βa-GFP is membrane bound, and that the soluble mCherry luminal marker dominates the more voluminous cisternal ER. In conclusion, our observations indicate that *Hv*Sec61βa is localized to all parts of the ER.

Since we confirmed the ER localisation of *Hv*Sec61βa-GFP in barley and have observed increased resistance after silencing this gene, we were interested in knowing how the ER changes its location after pathogen attack. It is often described that infected host cells re-localize organelles and specific proteins, which results in their accumulation at the pathogen attack site (Takemoto et al., 2003; Koh et al., 2005; Caillaud et al., 2012). We used the 35S promoter-driven SP-mCherry-HDEL construct to study the localization of the ER after attack by *Bgh*. Confocal imaging of an infected barley cell revealed that the mCherry ER-luminal marker was located around the body of the *Bgh* haustorium. Meanwhile, this ER marker was most often not present around the haustorial fingers (**Figures 3A,B**). In a 3D projection (**Figure 3C**) of the mCherry fluorescent signal, this distinction between the haustorial body and fingers is clearly visible. These observations revealed that the ER network is in close proximity to the EHM around the haustorial body.

Similar to the mCherry ER-luminal marker (**Figure 3**), the *Hv*Sec61βa-GFP signal was present in the ER network around the *Bgh* haustorial body as well (**Figure 4**). Contiguous accumulation of *Hv*Sec61βa-GFP was detected around the nucleus, which was

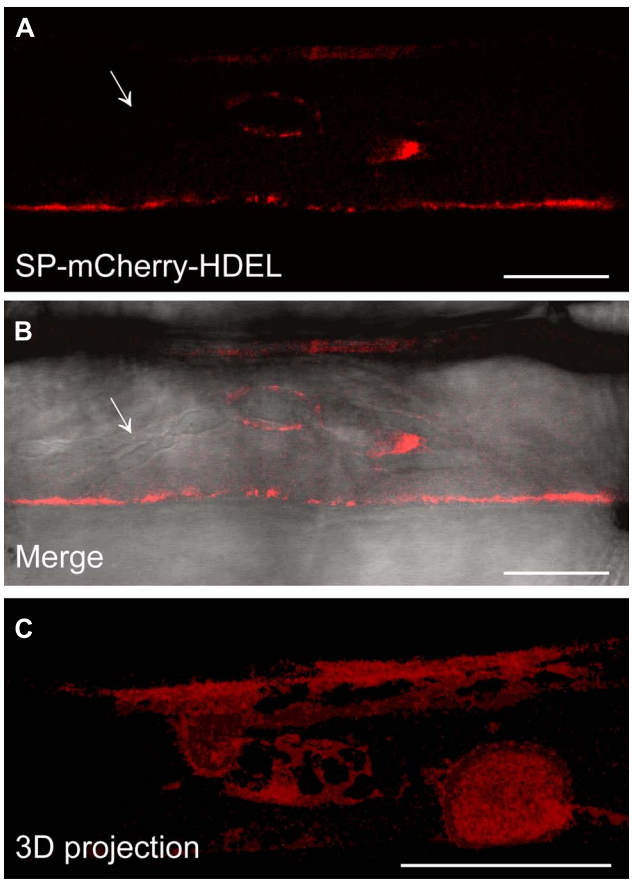


FIGURE 3 | The SP-mCherry-HDEL ER marker localizes around the *Bgh* haustorial body. (A,B) Confocal images of infected barley epidermal cell 48 h after inoculation with *Bgh*. The fluorescent signal of SP-mCherry-HDEL **(A)** localizes to the ER and surrounds the haustorium inhomogeneously. No fluorescence signal of SP-mCherry-HDEL was observed along the haustorial fingers (arrow). The merged image **(B)** displays the haustorium structure in bright field, overlaid with the fluorescence signal. To visualize the ER tubules around the haustorial body, a 3D projection of a z-series of confocal images **(C)** was generated (Image Surfer 1.2). Scale bar, 10 μm.

observed close to the haustorium, supporting the re-localization of this organelle upon pathogen attack (**Figures 4A–E**), as previously described (Schmelzer, 2002). As for the ER-luminal marker, *Hv*Sec61βa-GFP confirmed that the ER and EHM are in close proximity around the haustorial body. In summary, these confocal microscopy results suggest that the *Hv*Sec61βa-GFP-labeled ER is differentially recruited to the proximity of the EHM around the haustorial body, but not around the fingers.

DISCUSSION

The fact that silencing of *HvSec61βa* causes the barley cells to become resistant to powdery mildew suggests that *Hv*Sec61βa either is a negative regulator of defense or a susceptibility factor required for disease. Sec61β is, as described above, associated with protein-transmitting pores in the ER. While it has been barely studied in plants, yeast data suggest that one of its activities is to be part of a post-translational translocon complex, but that this role is not essential under non-stressed conditions (Finke et al., 1996). Furthermore, Sec61β has also been associated with protein retrotranslocation from the ER (Kawaguchi and Ng, 2007; Nakatsukasa and Brodsky, 2008; Willer et al., 2008), and the question is, which of these activities is important in barley cells attacked by *Bgh*.

Silencing of *HvSec61βa* would result in inhibition of secretion if this protein is generally required for co- or post-translational

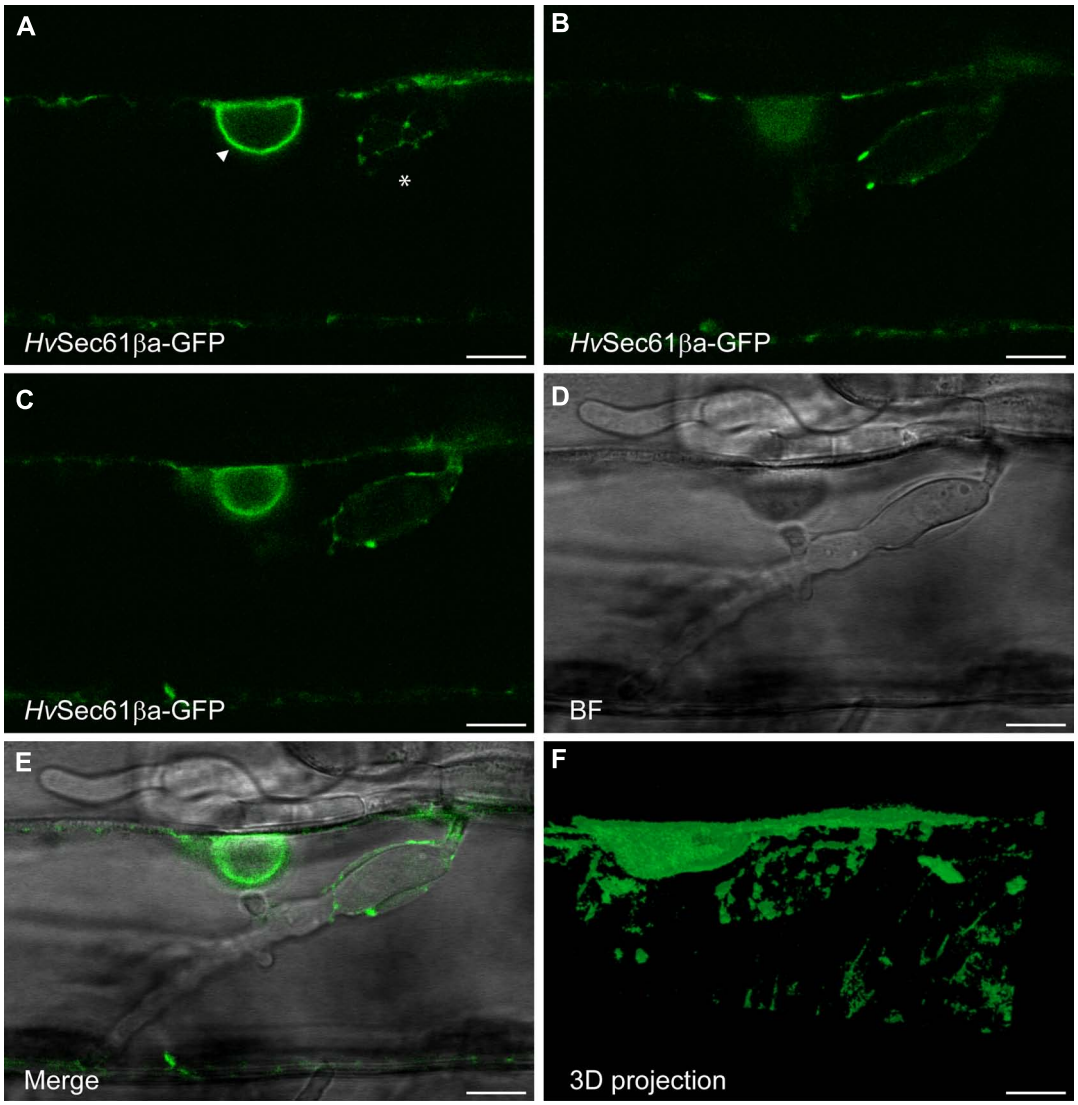


FIGURE 4 | *Hv*Sec61βa-GFP localizes around the *Bgh* haustorial body. Confocal image of an epidermal cell, transformed with the *Hv*Sec61βa-GFP construct, taken 48 h after *Bgh* inoculation. **(A–C)** Three different focal planes from an image series of an infected cell with a haustorium. *Hv*Sec61βa-GFP localizes to the ER around the nucleus (arrow head, **A**) and surrounds the haustorium in an ER-like tubular pattern (asterisk, **A**). **(C–E)** GFP fluorescence **(C)**, bright field (BF) **(D)** and merged image **(E)** show *Hv*Sec61βa-GFP localization at the surface of the haustorial body. *Hv*Sec61βa-GFP labels the tubular ER network, which is further illustrated in the 3D projection **(F)** (Image Surfer 1.2). Scale bar, 10 μm.

protein translocation into the ER. This can hardly explain our phenotype, as inhibition of secretion in barley results in increased susceptibility to *Bgh* (Ostertag et al., 2013). A more likely explanation might be found in a specific *Hv*Sec61βa-function in post-translational translocation. This could involve the so-called "unfolded protein response" (UPR), which results from ER stress due to accumulation of unfolded proteins. During UPR, ER chaperones and components of the ERAD system are up-regulated to prevent the cell from undergoing programmed cell death (Travers et al., 2000). Similarly, ER stress induced by, e.g., tunicamycin (an N-glycosylation inhibitor) increases transcript levels of genes encoding proteins of the ER-QC machinery and the secretory pathway (Martinez and Chrispeels, 2003; Huttner and Strasser, 2012). Recently, a functional link has been established between UPR and pathogen defense in plants. *Arabidopsis* plants mutated in the *IRE1a* gene, encoding a key positive regulator of UPR, were found to have reduced resistance to bacteria (Moreno et al., 2012). An important chaperone that counter acts UPR is the ER-luminal protein, BiP, which is taken up post-translationally through the translocon complex in a Sec61β-dependent manner (Finke et al., 1996). Therefore, a model could be that *HvSec61βa* silencing causes ER-deprivation of BiP, in turn resulting in UPR as well as increased resistance. An *Arabidopsis BiP* knock-out line has previously been suggested to be prone for UPR. However, in disagreement with the model, the *BiP* knock-out line had reduced resistance (Wang et al., 2005). This may indirectly suggest that reduced BiP import into the ER is not the cause of the Sec61βa phenotype we observe, while *Bgh* resistance increases in this situation. We therefore favor a function for Sec61βa in protein retrotranslocation in the interaction with the powdery mildew fungus.

In the meantime, we had an indication of active recruitment of ER by the fungus, supporting that *Hv*Sec61βa functions as a susceptibility component. We observed a close association of the ER, labeled by *Hv*Sec61βa-GFP, and the *Bgh* haustorial body. The ER has also in other cases been found to be closely associated with haustoria (Koh et al., 2005; Micali et al., 2011). However, only *Blumeria* haustoria differentiate in two parts and provide a chance to distinguish variations in ER association. Interestingly, there is little ER association with the haustorial fingers, which could suggest that the ER proximity to the haustorial body is not due to ER being present wherever there is cytosol. Therefore, it is possible that the fungus controls the ER-haustorium association. Voegele et al. (2009) proposed that effector proteins are transferred to the cytosol via the ER. Effectors need to cross a membrane in order to reach the host cytosol, and the ER retrotranslocon pore offers an escape route for this. The resistance phenotype seen after *HvSec61βa* silencing is in agreement with a model, where this protein is necessary for pore function. As illustrated in **Figure 5**, we suggest that vesicle trafficking transfers the effectors from the EHMx to the ER in order for them subsequently to employ the retrotranslocon to enter the cytosol. While we consider the model in **Figure 5** to describe the most likely mode of action of Sec61βa in plant powdery mildew interactions, other scenarios are possible. An unexpected function has for instance been described for *Drosophila* Sec61β, which is

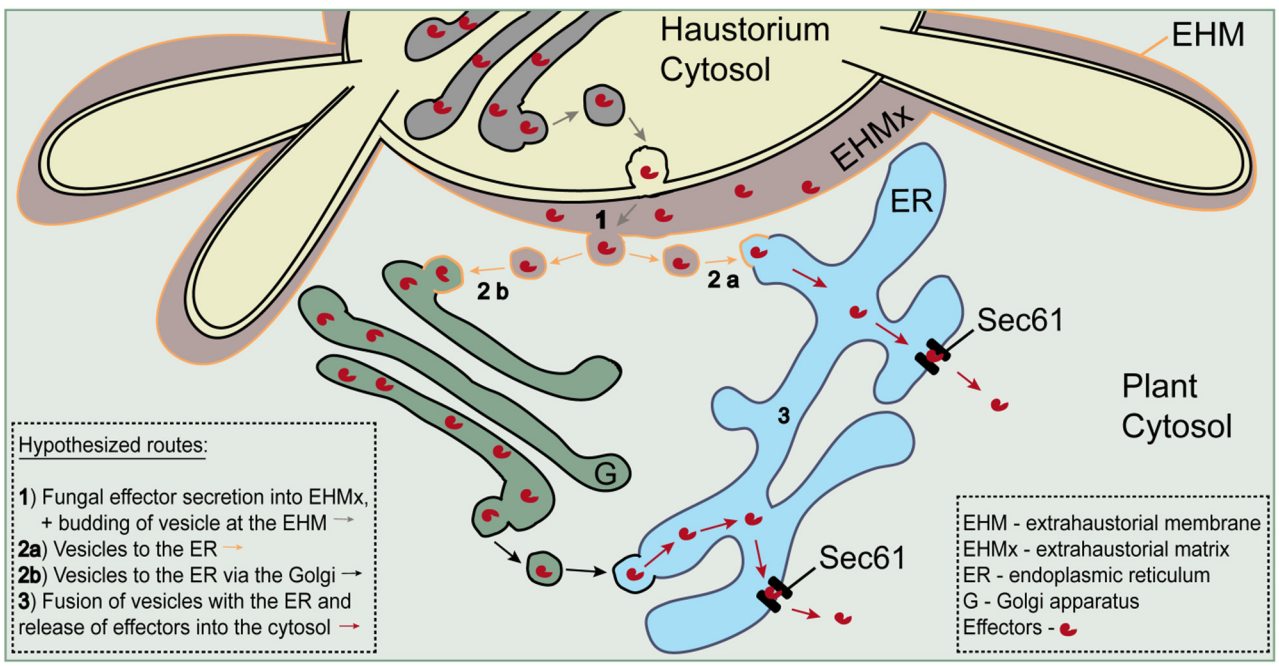


FIGURE 5 | Schematic model for a possible Sec61-dependent route of effector release into the host cytosol. Effectors are hypothesized to be transferred from the extrahaustorial matrix to the cytosol through Sec61 retrotranslocon pores in the ER. Trafficking from the matrix to the ER is envisaged to take place in vesicles dependent or independent of Golgi. Adapted from Voegele et al. (2009).

important for the secretion of the Gurken protein (Kelkar and Dobberstein, 2009). After silencing of *Sec61*β, Gurken left the ER as it normally did in control cells, but subsequently became stalled in the Golgi. Since a control protein still was observed to be secreted after silencing of *Sec61*β, this suggests that Sec61β is required for the Golgi-processing of a subset of the secreted proteins, including Gurken (Kelkar and Dobberstein, 2009). While this cannot be excluded to be due to a retrotranslocon defect, it may indicate that Sec61β also has a function in secretion, which is unrelated to the Sec61 protein pore. Future work will determine which function makes Sec61β important for plant's susceptibility to the powdery mildew fungus, and whether modification of the gene can be exploited for a disease resistance purpose.

ACKNOWLEDGMENTS

We are grateful to Dr. Patrick Schweizer for providing the pIPKTA30N vector, to Prof. Robert Dudler for providing the pBC17 construct and to Prof. Andreas Nebenführ for the 35S-SP-mCherry-HDEL construct. This work was supported by the Danish Strategic Research Council and The Faculty of Science, University of Copenhagen. Fluorescence imaging was performed using the facilities of the Center for Advanced Bioimaging (CAB) Denmark, University of Copenhagen.

REFERENCES

Blanke, S. R. (2006). Portals and pathways: principles of bacterial toxin entry into host cells. *Microbe* 1, 26–32.

Bozkurt, T. O., Schornack, S., Banfield, M. J., and Kamoun, S. (2012). Oomycetes, effectors, and all that jazz. *Curr. Opin. Plant Biol.* 15, 483–292.

Caillaud, M. C., Piquerez, S. J., Fabro, G., Steinbrenner, J., Ishaque, N., Beynon, J., et al. (2012). Subcellular localization of the Hpa RxLR effector repertoire identifies a tonoplast-associated protein HaRxL17 that confers enhanced plant susceptibility. *Plant J.* 69, 252–265.

Carvalho, P., Goder, V., and Rapoport, T. A. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126, 361–373.

Carvalho, P., Stanley, A. M., and Rapoport, T. A. (2010). Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell* 143, 579–591.

Curtis, M. D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* 133, 462–469.

Dash, S., Van Hemert, J., Hong, L., Wise, R. P., and Dickerson, J. A. (2012). PLEXdb: gene expression resources for plants and plant pathogens. *Nucleic Acids Res.* 40, D1194–D1201.

Deng, W., Nickle, D. C., Learn, G. H., Maust, B., and Mullins, J. I. (2007). ViroBLAST: a stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. *Bioinformatics* 23, 2334–2336.

Douchkov, D., Nowara, D., Zierold, U., and Schweizer, P. (2005). A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells. *Mol. Plant Microbe Interact.* 18, 755–761.

Feng, D., Marshburn, D., Jen, D., Weinberg, R. J., Taylor, R. M. II, and Burette, A. (2007). Stepping into the third dimension. *J. Neurosci.* 27, 12757–12760.

Finke, K., Plath, K., Panzner, S., Prehn, S., Rapoport, T. A., Hartmann, E., et al. (1996). A second trimeric complex containing homologs of the Sec61p complex functions in protein transport across the ER membrane of *S. cerevisiae*. *EMBO J.* 15, 1482–1494.

Gan, P. H. P., Dodds, P. N., and Hardham, A. R. (2012). "Plant infection by biotrophic fungal and oomycete pathogens," in *Signaling and Communication in Plant Symbiosis, Signaling and Communication in Plants*, Vol. 11, eds S. Perotto and F. Baluska (Berlin: Springer-Verlag), 183–212.

Gomord, V., Denmat, L. A., Fitchette-Laine, A. C., Satiat-Jeunemaitre, B., Hawes, C., and Faye, L. (1997). The C-terminal HDEL sequence is sufficient for retention of secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. *Plant J.* 11, 313–325.

Guerriero, C. J., and Brodsky, J. L. (2012). The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiol. Rev.* 92, 537–576.

Huttner, S., and Strasser, R. (2012). Endoplasmic reticulum-associated degradation of glycoproteins in plants. *Front. Plant Sci.* 3:67. doi: 10.3389/fpls.2012.00067

Kawaguchi, S., and Ng, D. T. (2007). SnapShot: ER-associated protein degradation pathways. *Cell* 129, 1230.

Kelkar, A., and Dobberstein, B. (2009). Sec61beta, a subunit of the Sec61 protein translocation channel at the endoplasmic reticulum, is involved in the transport of Gurken to the plasma membrane. *BMC Cell Biol.* 10:11. doi: 10.1186/1471-2121-10-11

Koh, S., Andre, A., Edwards, H., Ehrhardt, D., and Somerville, S. (2005). *Arabidopsis thaliana* subcellular responses to compatible *Erysiphe cichoracearum* infections. *Plant J.* 44, 516–529.

Liao, H. J., and Carpenter, G. (2007). Role of the Sec61 translocon in EGF receptor trafficking to the nucleus and gene expression. *Mol. Biol. Cell* 18, 1064–1072.

Martinez, I. M., and Chrispeels, M. J. (2003). Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. *Plant Cell* 15, 561–576.

Mayer, K. F., Waugh, R., Brown, J. W., Schulman, A., Langridge, P., Platzer, M., et al. (2012). A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491, 711–717.

Micali, C. O., Neumann, U., Grunewald, D., Panstruga, R., and O'Connell, R. (2011). Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. *Cell. Microbiol.* 13, 210–226.

Moreno, A. A., Mukhtar, M. S., Blanco, F., Boatwright, J. L., Moreno, I., Jordan, M. R., et al. (2012). IRE1/bZIP60-mediated unfolded protein response plays distinct roles in plant immunity and abiotic stress responses. *PLoS ONE* 7:e31944. doi: 10.1371/journal.pone.0031944

Nakatsukasa, K., and Brodsky, J. L. (2008). The recognition and retrotranslocation of misfolded proteins from the endoplasmic reticulum. *Traffic* 9, 861–870.

Nelson, B. K., Cai, X., and Nebenführ, A. (2007). A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J.* 51, 1126–1136.

Ostertag, M., Stammler, J., Douchkov, D., Eichmann, R., and Huckelhoven, R. (2013). The conserved oligomeric Golgi complex is involved in penetration resistance of barley to the barley powdery mildew fungus. *Mol. Plant Pathol.* 14, 230–240.

Pedersen, C., van Themaat, E. V., McGuffin, L. J., Abbott, J. C., Burgis, T. A., Barton, G., et al. (2012). Structure and evolution of barley powdery mildew effector candidates. *BMC Genomics* 13:694. doi: 10.1186/1471-2164-13-694

Rodighiero, C., Tsai, B., Rapoport, T. A., and Lencer, W. I. (2002). Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. *EMBO Rep.* 3, 1222–1227.

Rolls, M. M., Stein, P. A., Taylor, S. S., Ha, E., McKeon, F., and Rapoport, T. A. (1999). A visual screen of a GFP-fusion library identifies a new type of nuclear envelope membrane protein. *J. Cell Biol.* 146, 29–44.

Saunders, D. G., Win, J., Cano, L. M., Szabo, L. J., Kamoun, S., and Raffaele, S. (2012). Using hierarchical clustering of secreted protein families to classify and rank candidate effectors of rust fungi. *PLoS ONE* 7:e29847. doi: 10.1371/journal.pone.0029847

Schmelzer, E. (2002). Cell polarization, a crucial process in fungal defence. *Trends Plant Sci.* 7, 411–415.

Schmitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol.* 148, 1203–1212.

Schweizer, P., Pokorny, J., Schulze-Lefert, P., and Dudler, R. (2000). Technical advance. Double-stranded RNA interferes with gene function at the single-cell level in cereals. *Plant J.* 24, 895–903.

Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stuber, K., et al. (2010). Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* 330, 1543–1546.

Takemoto, D., Jones, D. A., and Hardham, A. R. (2003). GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens. *Plant J.* 33, 775–792.

Teter, K., Allyn, R. L., Jobling, M. G., and Holmes, R. K. (2002). Transfer of the cholera toxin A1 polypeptide from the endoplasmic reticulum to the cytosol is a rapid process facilitated by the endoplasmic reticulum-associated degradation pathway. *Infect. Immun.* 70, 6166–6171.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249–258.

Van den Berg, B., Clemons, W. M. Jr., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., et al. (2004). X-ray structure of a protein-conducting channel. *Nature* 427, 36–44.

Voegele, R. T., Hahn, M., and Mendgen, K. (2009). "The uredinales: cytology, biochemistry, and molecular biology," in *Plant Relationships, The Mycota V*, ed. H. Deising (Berlin: Springer-Verlag), 69–98.

Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M., and Rapoport, T. A. (2006). A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* 124, 573–586.

Wang, D., Weaver, N. D., Kesarwani, M., and Dong, X. (2005). Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308, 1036–1040.

Wang, Y. N., Yamaguchi, H., Huo, L., Du, Y., Lee, H. J., Lee, H. H., et al. (2010). The translocon Sec61beta localized in the inner nuclear membrane transports membrane-embedded EGF receptor to the nucleus. *J. Biol. Chem.* 285, 38720–38729.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450, 115–118.

Willer, M., Forte, G. M., and Stirling, C. J. (2008). Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY. *J. Biol. Chem.* 283, 33883–33888.

Zhao, X., and Jantti, J. (2009). Functional characterization of the transmembrane domain interactions of the Sec61 protein translocation complex beta-subunit. *BMC Cell Biol.* 10:76. doi: 10.1186/1471-2121-10-76

Zimmermann, R., Eyrisch, S., Ahmad, M., and Helms, V. (2011). Protein translocation across the ER membrane. *Biochim. Biophys. Acta* 1808, 912–924.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 February 2013; accepted: 18 April 2013; published online: 16 May 2013.

*Citation: Zhang W-J, Hanisch S, Kwaaitaal M, Pedersen C and Thordal-Christensen H (2013) A component of the Sec61 ER protein transporting pore is required for plant susceptibility to powdery mildew. Front. Plant Sci. **4**:127. doi: 10.3389/fpls.2013.00127*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Zhang, Hanisch, Kwaaitaal, Pedersen and Thordal-Christensen. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 07 May 2013
doi: 10.3389/fpls.2013.00124

Lectin receptor kinases in plant innate immunity

Prashant Singh*[1,2] *and Laurent Zimmerli*[1,2]*

[1] *Department of Life Science, National Taiwan University, Taipei, Taiwan*
[2] *Institute of Plant Biology, National Taiwan University, Taipei, Taiwan*

Edited by:
Corné M. Pieterse, Utrecht University, Netherlands

Reviewed by:
Klaas Bouwmeester, Wageningen University, Netherlands
Maeli Melotto, University of Texas at Arlington, USA

****Correspondence:***
Laurent Zimmerli, Institute of Plant Biology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan.
e-mail: lauzim2@ntu.edu.tw

A key feature of innate immunity is the ability to recognize and respond to potential pathogens in a highly sensitive and specific manner. In plants, the first layer of defense is induced after recognition by pattern recognition receptors of microbe-associated molecular patterns. This recognition elicits a defense program known as pattern-triggered immunity. Pathogen entry into host tissue is a critical early step in causing infection. For foliar bacterial pathogens, natural surface openings such as stomata, are important entry sites. Stomata in contact with bacteria rapidly close and can thus restrict bacterial entry into leaves. The molecular mechanisms regulating stomatal closure upon pathogen perception are not yet well-understood. Plant lectin receptor kinases are thought to play crucial roles during development and in the adaptive response to various stresses. Although the function of most plant lectin receptor kinases is still not clear, a role for this kinase family in plant innate immunity is emerging. Here, we summarize recent progresses in the identification of lectin receptor kinases involved in plant innate immunity. We also discuss the role of lectin receptor kinases in stomatal innate immunity signaling.

Keywords: plant, receptor-like kinase, lectin receptor kinase, innate immunity, stomatal innate immunity, bacteria

INTRODUCTION

Plants face threats from various pathogenic microbes and resist attacking pathogens through both constitutive and inducible defenses (Jones and Dangl, 2006). The pattern-triggered immunity (PTI) defense response represents the front line of plant innate immunity. PTI is activated upon recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) via pattern recognition receptors (PRRs; Jones and Dangl, 2006; Zipfel, 2009; Tsuda and Katagiri, 2010; Zhang and Zhou, 2010). Examples of MAMPs comprise the lipopolysaccharide envelope of Gram-negative bacteria, peptidoglycans from Gram-positive bacteria, eubacterial flagellin, eubacterial elongation factor (EF), methylated bacterial DNA fragments, and fungal cell wall derived glucans, chitins, and proteins (Girardin et al., 2002; Cook et al., 2004; Ausubel, 2005; Boller and Felix, 2009). MAMP perception results in PTI activation which includes downstream defense responses such as production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, changes in gene expression, and production of defense compounds together leading to broad resistance to pathogens (Boller and Felix, 2009). In addition, MAMP perception at stomatal guard cells induces stomatal closure, thus activating stomatal innate immunity (Melotto et al., 2006; Zeng et al., 2010).

Pathogen entry into host tissue is a critical, first step in causing plant infection. Stomata at the leaf epidermis are natural openings that bacteria use to enter into leaves. Typically, *Arabidopsis* stomata close when in contact with bacteria, thus functioning as innate immunity gates to actively prevent bacteria entry into plants (Melotto et al., 2006, 2008, Schulze-Lefert and Robatzek, 2006; Zeng et al., 2010; Faulkner and Robatzek, 2012). Usually, 1 h after exposure to *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) bacteria, *Arabidopsis* stomata close as a result of stomatal innate immunity activation. Virulent bacteria such as *Pst* DC3000 can re-open *Arabidopsis* Col-0 stomata 3–4 h after infection through the action of the chemical effector coronatine (COR) suggesting that plant pathogens have evolved virulence factors to suppress innate immunity functions of stomata (Melotto et al., 2006; Schulze-Lefert and Robatzek, 2006). The ability of COR to inhibit stomatal closure is dependent on the *COI1* gene (Melotto et al., 2006) and the priming compound beta-aminobutyric acid (BABA) blocks the COR-dependent re-opening of stomata during *Pst* DC3000 and *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) infection (Tsai et al., 2011; Po-Wen et al., 2013). Stomatal closure in response to treatments with flg22, a peptide representing the most conserved domain of bacterial flagellin, is dependent on the flagellin receptor FLS2 (FLAGELLIN SENSITIVE2), demonstrating that perception of bacterial MAMPs through PRRs leads to closure of *Arabidopsis* stomata (Zipfel et al., 2004; Zeng and He, 2010). The chloroplastic enzyme ASPARTATE OXIDASE that catalyzes *de novo* biosynthesis of nicotinamide adenine dinucleotide is also a critical player during activation of stomatal innate immunity in response to *Pst* infection (Macho et al., 2012). In addition, both salicylic acid (SA) and abscisic acid (ABA) signaling pathways are required during bacteria- and MAMP-induced stomatal closure in *Arabidopsis* (Melotto et al., 2006; Zeng et al., 2010). Recent works emphasized the lectin receptor kinases in plant innate immunity. In this review, we will thus focus on the role of this emerging family of receptor kinases in plant innate immunity, with highlights on stomatal innate immunity.

LECTIN RECEPTOR KINASES IN PLANT DEFENSE

In plants, perception and transduction of environmental stimuli are largely governed by receptor-like kinases (RLKs; Mahajan and Tuteja, 2005). RLKs belong to a vast protein family found in

higher plants that is represented by 610 genes in the *Arabidopsis* genome (Shiu and Bleecker, 2001, 2003).Lectin receptor kinases are RLKs characterized by an extracellular lectin motif. These lectin receptor kinases are classified into three types: G, C, and L (Bouwmeester and Govers, 2009; Vaid et al., 2012). G-type lectin receptor kinases are known as S-domain RLKs and are involved in self-incompatibility in flowering plants (Kusaba et al., 2001; Sherman-Broyles et al., 2007). C-type (calcium-dependent) lectin motifs can be found in a large number of mammalian proteins that mediate innate immune responses and play a major role in pathogen recognition (Cambi et al., 2005), but are rare in plants. *Arabidopsis* has only a single gene encoding a protein with a C-type lectin motif but so far its function has not been elucidated (Bouwmeester and Govers, 2009). *Arabidopsis* contains 45 L-type lectin receptor kinases (LecRKs) that are characterized by an extracellular legume lectin-like domain, a transmembrane domain and an intracellular kinase domain (Herve et al., 1996; Barre et al., 2002; Bouwmeester and Govers, 2009). LecRKs were suggested to play a role in abiotic stress signal transduction (Garcia-Hernandez et al., 2002; Nishiguchi et al., 2002; Riou et al., 2002; He et al., 2004; Deng et al., 2009; Joshi et al., 2010). Notably, LecRK members of the *Arabidopsis* LecRK-VI clade (Bouwmeester and Govers, 2009), are redundant negative regulators of the ABA response during seed germination (Xin et al., 2009).

Due to the resemblance of the extracellular domain with lectin proteins known to bind to fungal and bacterial cell wall components, lectin receptor kinases are predominantly hypothesized to participate in biotic stress tolerance (Bouwmeester and Govers, 2009). Some lectin receptor kinases were indeed reported to be involved in plant resistance to pathogens. For example, *Pi-d2*, a G-type lectin receptor kinase from rice, provides resistance against the fungal pathogen *Magnaporthe grisea*, the causal agent of rice blast (Chen et al., 2006). In tobacco, the expression of another G-type lectin receptor kinase was recently shown to be up-regulated by lipopolysaccharides (Sanabria et al., 2012). In *Nicotiana benthamiana,* the LecRK *NbLRK1* was suggested to be a component of the *N. benthamiana* protein complex that recognizes the *Phytophthora infestans* INF1 elicitor and mediates INF1-induced cell death (Kanzaki et al., 2008).

Like few other RLK proteins, such as PERK (proline-rich extensin-like receptor protein kinase), WAK (wall-associated kinase) and CrRLK (*Catharanthus roseus*-like RLK), LecRK-I.9 mediates cell wall–plasma membrane (CW–PM) continuum (Bouwmeester and Govers, 2009). The maintenance of structural CW–PM continuity is a critical factor that governs plants response to various stimuli and is essential for defense against pathogens (Bouwmeester and Govers, 2009; Bouwmeester et al., 2011). The association of RGD (arginine–glycine–aspartic acid) motif containing proteins with cellular proteins is a key mechanism that maintains the structural integrity of CW–PM contacts (Gouget et al., 2006). The RGD motif present in IPI-O (*in planta* induced-O), a secreted effector protein of the oomycete pathogen *Phytophthora infestans*, disrupts CW–PM adhesions upon interaction with a variety of cellular proteins, including LecRKs (Gouget et al., 2006). Further analysis revealed that deficiency in LecRK-I.9, earlier found to interact with RGD motif containing proteins (Gouget et al., 2006), leads to a gain of susceptibility phenotype toward the oomycete *Phytophthora brassicae* (Bouwmeester et al., 2011). These results imply that LecRKs may be involved in protein–protein interactions with RGD-containing proteins as potential ligands, and may play a structural and signaling role at the plant cell surfaces upon pathogen infection.

LecRK-VI.2 is critical for resistance against hemibiotrophic *Pst* DC3000 and necrotrophic *Pcc* bacteria (Singh et al., 2012). Increased susceptibility of the transferred DNA (T-DNA) insertion mutant line *lecrk-VI.2-1* is correlated with defective bacteria- and MAMP-induced MPK3 (Mitogen-activated protein kinase 3) and MPK6 (Mitogen-activated protein kinase 6) activities, PTI-responsive gene expression, and callose deposition (Singh et al., 2012). Transcriptome analysis of a *LecRK-VI.2* over-expression line revealed transcription up-regulation of numerous genes responsive to virulent or avirulent bacteria, the MAMP flg22, or to the SA functional analog benzothiadiazole further suggesting a role for LecRK-VI.2 in the *Arabidopsis* PTI response (Singh et al., 2012). BAK1 (Brassinosteroid insensitive1-associated kinase 1) and FLS2 association, BIK1 (BOTRYTIS-INDUCED KINASE1) phosphorylation, and ROS production that are usually considered as early PTI responses (Zipfel and Robatzek, 2010), were not compromised in the mutant *lecrk-VI.2-1*. These data suggest that LecRK-VI.2 positively modulates PTI signaling upstream of MPK3 and MPK6 and downstream of FLS2 (Singh et al., 2012). In addition, LecRK-VI.2 is a key modulator of BABA-mediated priming and BABA-induced resistance (Singh et al., 2012). Further analyses of the function of LecRK-VI.2 revealed that LecRK-VI.2 possesses a functional kinase domain and is not critical for resistance to the necrotrophic fungal pathogen *Botrytis cinerea* (Singh et al., 2013). By contrast, over-expression of the plasma membrane-localized L-type lectin-like protein kinase 1, AtLPK1 (LecRK-IV.3) induces *Arabidopsis* resistance to *B. cinerea* (Huang et al., 2013).

Lectin receptor kinases are also critical for plant resistance to insects. The lectin receptor kinase 1 (LecRK1) is important during herbivory by *Manduca sexta* larvae to suppress insect-mediated inhibition of jasmonic acid-induced defense responses in *Nicotiana attenuata* (Gilardoni et al., 2011). Importantly, reduction of *LecRK1* expression in *N. attenuata* induces increased *Manduca sexta* folivory (Gilardoni et al., 2011). The insect-induced accumulation of protease inhibitors, as well as the expression of the gene encoding threonine deaminase, two critical defense responses were also several fold reduced in *N. attenuata* with a silenced *LecRK1* when compared to non-silenced controls (Gilardoni et al., 2011). Inhibition of SA accumulation through the expression of nahG in silenced *lecRK1* plants restores wild-type levels of resistance against *Manduca sexta* herbivory, suggesting that LecRK1 inhibits the accumulation of SA during herbivory (Gilardoni et al., 2011). More recently, LecRK-I.8 was suggested to be important for the perception of insect egg-derived elicitors in *Arabidopsis* (Gouhier-Darimont et al., 2013).

LecRK-VI.2 AND LecRK-V.5 IN *Arabidopsis* STOMATAL INNATE IMMUNITY

In addition to positively regulating apoplastic PTI, LecRK-VI.2 is also critical for *Arabidopsis* stomatal innate immunity

(Singh et al., 2012). Notably and similarly to the PRR mutant *fls2* (Zeng and He, 2010), *lecrk-VI.2-1* mutants demonstrate a high sensitivity to *Pst* DC3000 COR$^-$ deficient bacterial mutants that cannot re-open stomata upon infection. Since *Arabidopsis* is resistant to these bacterial mutants (Melotto et al., 2006), LecRK-VI.2 may play a positive role in bacteria-mediated stomatal closure (Singh et al., 2012). Consistent with this observation, stomatal closure upon bacterial inoculation and MAMPs treatments were found to be defective in the mutant *lecrk-VI.2-1* (Singh et al., 2012). This suggests that LecRK-VI.2 plays a positive role during stomatal innate immunity activation at a signaling node downstream of MAMP perception. In addition, transgenic lines over-expressing *LecRK-VI.2* demonstrate constitutive stomatal closure, further suggesting a positive role for LecRK-VI.2 in stomatal innate immunity (Singh et al., 2012). The mutant *lecrk-VI.2-1* demonstrates wild-type stomatal closure levels in response to ABA indicating that LecRK-VI.2 acts upstream or independently of ABA signaling during stomatal closure (Singh et al., 2012).

Another LecRK involved in *Arabidopsis* stomatal innate immunity is LecRK-V.5. However, in contrary to LecRK-VI.2 that positively regulates stomatal innate immunity, LecRK-V.5 negatively regulates stomatal closure upon bacterial infection. Plants lacking a functional LecRK-V.5 are resistant to *Pst* DC3000 and *Pcc* surface inoculation, but are normally sensitive to infiltration inoculation (Arnaud et al., 2012; Desclos-Theveniau et al., 2012). These observations suggest that disruption of LecRK-V.5 affects early *Arabidopsis* defenses by restricting bacterial entry into leaves and point to a role of LecRK-V.5 in stomatal innate immunity (Desclos-Theveniau et al., 2012). Analyses of stomatal apertures in *lecrk-V.5* indeed revealed that this mutant possesses constitutively closed stomata (Desclos-Theveniau et al., 2012). Transgenic lines over-expressing *LecRK-V.5* are less resistant to *Pst* DC3000 COR$^-$ and this is correlated with a re-opening of stomata in *LecRK-V.5* over-expression lines even in the absence of COR. These observations suggest the existence of a stomatal re-opening mechanism positively modulated by LecRK-V.5 (Desclos-Theveniau et al., 2012). Interestingly, LecRK-V.5 over-expression lines are also defective in MAMP-induced stomatal closure. Together these data indicate that LecRK-V.5 negatively regulates *Arabidopsis* resistance to bacteria through fine-tuning of stomatal innate immunity (Desclos-Theveniau et al., 2012). Localized expression of LecRK-V.5 upon PTI activation at stomatal guard cells further supports a role for LecRK-V.5 in stomatal innate immunity (Desclos-Theveniau et al., 2012). Similarly to the *scord5* mutant that shows a defective stomatal innate immunity but exhibits wild-type apoplastic immunity (Zeng et al., 2011), apoplastic PTI responses such as flg22-triggered oxidative burst, bacteria-mediated callose deposition and up-regulation of PTI marker genes are not affected in *lecrk-V.5* mutants. COR treatments re-open closed stomata in *lecrk-V.5* mutants (Desclos-Theveniau et al., 2012), suggesting that LecRK-V.5 acts upstream of COR. *lecrk-V.5* mutants accumulate high levels of ROS in guard cells and chemical inhibition of ROS accumulation in *lecrk-V.5* guard cells re-opens closed stomata (Desclos-Theveniau et al., 2012). By contrast, treatments with PAMPs increase guard cell ROS levels in wild-type, but no increase of ROS production was observed in *Arabidopsis* over-expressing *LecRK-V.5* (Desclos-Theveniau et al., 2012). Since ROS induce stomatal closure, high levels of ROS, and defective ROS accumulation may explain constitutive stomatal closure in *lecrk-V.5* mutants and deficient stomatal closure in *LecRK-V.5* over-expression lines, respectively. In addition, lines over-expressing *LecRK-V.5* demonstrate a compromised ABA-mediated stomatal closure (Desclos-Theveniau et al., 2012), thus LecRK-V.5 functions in guard cell ABA signaling pathway downstream of MAMP perception. LecRK-V.5 may thus act at a specific branch involving ABA for the control of stomatal innate immunity and may negatively regulate ABA-mediated stomatal responses (Desclos-Theveniau et al., 2012). Negative regulation of stomatal innate immunity may have evolved in order to avoid the deleterious effects of a prolonged inhibition of photosynthesis that would be caused by decreased CO_2 availability following prolonged stomatal closure.

CONCLUSION

Although new knowledge about lectin receptor kinases function and signaling has emerged recently, many questions still remain unanswered. For example, what are the potential ligands and downstream partners that modulate lectin receptor kinase-dependent innate immunity responses are critical points that need to be solved. Importantly, the unraveling of the mechanisms modulating ligands perception by lectin receptor kinases will provide further insights into how LecRKs affect the plant response to pathogens. This may clarify whether these receptor kinases function as PRRs. Knowledge derived from such studies could lead to novel methods for managing plant disease resistance.

ACKNOWLEDGMENTS

This work was supported by grants to Laurent Zimmerli from the National Science Council of Taiwan grants 98-2311-B-002-008-MY3 and 99-2628-B-002-053-MY3 and the Frontier and Innovative Research grant of the National Taiwan University code number 99R70436.

REFERENCES

Arnaud, D., Desclos-Theveniau, M., and Zimmerli, L. (2012). Disease resistance to *Pectobacterium carotovorum* is negatively modulated by the *Arabidopsis* Lectin Receptor Kinase LecRK-V.5. *Plant Signal Behav.* 7, 1070–1072.

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973–979.

Barre, A., Herve, C., Lescure, B., and Rouge, P. (2002). Lectin receptor kinases in plants. *Crit. Rev. Plant Sci.* 21, 379–399.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406.

Bouwmeester, K., de Sain, M., Weide, R., Gouget, A., Klamer, S., Canut, H., et al. (2011). The lectin receptor kinase LecRK-I. 9 is a novel *Phytophthora* resistance component and a potential host target for a RXLR effector. *PLoS Pathog.* 7:e1001327. doi: 10.1371/journal.ppat.1001327

Bouwmeester, K., and Govers, F. (2009). *Arabidopsis* L-type lectin receptor kinases: phylogeny, classification, and expression profiles. *J. Exp. Bot.* 60, 4383–4396.

Cambi, A., Koopman, M., and Figdor, C. G. (2005). How C-type lectins detect pathogens. *Cell. Microbiol.* 7, 481–488.

Chen, X., Shang, J., Chen, D., Lei, C., Zou, Y., Zhai, W., et al. (2006). A B-lectin receptor kinase gene conferring rice blast resistance. *Plant J.* 46, 794–804.

Cook, D. N., Pisetsky, D. S., and Schwartz, D. A. (2004). Toll-like receptors in the pathogenesis of

human disease. *Nat. Immunol.* 5, 975–979.

Deng, K., Wang, Q., Zeng, J., Guo, X., Zhao, X., Tang, D., et al. (2009). A lectin receptor kinase positively regulates ABA response during seed germination and is involved in salt and osmotic stress response. *J. Plant Biol.* 52, 493–500.

Desclos-Theveniau, M., Arnaud, D., Huang, T. Y., Lin, G. J. C., Chen, W. Y., Lin, Y. C., et al. (2012). The *Arabidopsis* lectin receptor kinase LecRK-V.5 represses stomatal immunity induced by *Pseudomonas syringae pv. tomato DC*3000. *Plos Pathog.* 8:e1002513. doi: 10.1371/journal.ppat.1002513

Faulkner, C., and Robatzek, S. (2012). Plants and pathogens: putting infection strategies and defense mechanisms on the map. *Curr. Opin. Plant Biol.* 15, 699–707.

Garcia-Hernandez, M., Berardini, T. Z., Chen, G., Crist, D., Doyle, A., Huala, E., et al. (2002). TAIR: a resource for integrated *Arabidopsis* data. *Funct. Integr. Genomics* 2, 239–253.

Gilardoni, P. A., Hettenhausen, C., Baldwin, I. T., and Bonaventure, G. (2011). *Nicotiana attenuata LECTIN RECEPTOR KINASE1* suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. *Plant Cell* 23, 3512–3532.

Girardin, S. E., Sansonetti, P. J., and Philpott, D. J. (2002). Intracellular vs extracellular recognition of pathogens – common concepts in mammals and flies. *Trends Microbiol.* 10, 193–199.

Gouget, A., Senchou, V., Govers, F., Sanson, A., Barre, A., Rougé, P., et al. (2006). Lectin receptor kinases participate in protein–protein interactions to mediate plasma membrane–cell wall adhesions in *Arabidopsis*. *Plant Physiol.* 140, 81–90.

Gouhier-Darimont, C., Schmiesing, A., Bonnet, C., Lassueur, S., and Reymond, P. (2013). Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity. *J. Exp. Bot.* 64, 665–674.

He, X. J., Zhang, Z. G., Yan, D. Q., Zhang, J. S., and Chen, S. Y. (2004). A salt-responsive receptor-like kinase gene regulated by the ethylene signaling pathway encodes a plasma membrane serine/threonine kinase. *Theor. Appl. Genet.* 109, 377–383.

Herve, C., Dabos, P., Galaud, J. R., Rouge, P., and Lescure, B. (1996). Characterization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. *J. Mol. Biol.* 258, 778–788.

Huang, P., Ju, H.-W., Min, J.-H., Zhang, X., Kim, S.-H., Yang, K.-Y., et al. (2013). Overexpression of L-type lectin-like protein kinase 1 confers pathogen resistance and regulates salinity response in *Arabidopsis thaliana. Plant Sci.* 203–204, 98–106.

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 16, 323–329.

Joshi, A., Hung, D. Q., Vaid, N., and Tuteja, N. (2010). Pea lectin receptor-like kinase promotes high salinity stress tolerance in bacteria and expresses in response to stress in planta. *Glycoconj. J.* 27, 133–150.

Kanzaki, H., Saitoh, H., Takahashi, Y., Berberich, T., Ito, A., Kamoun, S., et al. (2008). NbLRK1, a lectin-like receptor kinase protein of *Nicotiana benthamiana*, interacts with *Phytophthora infestans* INF1 elicitin and mediates INF1-induced cell death. *Planta* 228, 977–987.

Kusaba, M., Dwyer, K., Hendershot, J., Vrebalov, J., Nasrallah, J. B., and Nasrallah, M. E. (2001). Self-incompatibility in the genus *Arabidopsis*: characterization of the *S* locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana. Plant Cell* 13, 627–643.

Macho, A. P., Boutrot, F., Rathjen, J. P., and Zipfel, C. (2012). Aspartate oxidase plays an important role in *Arabidopsis* stomatal immunity. *Plant Physiol.* 159, 1845–1856.

Mahajan, S., and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys.* 444, 139–158.

Melotto, M., Underwood, W., and He, S. Y. (2008). Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* 46, 101–122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980.

Nishiguchi, M., Yoshida, K., Sumizono, T., and Tazaki, K. (2002). A receptor-like protein kinase with a lectin-like domain from lombardy poplar: gene expression in response to wounding and characterization of phosphorylation activity. *Mol. Genet. Genomics* 267, 506–514.

Po-Wen, C., Singh, P., and Zimmerli, L. (2013). Priming of the *Arabidopsis* pattern-triggered immunity response upon infection by necrotrophic *Pectobacterium carotovorum* bacteria. *Mol. Plant Pathol.* 14, 8–70.

Riou, C., Herve, C., Pacquit, V., Dabos, P., and Lescure, B. (2002). Expression of an *Arabidopsis* lectin kinase receptor gene, lecRK-al, is induced during senescence, wounding and in response to oligogalacturonic acids. *Plant Physiol. Biochem.* 40, 431–438.

Sanabria, N. M., van Heerden, H., and Dubery, I. A. (2012). Molecular characterization and regulation of a *Nicotiana tabacum* S-domain receptor-like kinase gene induced during an early rapid response to lipopolysaccharides. *Gene* 501, 39–48.

Schulze-Lefert, P., and Robatzek, S. (2006). Plant pathogens trick guard cells into opening the gates. *Cell* 126, 831–834.

Sherman-Broyles, S., Boggs, N. A., Farkas, A., Liu, P., Vrebalov, J., Nasrallah, M. E., et al. (2007). *S* locus genes and the evolution of self-fertility in *Arabidopsis thaliana. Plant Cell* 19, 94–106.

Shiu, S. H., and Bleecker, A. B. (2001). Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10763–10768.

Shiu, S. H., and Bleecker, A. B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis. Plant Physiol.* 132, 530–543.

Singh, P., Chien, C. C., Mishra, S., Tsai, C. H., and Zimmerli, L. (2013). The *Arabidopsis* LECTIN RECEPTOR KINASE-VI.2 is a functional protein kinase and is dispensable for basal resistance to *Botrytis cinerea. Plant Signal. Behav.* 8, e22611.

Singh, P., Kuo, Y. C., Mishra, S., Tsai, C. H., Chien, C. C., Chen, C. W., et al. (2012). The lectin receptor kinase-VI.2 is required for priming and positively regulates *Arabidopsis* pattern-triggered immunity. *Plant Cell* 24, 1256–1270.

Tsai, C. H., Singh, P., Chen, C. W., Thomas, J., Weber, J., Mauch-Mani, B., et al. (2011). Priming for enhanced defense responses by specific inhibition of the *Arabidopsis* response to coronatine. *Plant J.* 65, 469–479.

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465.

Vaid, N., Pandey, P. K., and Tuteja, N. (2012). Genome-wide analysis of lectin receptor-like kinase family from *Arabidopsis* and rice. *Plant Mol. Biol.* 80, 365–388.

Xin, Z. Y., Wang, A. Y., Yang, G. H., Gao, P., and Zheng, Z. L. (2009). The *Arabidopsis* A4 subfamily of lectin receptor kinases negatively regulates abscisic acid response in seed germination. *Plant Physiol.* 149, 434–444.

Zeng, W., Brutus, A., Kremer, J. M., Withers, J. C., Gao, X., Jones, A. D., et al. (2011). A genetic screen reveals *Arabidopsis* stomatal and/or apoplastic defenses against *Pseudomonas syringae* pv. *tomato* DC3000. *PLoS Pathog.* 7:e1002291. doi: 10.1371/journal.ppat.1002291

Zeng, W., and He, S. Y. (2010). A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv *tomato* DC3000 in *Arabidopsis. Plant Physiol.* 3, 1188–1198.

Zeng, W., Melotto, A. M., and He, S. Y. (2010). Plant stomata: a checkpoint of host immunity and pathogen virulence. *Curr. Opin. Biotechnol.* 21, 1–5.

Zhang, J., and Zhou, J. M. (2010). Plant immunity triggered by microbial molecular signatures. *Mol. Plant.* 3, 783–793.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* 12, 414–420.

Zipfel, C., and Robatzek, S. (2010). Pathogen-associated molecular pattern-triggered immunity: Veni, vidi...? *Plant Physiol.* 154, 551–554.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G., et al. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428, 764–767.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2013; accepted: 16 April 2013; published online: 07 May 2013.

Citation: Singh P and Zimmerli L (2013) Lectin receptor kinases in plant innate immunity. Front. Plant Sci. ***4****:124. doi: 10.3389/fpls.2013.00124*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Singh and Zimmerli. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 23 April 2013
doi: 10.3389/fpls.2013.00097

The xylem as battleground for plant hosts and vascular wilt pathogens

Koste A. Yadeta[1†] and Bart P. H. J. Thomma[1,2]*

[1] *Laboratory of Phytopathology, Wageningen University, Wageningen, Netherlands*
[2] *Centre for BioSystems Genomics, Wageningen, Netherlands*

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Sheng Yang He, Michigan State University, USA
Dale Ronald Walters, Scottish Agricultural College, Scotland

***Correspondence:**
Bart P. H. J. Thomma, Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, Netherlands.
e-mail: bart.thomma@wur.nl

[†]***Present address:***
Koste A. Yadeta, Department of Plant Pathology, University of California, Davis, 573 Hutchison Hall, Davis, CA, USA.

Vascular wilts are among the most destructive plant diseases that occur in annual crops as well as in woody perennials. These diseases are generally caused by soil-borne bacteria, fungi, and oomycetes that infect through the roots and enter the water-conducting xylem vessels where they proliferate and obstruct the transportation of water and minerals. As a consequence, leaves wilt and die, which may lead to impairment of the whole plant and eventually to death of the plant. Cultural, chemical, and biological measures to control this group of plant pathogens are generally ineffective, and the most effective control strategy is the use of genetic resistance. Owing to the fact that vascular wilt pathogens live deep in the interior of their host plants, studies into the biology of vascular pathogens are complicated. However, to design novel strategies to combat vascular wilt diseases, understanding the (molecular) biology of vascular pathogens and the molecular mechanisms underlying plant defense against these pathogens is crucial. In this review, we discuss the current knowledge on interactions of vascular wilt pathogens with their host plants, with emphasis on host defense responses against this group of pathogens.

Keywords: xylem, defense response, immunity, innate, pathogen, fungi, bacteria

INTRODUCTION

Plants are continuously confronted with potential pests and pathogens that include insects, nematodes, viruses, bacteria, fungi, and oomycetes. While many of these organisms evolved to infect aerial plant parts, such as leaves, stems, flowers, and fruits, others target below-ground organs, such as roots and tubers. Specific pathogens target the vascular system that is composed of xylem vessels, tracheary elements that transport water and minerals that are absorbed by the roots to the photosynthetic organs, and phloem elements, the living tissue that transports organic photosynthesis products. Paradoxically, although the phloem is rich in sugars, most vascular pathogens colonize the nutrient-poor xylem vessels. This may be explained by the accessibility of both types of vessel elements, as the phloem is characterized by living cells with a high osmotic pressure which makes penetration difficult, while the xylem is composed of dead tracheary elements with relatively low osmotic pressure. Consequently, phloem pathogens comprise rickettsias, spiroplasmas, and phytoplasmas that are introduced by vectors such as phloem feeding insects, or by cultural practices like grafting.

Xylem-invading pathogens comprise bacterial, fungal, and oomycete microorganisms that cause vascular wilt diseases. Vascular wilt pathogens are among the most destructive plant pathogens that can wipe out entire crops. Vascular wilt diseases occur worldwide and affect annual crops as well as woody perennials, thus not only impacting food and feed production, but also natural ecosystems. Most of the symptoms caused by vascular wilt pathogens develop in acropetal direction: from bottom to top. Epinasty is the primary disease symptom, followed by flaccidity, chlorosis, vascular browning, and necrosis of the terminal leaflets (Agrios, 2005). A large range of symptoms is caused by vascular wilt pathogens, and the same pathogen may cause different symptoms on different host plants. Depending on the pathogen species and the host, plants may become stunted, wilt partially or completely, and ultimately die. Plant death may occur within days to weeks or, in case of perennials, months to years (Purcell and Hopkins, 1996; Fradin and Thomma, 2006; Niño-Liu et al., 2006; Juzwik et al., 2008; Klosterman et al., 2009, 2011; Michielse and Rep, 2009; Genin, 2010; Janse and Obradovic, 2010; Harwood et al., 2011). Age, fitness, and the nutritional status of the host, environmental conditions, and virulence of the pathogen can all determine the speed and severity at which symptoms develop (Tjamos and Beckman, 1989; Hayward, 1991; Roncero et al., 2003; Niño-Liu et al., 2006; Chatterjee et al., 2008). In all cases where it is observed, wilting symptoms represent a transitory phase of the disease.

Vascular wilt pathogens generally overwinter in soil, plant debris, watercourses, or in insect vectors (Fradin and Thomma, 2006; Niño-Liu et al., 2006; Juzwik et al., 2008; Klosterman et al., 2009, 2011; Michielse and Rep, 2009; Genin, 2010; Janse and Obradovic, 2010; Nadarasah and Stavrinides, 2011). While most vascular wilt pathogens are soil-borne and enter their hosts through the roots by penetration via wounds or cracks that appear at the sites of lateral root formation (Vicente et al., 2001; Di Pietro et al., 2003; Fradin and Thomma, 2006; Klosterman et al., 2009; Michielse and Rep, 2009; Genin, 2010), some enter leaves via natural openings such as stomata and hydathodes, such as the bacterial leaf blight pathogen of rice, *Xanthomonas oryzae* (Niño-Liu et al., 2006). Furthermore, some vascular wilt pathogens are delivered directly into the xylem by insect vectors that feed on xylem sap,

such as *Xylella fastidiosa* bacteria that are transmitted by leafhoppers, or by chewing insects, such as *Ophiostoma* fungi that are transmitted by bark beetles (Purcell and Hopkins, 1996; Chatterjee et al., 2008; Moser et al., 2010; Nadarasah and Stavrinides, 2011). Regardless of the mechanism used by vascular wilt pathogens to enter their hosts, they subsequently colonize the xylem vessels where they proliferate (Tjamos and Beckman, 1989; Purcell and Hopkins, 1996; Agrios, 2005; Niño-Liu et al., 2006; Klosterman et al., 2009; Genin, 2010).

CONTROL OF VASCULAR WILT DISEASES

Controlling vascular wilt pathogens is difficult for several reasons. First of all, no efficient treatments exist to cure infected plants, and growers generally have to remove them from their crops. Secondly, many vascular wilt pathogens are soil-borne and produce persistent resting structures that are able to survive for long periods of time in the absence of host plants. Thirdly, some of these pathogens can infect a broad range of host plants and as a consequence, cultural control measures such as crop rotation are generally not very effective. Resting structures are desirable targets for control by soil solarization and chemical fumigation. However, limitations in large-scale applicability and ban on chemical fumigants because of public health and environmental issues render these approaches unsuitable. Biological agents and organic soil amendments are used to control vascular wilt diseases (Tsuda et al., 2001; Spadaro and Gullino, 2005; Suárez-Estrella et al., 2007; Ji et al., 2008; Markakis et al., 2008). For instance, injection of the Dutch trig, a bio-control vaccine that contains conidia of a non-pathogenic strain of the vascular wilt fungus *Verticillium albo-atrum* isolate, into elm trees is used to induce the natural defense against Dutch elm disease caused by the fungi *O. ulmi* and *O. novo-ulmi* (Scheffer et al., 2008). However, since biological agents are often affected by biotic and abiotic factors, performance of bio-control microorganisms in the field is often inconsistent (Tsuda et al., 2001).

The most effective strategy to control vascular wilt diseases thus far is the use of genetic resistance in host plants. Due to the fact that vascular wilt pathogens live deep in the interior of their host plants, studies into the biology of vascular pathogens is complicated. However, their high economic impact, combined with the absence of curative treatments, justifies increased attention. The recent availability of a number of genome sequences of vascular pathogens has inspired novel research efforts to unravel the molecular basis of vascular wilt diseases (**Table 1**). To design novel strategies to combat vascular wilt diseases, understanding the (molecular) biology of vascular pathogens and the molecular mechanisms underlying plant defense against these pathogens is crucial.

Table 1 | Publically available genome sequences of vascular wilt pathogens.

Organism	Species	Reference
Fungus	*Verticillium dahliae*	Klosterman et al. (2011)
	Verticillium albo-atrum	Klosterman et al. (2011)
	Fusarium oxysporum f. sp. *lycopersici*	Ma et al. (2010)
Bacterium	*Ralstonia solanacearum*	Salanoubat et al. (2002)
	Xanthomonas oryzae pv. *oryzae*	Lee et al. (2005)
	Xanthomonas campestris pv. *campestris*	Qian et al. (2005)
	Xylella fastidiosa	Simpson et al. (2000)
	Clavibacter michiganensis ssp. *michiganensis*	Gartemann et al. (2008)
	Erwinia amylovora	Sebaihia et al. (2010), Smits et al. (2010), Powney et al. (2011)
Oomycete	*Pythium ultimum*	Lévesque et al. (2010)

CAUSAL AGENTS OF VASCULAR WILT DISEASES

FUNGAL VASCULAR WILT PATHOGENS

There are four fungal genera containing the major vascular wilt pathogens: *Ceratocystis* (vascular wilts of oak, cocoa, and eucalyptus), *Ophiostoma* (vascular wilts of elm trees), *Verticillium* (broad host range), and *Fusarium* (broad host range; Tjamos and Beckman, 1989; Agrios, 2005; Juzwik et al., 2008; Schumann and D'Arcy, 2010; Harwood et al., 2011; López-Escudero and Mercado-Blanco, 2011). In contrast to the other three genera, the vast majority of *Fusarium* vascular wilt pathogens all belong to a single species, *F. oxysporum*, which contains morphologically indistinguishable pathogenic and non-pathogenic strains (Lievens et al., 2008). The pathogenic strains cause vascular wilts or root rot in over 100 different host species (Di Pietro et al., 2003; Roncero et al., 2003; Michielse and Rep, 2009). Despite the broad host range of these species, individual strains typically infect only a single or a few hosts and are assigned to *formae speciales* (Michielse and Rep, 2009). Interestingly, it was experimentally demonstrated that the transfer of two lineage specific (LS) chromosomes from a tomato pathogenic *F. oxysporum* f. sp. *lycopersici* strain to a non-pathogenic strain converted the latter into a tomato pathogen, suggesting that host specificity within *F. oxysporum* may be determined by pathogenicity chromosomes (Ma et al., 2010). Such pathogenicity chromosomes have not been identified in vascular wilt pathogens of the *Verticillium* genus for which genome sequences have recently been determined as well (Klosterman et al., 2011).

Most fungal vascular wilt pathogens overwinter as resting structures in the soil or on dead host tissues. These include microsclerotia, chlamydospores, thick-walled mycelium, and spore-bearing coremia that all can survive for an extended period of time without losing viability. Compounds released from host plants, referred to as exudates, trigger germination of these resting structures. Except for *Ophiostoma* spp. and the oak wilt pathogen *Ceratocystis fagacearum* that are transmitted by beetles (Hayslett et al., 2008; Juzwik et al., 2008; Harwood et al., 2011), fungal vascular wilt pathogens enter their host plants through the roots. Following penetration, the fungi colonize the cortical cells from where

hyphae migrate intercellularly toward the vascular parenchyma cells and invade the xylem vessels (Di Pietro et al., 2003; Klosterman et al., 2009; Schumann and D'Arcy, 2010; Nadarasah and Stavrinides, 2011). In the xylem, conidiospores are produced which are disseminated acropetally with xylem sap movement. Fungal vascular wilt pathogens are mostly restricted to xylem vessels, but once host tissues become necrotized also these are colonized and the fungus starts to produce resting structures which are released into the soil eventually (Di Pietro et al., 2003; Agrios, 2005; Fradin and Thomma, 2006).

BACTERIAL VASCULAR WILT PATHOGENS

Seven bacterial genera contain vascular wilt pathogens: *Clavibacter* (causing ring rot of potato and bacterial canker and wilt of tomato), *Curtobacterium* (bacterial wilt of beans), *Erwinia* (bacterial wilt of cucurbits), *Pantoea* (stewart's wilt of corn), *Ralstonia* (southern bacterial wilt of Solanaceous crops and Moko disease of banana), *Xanthomonas* (black rot of crucifers, bacterial blight of rice), and *Xylella* (Pierce's disease of grape, citrus variegation chlorosis; Tjamos and Beckman, 1989; Agrios, 2005; Chatterjee et al., 2008; Schumann and D'Arcy, 2010; Nadarasah and Stavrinides, 2011; Roper, 2011). Bacterial vascular wilt pathogens overwinter in plant debris in soil, in seeds, in vegetative propagules, or in their insect vectors as dormant cells (Agrios, 2005). They enter host tissues only passively, via wounds, cracks, or natural openings such as stomata and hydathodes, while others are directly delivered into the xylem by insect vectors, such as *Xylella fastidiosa* by sharpshooter leafhoppers and spittlebugs, *Pantoea stewartii* by corn flea beetles and *E. tracheiphila* by cucumber beetles (Schumann and D'Arcy, 2010; Nadarasah and Stavrinides, 2011; Roper, 2011). After entrance, they rapidly multiply and invade the root cortex and vascular parenchyma intercellularly, from where they spread to the xylem vessels that are used as avenues for passive spread to aerial plant parts. During colonization, bacterial wilt pathogens degrade xylem cell wall components, parenchyma cells, and pit membranes, resulting in slimy masses of bacteria and cellular debris (Agrios, 2005; Schumann and D'Arcy, 2010).

OOMYCETE VASCULAR WILT PATHOGENS

Only one oomycete genus, *Pythium*, contains vascular wilt pathogens. *Pythium* mainly infects seeds or seedlings in the soil, causing pre-emergence or post-emergence seedling damping-off disease, and young and juvenile plant tissues (Martin and Loper, 1999; Oliver et al., 2009). The genus *Pythium* comprises many complex species, most of which are plant pathogens, while others are saprophytes or animal parasites (Martin and Loper, 1999). *Pythium* species survive in soil or in organic substrates for long periods of time as dormant oospores; thick-walled sexual spores that can withstand harsh environmental conditions (Martin and Loper, 1999). Oospores germinate upon stimulation by exudates released from plants, and often produce a sporangium containing zoospores that are released and encysted after host contact. Alternatively, oospores produce germinating hyphae to penetrate the root epidermis, migrate through the cortex, endodermis, and parenchyma cells, and eventually invade the vascular stele causing typical damping-off symptoms (Rey et al., 1998).

XYLEM STRUCTURE AND DEVELOPMENT

The xylem consists of distinct cells with special wall structures that allow efficient transport of water and solutes from the roots to upper plant parts. The xylem functions not only in long distance transport, but also provides physical strength to the plant. Xylem development occurs in two phases during which primary and secondary xylem is produced (Fukuda, 1997, 2004; Ye, 2002; Zhang et al., 2011). Primary development involves the formation of primary xylem from procambium cells which are derived from the apical meristem. Procambium cells give rise to xylem precursor cells that eventually differentiate into treachery elements, xylem parenchyma cells or fiber cells; collectively called the xylem (Ye, 2002; Fukuda, 2004). Treachery elements, which consist of tracheid and vessel elements, are the main conductive tissues. While the xylem parenchyma cells are metabolically active and adapted for storage and transport, the xylem fiber cells together with treachery elements provide physical support (Nieminen et al., 2004). Following xylem differentiation, the treachery elements undergo cell elongation before the initiation of secondary xylem wall development (Ye, 2002; Fukuda, 2004; Nieminen et al., 2004; Zhang et al., 2011). The secondary xylem walls, which are derived from vascular cambium, are deposited onto the primary xylem walls (Fukuda, 1997; De Boer and Volkov, 2003). Secondary xylem is made of cellulose microfibrils, crystalline aggregates of linear polymers of D-glucopyranosyl residues linked in β-(1-4) conformation (Brett, 2000; Emons and Mulder, 2000). The secondary xylem walls are further impregnated with different polysaccharides, such as lignin, hemicellulose, pectin, and structural proteins that add strength and rigidity to the wall (Ye, 2002; Fukuda, 2004; Yokoyama and Nishitani, 2006). Subsequently, the secondary xylem walls are lignified, cross-linked, and eventually waterproofed by polymerization of the aromatic compound monolignol (Fukuda, 1996; De Boer and Volkov, 2003). The patterned secondary xylem walls provide physical strength to the treachery elements to withstand the negative pressure generated during transpiration and by the compressive pressure from surrounding cells (Ye, 2002; Nieminen et al., 2004; Choat and Pittermann, 2009; Zhang et al., 2011).

The final step of xylem development is the induction of programed cell death (PCD) that destroys the cellular contents of treachery elements, leaving behind hollow tube-like vessels through which water and nutrients flow (Fukuda, 1997; Zhang et al., 2011). The PCD is developmentally regulated and is strongly associated with secondary xylem wall formation (Fukuda, 2004). The vessel tubes are dedicated to the unrestricted water and solute movement throughout the plant and individual vessels are interconnected through small openings called pits (De Boer and Volkov, 2003; Choat and Pittermann, 2009). Pits between vessels typically have overarching secondary walls that form a bowl-shaped chamber, referred to as a border pit (De Boer and Volkov, 2003; Jansen et al., 2004). Border pit exists in pairs and contain a pit membrane at the center, which is formed from primary walls and the intervening middle lamella (De Boer and Volkov, 2003). The pit membrane is made of cellulose microfibrils embedded in polysaccharide matrix of hemicellulose and pectin (Tyree and Zimmermann, 2002; Pérez-Donoso et al., 2010). This fine mesh-like and tightly interlocked polysaccharide structure has minute

openings through which water and solutes can move with a minimal resistance between vessels or to neighboring parenchyma cells (Choat and Pittermann, 2009). In angiosperm trees, the pit pore diameter varies between 5 and 20 nm (Choat et al., 2003, 2004), thus acting as a safety mechanism to limit the spread of embolism within xylem vessels (Tyree and Zimmermann, 2002; De Boer and Volkov, 2003; Choat et al., 2008; Pérez-Donoso et al., 2010).

All vascular wilt pathogens have to breach the highly structured and rigid secondary xylem walls to enter the vessels. Also the pit membranes are a major barriers for vascular pathogens as vascular wilt pathogens are too large to pass pit membrane pores (Choat et al., 2003, 2004). For example, the rod-shaped bacterium *Xylella fastidiosa* has a cell size of 0.25–0.5 μm in diameter (Mollenhauer and Hopkins, 1974), while the conidia of *Verticillium* species have a diameter of about 2.2 μm (Qin et al., 2008).

THE XYLEM AS A NICHE FOR VASCULAR WILT PATHOGENS

The xylem is a nutritionally poor environment, which could be an important reason why only a limited number of plant pathogens are able to thrive in this environment. Possibly, vascular wilt pathogens exploit this niche to avoid competition with other microbes (McCully, 2001). However, as they reside in the xylem for the major part of their lifecycle, vascular pathogens need to be able to obtain all factors required for growth, reproduction, and survival.

NUTRIENT COMPOSITION OF XYLEM SAP

Nitrate, sulfate, and phosphate are among the most abundant inorganic anions in the xylem sap, whereas calcium, potassium, magnesium, and manganese are the most predominate inorganic cations present in the xylem sap of oilseed rape (Nakamura et al., 2008). Although only in relatively low amounts, xylem sap also contains various carbohydrates, such as glucose, fructose, saccharose, maltose, raffinose, trehalose, and ribose (Alvarez et al., 2008; Nakamura et al., 2008; Fernandez-Garcia et al., 2011; Krishnan et al., 2011). Of these, glucose, fructose, and saccharose are predominant and are utilized as a carbon source for growth. Xylem sap furthermore contains various proteins, amino acids, and organic acids, which can also act as source of organic and inorganic nutrients (Alvarez et al., 2008; Nakamura et al., 2008; Fernandez-Garcia et al., 2011; Krishnan et al., 2011). For instance, the sulfur-containing amino acids methionine and cysteine can be used as a source of inorganic sulfur (Divon and Fluhr, 2007; Krishnan et al., 2011). Nevertheless, the quantities of the organic and inorganic compounds in the xylem sap are extremely low and fluctuate with day time, growth condition, and plant species (Siebrecht et al., 2003).

NUTRIENT ACQUISITION BY VASCULAR WILT PATHOGENS

Vascular wilt pathogens satisfy their nutritional requirements by efficiently acquiring the scarce nutrients available in the xylem sap, by enzymatic digestion of host cell walls, by invading neighboring cells, or by inducing nutrient leakage from surrounding tissues (Divon et al., 2005; Möbius and Hertweck, 2009; Klosterman et al., 2011).

Nitrogen is one of the limiting nutrients in the xylem sap for vascular wilt pathogens (Divon et al., 2005). The preferred primary nitrogen sources for fungal pathogens, ammonia, glutamine, and glutamate (Marzluf, 1997; Divon et al., 2006), are scarce in xylem sap. In absence of primary nitrogen sources, fungi can utilize secondary nitrogen sources such as nitrate, nitrite, purines, amides, amino acids, and proteins (Marzluf, 1997; Divon et al., 2005, 2006). GATA transcription factors, such as the *F. oxysporum* f. sp. *lycopersici* global nitrogen regulator (*FNR1*), are known to regulate utilization of secondary nitrogen sources (Marzluf, 1997; Divon and Fluhr, 2007; Bolton and Thomma, 2008; Donofrio et al., 2009). *FNR1* mutants grow normally on primary nitrogen sources, but fail to utilize secondary nitrogen sources such as amino acids, hypoxanthine, and uric acid (Divon et al., 2006). Disruption of *FNR1* not only affected virulence of *F. oxysporum* f. sp. *lycopersici* on tomato, but also regulation of three nitrogen acquisition genes, *Gap1*, *Mtd1*, and *Uricase* during growth *in planta*, suggesting that *FNR1* regulates the utilization of secondary nitrogen sources *in planta* (Divon et al., 2006).

Analysis of the whole genome sequences of *F. oxysporum* f. sp. *lycopersici*, *V. dahliae*, and *V. albo-atrum* showed that these genomes are enriched in genes that encode cell wall-degrading enzymes (CWDEs) that may be used for the enzymatic digestion of xylem walls and pit membranes (Ma et al., 2010; Klosterman et al., 2011). Also other vascular wilt pathogens are known as CWDE producers (Di Pietro et al., 2003; Jha et al., 2005; Sun et al., 2005; Fradin and Thomma, 2006; Michielse and Rep, 2009; Klosterman et al., 2011). While degrading cell wall components, these enzymes liberate sugars that may be used as nutrient sources.

Various vascular wilt pathogens produce high- and low-molecular weight phytotoxins during host colonization that have often been associated with wilt symptom development (Temple and Horgen, 2000; Wang et al., 2004; Palmer et al., 2005; Stipanovic et al., 2011; Zhou et al., 2012; Santhanam et al., 2013). As several phytotoxins disturb plant cell membrane integrity (Möbius and Hertweck, 2009), leakage of nutrients may occur from cells surrounding the xylem vessels that can be utilized by vascular wilt pathogens. For instance, two *Verticillium* necrosis- and ethylene inducing-like proteins, NLP1 and NLP2, were shown to display cytotoxic activity and differentially contribute to virulence on various host plant species, although the mechanism through which these NLPs contribute to virulence remains unclear (Zhou et al., 2012; Santhanam et al., 2013). Interestingly, compared with other ascomycete plant pathogens that typically contain up to three *NLP* genes, the *NLP* gene family is expanded in the *V. dahliae* genome (Klosterman et al., 2011; Santhanam et al., 2013). A similar expansion has been reported for the *F. oxysporum* genome, and it has been speculated that this expansion has contributed to their broad host range among dicotyledonous plant hosts (Ma et al., 2010; Klosterman et al., 2011). However, in addition to NLP1 and NLP2, none of the other *V. dahliae* NLPs were found to display cytotoxic activity, and their potential role in fungal virulence still remains enigmatic (Zhou et al., 2012; Santhanam et al., 2013).

Finally, although most vascular wilt pathogens are confined to xylem vessels, some of them degrade xylem vessel walls to colonize adjacent parenchyma cells (Agrios, 2005). These pathogens may obtain nutrition by parasitizing parenchyma cells.

PLANT DEFENSE AGAINST VASCULAR WILT PATHOGENS

Plants deploy two types of defenses against invading pathogens: pre-existing and inducible plant defense responses. The pre-existing defenses are constitutive and provide physical and chemical barriers against attempted host penetration. Once successful pathogens breach pre-existing defenses, they encounter a spectrum of inducible defense responses with microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI) as two extreme ends (Jones and Dangl, 2006; Dodds and Rathjen, 2010). While MTI is activated upon recognition of conserved MAMPs, ETI is activated upon recognition of secreted effector proteins (**Figure 1**).

PERCEPTION OF VASCULAR WILT PATHOGENS

In general, plants sense invading pathogens by using two types of receptors: extra- and intracellular receptors (**Figure 1**). While extracellular receptors recognize pathogen molecules on the cellular surface as well as damage-associated host molecules that are released as a consequence of pathogen activity, intracellular receptors recognize pathogen molecules that are delivered inside host cells (**Figure 1**). This extra- and intracellular receptor-mediated recognition of pathogen molecules [microbe-associated molecular patterns (MAMPs)/pathogen-associated molecular patterns (PAMPs), effectors] leads to the activation of plant innate immunity that wards off invading pathogens. Consequently, failure of a host plant to perceive invading pathogens leads to susceptibility and successful pathogen infections (**Figure 2**).

Extracellular plant receptors

Several MAMP receptors have been characterized, including *Arabidopsis* FLS2 (flagellin-sensitive 2), EFR (elongation factor Tu receptor), and CERK1 (chitin elicitor receptor kinase 1), and rice CEBiP (chitin elicitor binding protein). While FLS2 and EFR encode receptor-like kinases (RLKs) that recognize the bacterial MAMPs flg22 and EF-Tu, respectively (Gómez-Gómez et al., 2001; Kunze et al., 2004; Zipfel et al., 2006), CERK1 and CEBiP encode LysM domain-containing receptors that recognize chitin, the main constituent of fungal cell walls (Gómez-Gómez et al., 2001; Kunze et al., 2004; Zipfel et al., 2006). These MAMP receptors are considered to display a low degree of specificity and broadly act in pathogen defense.

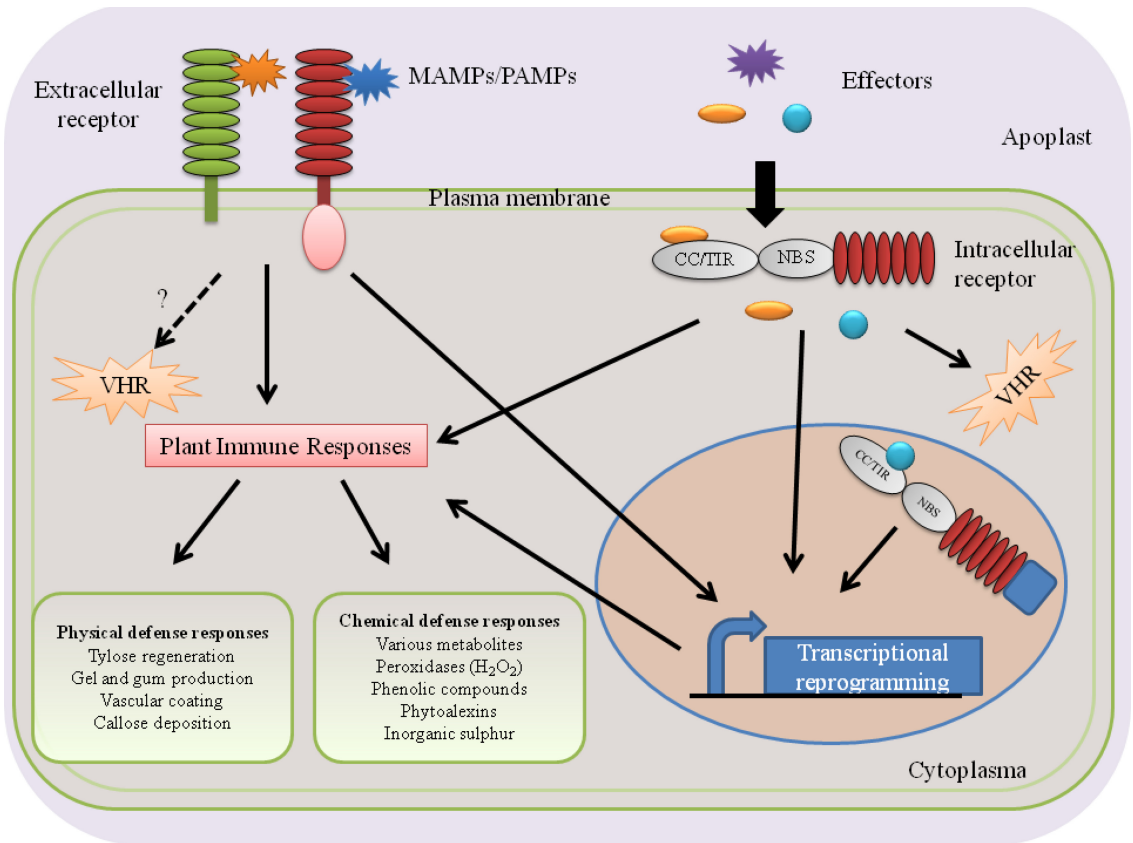


FIGURE 1 | Perception of vascular wilt pathogens and activation of subsequent plant immune responses. Plants perceive PAMPs/MAMPs or effector proteins of vascular wilt pathogens using extracellular or intracellular receptors and activate immune responses in the xylem. The tomato receptor-like protein Ve1 and the rice receptor-like kinase Xa21 are examples of extracellular receptors that recognize *Verticillium* Ave1 and *Xanthomonas oryzae* pv. *oryzae* Xa21, respectively. Tomato I-2 and *Arabidopsis* RRS1-R are examples of intracellular NB–LRR-type receptors that perceive the *F. oxysporum* f. sp. *lycopersici* Avr2 effector and the R. solanacearum effector PopP2, respectively. Presumably, these processes take place in the parenchyma cells surrounding the xylem vessels.

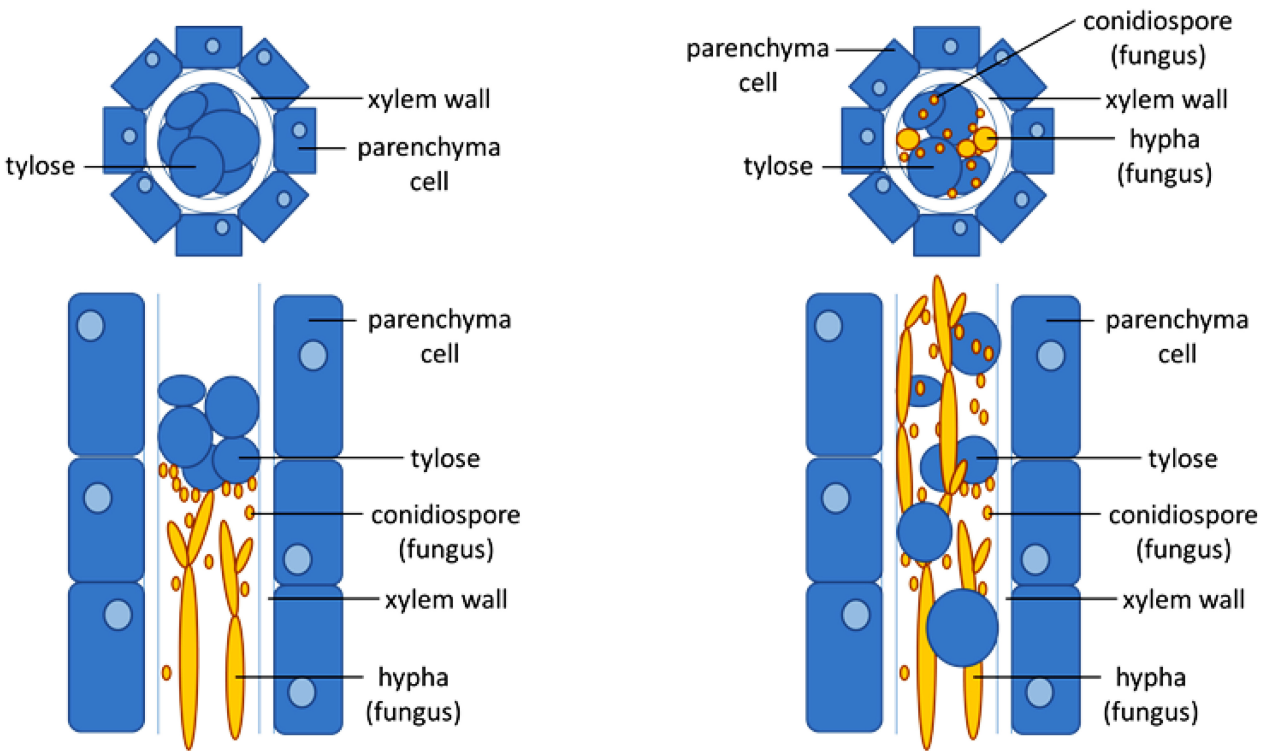


FIGURE 2 | Xylem occlusion limits pathogen growth in resistant plants. Schematic drawing of cross-sections (top) or longitudinal sections (bottom) of a fungal-infected xylem vessel of a resistant (left) and a susceptible (right) plant. In the resistant plant, timely induction of the formation of tyloses, bubble-like outgrowth of the parenchyma contact cells surrounding the xylem vessels that protrude into the lumen of the vessel, are able to trap the fungus after which elimination can occur. As long as the number of vessels that is closed by the tyloses is limited, the host plant will not suffer from droughts stress. In the susceptible plant, tylose formation cannot trap the pathogen which is able to spread and further colonize the xylem.

Extracellular plant receptors that play a role in plant defense against specific vascular wilt pathogens have also been described. Rice Xa21 confers resistance against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*; Song et al., 1995). Xa21 recognizes Ax21 (activator of Xa21), a type I-secreted sulfated protein (Song et al., 1995; Lee et al., 2009). Similar to *FLS2* and *EFR*, *Xa21* encodes a RLK (Song et al., 1995; Park et al., 2010b). Xa21 physically interacts with XB24, a protein with a C-terminal ATP synthase (ATPase) motif (Chen et al., 2010). XB24 promotes autophosphorylation of Ser/Thr residues on Xa21 through its ATPase activity, keeping Xa21 in an inactive state (Chen et al., 2010; Chen and Ronald, 2011). Upon Xa21-mediated Ax21 recognition, the Xa21 kinase becomes activated, triggering rice defense responses (Chen et al., 2010; Park et al., 2010b). XB25, plant-specific ankyrin-repeat family protein (Jiang et al., 2012), and BiP3, an endoplasmic reticulum chaperone protein (Park et al., 2010a), are also reported to be involved in Xa21-mediated rice immunity against *Xoo*.

Tomato Ve1 is another example of extracellular plant receptor that plays a role in xylem defense. Ve1 is an extracellular leucine-rich repeat (LRR) receptor-like protein (RLP; Kawchuk et al., 2001; Wang et al., 2008) that provides resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2009, 2011). Interestingly, Fradin et al. (2011) have recently shown that interfamily transfer of *Ve1* gene to *Arabidopsis* confers resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum*. Recently, the pathogen ligand that is perceived by Ve1 was identified as the Ave1 effector, a small (134 aa) effector protein with four cysteines that is required for full virulence on tomato plants lacking Ve1 (De Jonge et al., 2012). Ave1 is homologous to a widespread family of plant natriuretic peptides, mobile signaling molecules that play a role in the regulation of water and ion homeostasis, and it was suggested that *Ave1* was acquired by *Verticillium* through horizontal gene transfer from plants (De Jonge et al., 2012). Ave1 homologs are found in a few other plant pathogens, including the vascular wilt fungus *F. oxysporum* f. sp. *lycopersici*, but a role in virulence has not yet been demonstrated for these homologs (De Jonge et al., 2012). Intriguingly, the Ave1 homolog from *F. oxysporum* f. sp. *lycopersici* is recognized by Ve1 upon transient co-expression in tobacco, and Ve1 was found to mediate resistance toward *F. oxysporum* f. sp. *lycopersici* in tomato (De Jonge et al., 2012).

Recently, Zhang et al. (2013) have reported the presence of a functional *Ve1* ortholog in the tobacco species *Nicotiana glutinosa*, as *Ave1* expression in *N. glutinosa* causes an hypersensitive response (HR), rapid and localized cell death of plant tissue surrounding the site where recognition of pathogen effectors by host immune receptors occurs. Furthermore, *N. glutinosa* shows resistance against race 1 *V. dahliae* that is compromised upon inoculation with an *Ave1* deletion mutant of a race 1 *V. dahliae* isolate (Zhang et al., 2013).

Intracellular plant receptors

Also intracellular plant receptors that mediate plant defense against xylem-invading pathogens have been characterized. The tomato *I-2* gene is an intracellular receptor that contributes to resistance against race 2 isolates of *F. oxysporum* f. sp. *lycopersici* (Huang and Lindhout, 1997; Takken and Rep, 2010). It encodes a cytoplasmic CC (coiled-coil)–NBS (nucleotide-binding site)–LRR receptor protein that recognizes the effector protein Avr2, which was initially identified from the xylem sap of tomato infected by *F. oxysporum* f. sp. *lycopersici* and is taken up by tomato cells (Houterman et al., 2007, 2009; Takken and Rep, 2010).

The *Arabidopsis* RRS1-R resistance protein is an intracellular plant receptor that confers resistance against *R. solanacearum*. RRS1-R encodes a TIR (Toll/interleukin1 receptor)–NBS–LRR R-protein and contains a C-terminal nuclear localization signal (NLS) and a WRKY domain (Deslandes et al., 2002). It recognizes the *R. solanacearum* type III-secreted effector protein PopP2 (Deslandes et al., 2003). RRS1-R physically interacts with the effector PopP2 (Deslandes et al., 2003). RRS1-R requires RD19, a cysteine protease that also binds to PopP2 (Deslandes et al., 2003; Bernoux et al., 2008). RD19 is localized in the vacuole in absence of PopP2 and re-localizes to the nucleus in the presence of PopP2 (Deslandes et al., 2003; Bernoux et al., 2008). However, no direct interaction between RRS1-R and RD19 has been reported so far. Thus, the current notion is that RRS1-R potentially recognizes the RD19/PopP2 complex in the nucleus and activates the *Arabidopsis* ETI against *R. solanacearum*.

PLANT DEFENSE RESPONSES IN THE XYLEM

Comparison of the transcriptional changes in tomato induced by *Cladosporium fulvum*, a fungal foliar pathogen that causes leaf mold of tomato, with those induced by the vascular wilt pathogen *V. dahliae* revealed that the *Cladosporium fulvum*-induced transcriptional changes showed little overlap with those induced by *V. dahliae* (van Esse et al., 2009). Moreover, within the subset of genes that are regulated by both pathogens, many genes showed inverse regulation (van Esse et al., 2009).

Recognition of vascular wilt pathogens mediated by either extracellular or intracellular receptors leads to the activation of defense responses in the xylem vessels. These comprise physical defense responses which halt or contain the pathogen from further spread in the xylem vessels, and chemical defense responses that kill the pathogen or inhibit its growth (**Figure 2**).

A common defense mechanism in xylem vessels against vascular wilt pathogens is the formation of tyloses (Beckman, 1964; Talboys, 1972; Rahman et al., 1999; Fradin and Thomma, 2006). Tyloses are outgrowths of vessel-associated parenchyma cells which protrude into the xylem vessel through pits and block the spread of pathogens (Beckman, 1964; Talboys, 1972; Grimault et al., 1994; Agrios, 2005). They are formed during both compatible and incompatible interactions between the host and vascular wilt pathogens, although the time and extent of tylose formation significantly differs (**Figure 2**). Tyloses form much faster and more extensively in resistant plants when compared to susceptible plants (Grimault et al., 1994; Fradin and Thomma, 2006).

Often, the generation of tyloses is associated with the production of gels and gums around the differentiated tylose (Clérivet et al., 2000). Using immuno-gold labeling, strong accumulation of pectin-rich materials around the parenchyma cells, pit membrane, and the newly emerging tylose was observed in the xylem vessels of *Platanus acerifolia* cultivar infected by *Ceratocystis fimbriata* f. sp. *platani* (Clérivet et al., 2000). Plants potentially accumulate these pectin-rich gels and gums around tyloses to completely seal off a xylem vessel to prevent the vascular wilt pathogen from spread to adjacent xylem vessels (Rahman et al., 1999). However, complete sealing of xylem vessels can be disadvantageous for the plant as well. If tylose formation affects too many vessels and no new vessels are formed, tylose formation can result in drought stress (Fradin and Thomma, 2006).

Another typical symptom of *Verticillium* infection is vein clearing. Based on infections of *V. longisporum* on *Arabidopsis* and *Brassica napus*, it was recently reported that this vein clearing is caused by *Verticillium*-induced transdifferentiation of chloroplast-containing bundle sheath cells to functional xylem elements (Reusche et al., 2012). In addition, it was shown that infected *Arabidopsis* wild-type plants display enhanced drought stress tolerance compared with non-infected plants, suggesting that *Verticillium* infection activates a tissue-specific developmental program that compensates for compromised water transport (Reusche et al., 2012).

Another physical defense response observed during xylem colonization is vascular coating. A quick vascular wall coating around the initially infected and the adjacent xylem vessels, infusing the pit membrane and primary walls was observed in resistant chili pepper inoculated with *R. solanacearum*, whereas the xylem wall coating was not observed in susceptible chili pepper (Rahman et al., 1999). Similar coating of xylem parenchyma cells induced by *V. albo-atrum* was reported in tomato (Street et al., 1986) and alfalfa (Newcombe and Robb, 1988), indicating that infusion of pit membranes, primary walls and parenchyma cells with coating materials could prevent lateral and vertical spreading of vascular wilt pathogens in the xylem vessels. Furthermore, callose deposition and swelling of the primary walls of the xylem vessels was reported during the interaction of *R. solanacearum* with chili pepper (Rahman et al., 1999). Previously, a similar deposition of callose in resistant and susceptible tomato infected with *F. oxysporum* f. sp. *lycopersici* was reported (Beckman et al., 1982). However, the resistant cultivar maintains a stronger level of callose deposition during the course of the infection than the susceptible cultivar (Beckman et al., 1982). This high level deposition of callose in the resistant cultivar around the initially infected cells could inhibit pathogens from further spread.

Xylem colonization by *Xanthomonas campestris* pv. *campestris* has been reported to activate vascular immunity that triggers an HR, referred to as vascular HR (Xu et al., 2008). Vascular immunity was proposed based on the fact that $AvrAC_{Xcc8004}$ (also referred to as XopAC), a type III effector protein of *Xanthomonas campestris* pv. *campestris* that confers avirulence in *Arabidopsis* ecotype Col-0, provides resistance when exclusively targeted to the vascular system (Xu et al., 2008). Infiltration of $AvrAC_{Xcc8004}$ into leaf mesophyll tissue of Col-0 did not trigger resistance against *Xanthomonas*, implying that $AvrAC_{Xcc8004}$-mediated activation of defense (vascular immunity) occurs in the xylem. Castañeda et al. (2005) previously reported that the *Xanthomonas campestris*

pv. *campestris* effector protein AvrXccFM elicits vascular HR on Florida mustard seedlings. It is, however, important to note that unlike the HR occurring in leaf mesophyll cells, vascular HR is difficult to score (Castañeda et al., 2005; Xu et al., 2008).

WAT1 (*Walls Are Thin1*), which is involved in secondary cell wall deposition, is also implicated in vascular immunity (Denancé et al., 2012). *WAT1* mutant *Arabidopsis* plants are resistant to bacterial and fungal vascular wilt pathogens but not to foliar pathogens (Denancé et al., 2012). In leaf inoculation assays, *wat1* provides resistance to *R. solanacearum* and *Xanthomonas campestris* pv. *campestris* only when directly injected into the vascular system, but not when injected into mesophyll tissues (Denancé et al., 2012), demonstrating the tissue-specific immune response. Likely, *wat1* resistance involves root-localized metabolic channeling away from indole metabolites to salicylic acid (Denancé et al., 2012).

Arabidopsis AHL19, an AT-hook DNA binding protein, provides enhanced resistance to three plant pathogenic *Verticillium* spp. but not to the foliar pathogens *Botrytis cinerea*, *Plectosphaerella cucumerina*, and *Alternaria brassicicola* and enhanced susceptibility to *Pseudomonas syringae* pv. *tomato*, suggesting a role as positive regulator of xylem-specific plant immunity (Yadeta et al., 2011).

Xylem infection causes drastic metabolic changes in xylem parenchyma cells, which are located adjacent to the infected vessels. These metabolic changes lead to the accumulation of different proteins and secondary metabolites in the xylem sap. Some of the proteins and secondary metabolites that accumulate in the xylem sap during xylem colonization include PR-1, PR-2, PR-3, PR-4, PR-5, peroxidases, proteases, xyloglucan-endotransglycosylase (XET), and xyloglucan-specific endoglucanase inhibitor protein (XEGIP), phenols, phytoalexins, and lignin-like compounds (Cooper et al., 1996; Hilaire et al., 2001; Rep et al., 2002, 2003; Williams et al., 2002; Houterman et al., 2007; Basha et al., 2010; Gayoso et al., 2010). These compounds are known to contribute directly or indirectly to plant defense. The PR-1, PR-2, PR-3, and PR-5 were also among the proteins abundantly accumulated in xylem sap during compatible interaction between *Fusarium* and tomato (Rep et al., 2002; Houterman et al., 2007). For instance, PR-2 (β-1, 3-glucanase) and PR-3 (chitinase) hydrolyze the fungal cell wall component β-1,3-glucan and chitin, respectively (Leubner-Metzger and Meins, 1999; van Loon et al., 2006). In addition, antimicrobial activity of PR-5 proteins has also been demonstrated toward multiple pathogens (van Loon et al., 2006), implying that the presence of these proteins in xylem sap could inhibit or slow down the growth of the fungal vascular wilt pathogens in the xylem vessels.

Peroxidases are among the abundantly accumulated enzymes in xylem sap during host colonization of vascular wilt pathogens. The cationic peroxidase, PO-C1, accumulates in the cytoplasm, the primary and secondary walls of the xylem parenchyma, and lumen cells during incompatible interactions between *Xoo* and rice (Hilaire et al., 2001). Peroxidases are heme-containing enzymes that catalyze the oxidation of different substrates using hydrogen peroxides as an electron acceptor (Gayoso et al., 2010). Peroxidases are known to be involved in the production of reactive oxygen species through their enzymatic activity and reactive oxygen species are toxic compounds that can eliminate vascular wilt pathogens. Furthermore, peroxidases are implicated in the polymerization of cell wall compounds, lignin and suberin biosynthesis, and regulation of hydrogen peroxide levels, which all can contribute to defense (Hilaire et al., 2001; Passardi et al., 2005).

Plants accumulate different phenolic compounds in the xylem in response to infection. Olive trees accumulate phenols such as rutin, oleuropein, luteolin-7-glucoside, and tyrosol at the site of *V. dahliae* infection that were shown to have a toxic effect on *V. dahliae* (Báidez et al., 2007). Interestingly, exogenous treatment of Dutch elm trees with phenolic compounds induces accumulation of suberin-like compounds in the xylem tissue and thereby increases resistance to *O. novo-ulmi* (Martín et al., 2008). This indicates that, in addition to direct toxicity, phenolic compounds could also activate other defense responses against vascular wilt pathogens.

Plants employ not only complex organic phytoalexins as defense mechanism against vascular wilt pathogens, but also employ inorganic compounds such as elemental sulfur and sulfur-containing inorganic compounds (Williams et al., 2002; Cooper and Williams, 2004). During an incompatible interaction between *V. dahliae* and tomato elemental sulfur mainly accumulates in xylem parenchyma cells, xylem vessel walls, and around the vascular occluding gels (Williams et al., 2002). Similar accumulation of elemental sulfur has been observed in an incompatible interaction between *V. dahliae* and cacao (*Theobroma cacao*) or cotton (Cooper et al., 1996; Cooper and Williams, 2004). The accumulation of inorganic sulfur specifically in xylem vessel walls and around the vascular occluding gels might suggest its role in eliminating vascular wilt pathogens that are arrested by physical defense responses.

Overall, chemical defense responses play major roles in xylem defense. Some chemical compounds accumulated in xylem sap after infection modulate the morphology of xylem tissue and by doing so inhibit vertical and lateral colonization of the pathogens, whereas other compounds accumulate during xylem infection have antimicrobial activity and can eliminate vascular wilt pathogens contained by the physical defense responses.

CONCLUSION

Vascular wilt pathogens have adapted to thrive in the xylem, which is known as a nutrient-poor niche, causing vascular wilt diseases on hundreds of plant species. Recognition of vascular wilt pathogens by both extra- and intracellular plant receptors triggers plant innate immune responses that comprise physical and chemical defenses. Both types of defense responses occur in the xylem vessels in a coordinated manner, where physical defense responses mainly prevent the pathogens from spreading in the xylem vessels and chemical defense responses kill the pathogen or inhibit its growth.

Currently, little is known about the interaction between vascular wilt pathogens and their hosts. As this interaction takes place in xylem vessels which are located deep in the plant interior, the molecular basis underlying the interaction between vascular wilt pathogens and their hosts remains largely obscure. Genetic resistance is the best strategy for controlling vascular wilt pathogens. To develop genetic resistance, however, a deeper

understanding of the host defense mechanisms as well as the biology, evolution and pathogenicity of vascular wilt pathogens is required.

VASCULAR WILT PATHOGENS INDUCE DROUGHT STRESS

Wilting of plant parts as a consequence of xylem dysfunction is the most conspicuous symptom of vascular wilt disease. Daugherty et al. (2010) have nicely demonstrated using stable carbon isotope labeling that *Xylella fastidiosa* induces drought stress in alfalfa. Many factors can contribute to xylem occlusion, such as high- and low-molecular weight polysaccharides secreted by vascular wilt pathogens during xylem colonization and the presence of pathogen biomass (bacterial cells and fungal and oomycete mycelium and spores) in the xylem vessels. However, also plant defense responses can contribute to xylem occlusion, such as tyloses that are formed by the parenchyma cells and gum and gels that are secreted (Fradin and Thomma, 2006; Klosterman et al., 2009; Beattie, 2011). Embolism (the formation of air bubbles) in xylem vessels is another factor that can reduce the hydraulic conductivity of the xylem. Pérez-Donoso et al. (2007) have demonstrated using magnetic resonance imaging that *Xylella fastidiosa*-infected grape displayed early occurrence of embolism, which correlated with decreased xylem conductivity and drought stress. Although several research reports identify a correlation between xylem infection and drought stress, a recent report revealed enhanced drought tolerance in *Arabidopsis* upon *Verticillium* infection (Reusche et al., 2012). *Arabidopsis* plants infected with *V. longisporum* exhibited increased de novo xylem formation with newly transdifferentiated xylem vessels that were able to compensate for the occluded ones. Consequently, the plants showed higher drought stress tolerance when compared with non-infected plants.

REFERENCES

Agrios, G. N. (2005). *Plant Pathology*. Burlington, MA: Elsevier Academic Press.

Alvarez, S., Marsh, E. L., Schroeder, S. G., and Schachtman, D. P. (2008). Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant Cell Environ.* 31, 325–340.

Báidez, A. G., Gómez, P., Del Río, J. A., and Ortuño, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. role of phenolic compounds in plant defense mechanism. *J. Agric. Food Chem.* 55, 3373–3377.

Basha, S., Mazhar, H., and Vasanthaiah, H. (2010). Proteomics approach to identify unique xylem sap proteins in Pierce's disease-tolerant *Vitis* species. *Appl. Biochem. Biotechnol.* 160, 932–944.

Beattie, G. A. (2011). Water relations in the interaction of foliar bacterial pathogens with plants. *Annu. Rev. Phytopathol.* 49, 533–555.

Beckman, C. H. (1964). Host responses to vascular infection. *Annu. Rev. Phytopathol.* 2, 231–252.

Beckman, C. H., Mueller, W. C., Tessier, B. J., and Harrison, N. A. (1982). Recognition and callose deposition in response to vascular infection in *Fusarium* wilt-resistant or susceptible tomato plants. *Physiol. Plant Pathol.* 20, 1–10.

Bernoux, M., Timmers, T., Jauneau, A., Brière, C., de Wit, P. J. G. M., Marco, Y., et al. (2008). RD19, an *Arabidopsis* cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the *Ralstonia solanacearum* PopP2 effector. *Plant Cell* 20, 2252–2264.

Bolton, M. D., and Thomma, B. P. H. J. (2008). The complexity of nitrogen metabolism and nitrogen-regulated gene expression in plant pathogenic fungi. *Physiol. Mol. Plant Pathol.* 72, 104–110.

Brett, C. T. (2000). Cellulose microfibrils in plants: biosynthesis, deposition, and integration into the cell wall. *Int. Rev. Cytol.* 199, 161–199.

Castañeda, A., Reddy, J. D., El-Yacoubi, B., and Gabriel, D. W. (2005). Mutagenesis of all eight avr genes in *Xanthomonas campestris* pv. *campestris* had no detected effect on pathogenicity, but one avr gene affected race specificity. *Mol. Plant Microbe Interact.* 18, 1306–1317.

Chatterjee, S., Almeida, R. P. P., and Lindow, S. (2008). Living in two worlds: the plant and insect lifestyles of *Xylella fastidiosa*. *Annu. Rev. Phytopathol.* 46, 243–271.

Chen, X., Chern, M., Canlas, P. E., Ruan, D., Jiang, C., and Ronald, P. C. (2010). An ATPase promotes autophosphorylation of the pattern recognition receptor XA21 and inhibits XA21-mediated immunity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8029–8034.

Chen, X., and Ronald, P. C. (2011). Innate immunity in rice. *Trends Plant Sci.* 16, 451–459.

Choat, B., Ball, M., Luly, J., and Holtum, J. (2003). Pit membrane porosity and water stress-induced cavitation in four co-existing dry rainforest tree species. *Plant Physiol.* 131, 41–48.

Choat, B., Cobb, A. R., and Jansen, S. (2008). Structure and function of bordered pits: new discoveries and impacts on whole-plant hydraulic function. *New Phytol.* 177, 608–626.

Choat, B., Jansen, S., Zwieniecki, M. A., Smets, E., and Holbrook, N. M. (2004). Changes in pit membrane porosity due to deflection and stretching: the role of vestured pits. *J. Exp. Bot.* 55, 1569–1575.

Choat, B., and Pittermann, J. (2009). New insights into bordered pit structure and cavitation resistance in angiosperms and conifers. *New Phytol.* 182, 557–560.

Clérivet, A., Déon, V., Alami, I., Lopez, F., Geiger, J. P., and Nicole, M. (2000). Tyloses and gels associated with cellulose accumulation in vessels are responses of plane tree seedlings (*Platanus* × *acerifolia*) to the vascular fungus *Ceratocystis fimbriata* f. sp *platani*. *Trees Struct. Funct.* 15, 25–31.

Cooper, R. M., Resende, M. L. V., Flood, J., Rowan, M. G., Beale, M. H., and Potter, U. (1996). Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao*. *Nature* 379, 159–162.

Cooper, R. M., and Williams, J. S. (2004). Elemental sulphur as an induced antifungal substance in plant defence. *J. Exp. Bot.* 55, 1947–1953.

Daugherty, M., Lopes, J. S., and Almeida, R. P. (2010). Strain-specific alfalfa water stress induced by *Xylella fastidiosa*. *Eur. J. Plant Pathol.* 127, 333–340.

De Boer, A. H., and Volkov, V. (2003). Logistics of water and salt transport through the plant: structure and functioning of the xylem. *Plant Cell Environ.* 26, 87–101.

De Jonge, R., Van Esse, H. P., Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., et al. (2012). Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5110–5115.

Denancé, N., Ranocha, P., Oria, N., Barlet, X., Rivière, M.-P., Yadeta, K. A., et al. (2012). *Arabidopsis* wat1 (walls are thin1)-mediated resistance to the bacterial vascular pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and tryptophan metabolism. *Plant J.* doi: 10.1111/tpj.12027 [Epub ahead of print].

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., et al. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8024–8029.

Deslandes, L., Olivier, J., Theulières, F., Hirsch, J., Feng, D. X., Bittner-Eddy, P., et al. (2002). Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. *Proc. Natl. Acad. Sci. U.S.A.* 99, 2404–2409.

Di Pietro, A., Madrid, M. P., Caracuel, Z., Delgado-Jarana, J., and Roncero, M. I. G. (2003). *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Mol. Plant Pathol.* 4, 315–325.

Divon, H. H., and Fluhr, R. (2007). Nutrition acquisition strategies during fungal infection of plants. *FEMS Microbiol. Lett.* 266, 65–74.

Divon, H. H., Rothan-Denoyes, B., Davydov, O., Di Pietro, A., and Fluhr, R. (2005). Nitrogen-responsive genes are differentially regulated in planta during *Fusarium oxysporum* f. sp. *lycopersici* infection. *Mol. Plant Pathol.* 6, 459–470.

Divon, H. H., Ziv, C., Davydov, O., Yarden, O., and Fluhr, R. (2006). The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis. *Mol. Plant Pathol.* 7, 485–497.

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen

interactions. *Nat. Rev. Genet.* 11, 539–548.

Donofrio, N. M., Mitchell, T. K., Dean, R. A., Wang, G.-L., and Valent, B. (2009). "The significance of nitrogen regulation, source and availability on the interaction between rice and rice blast," in *Advances in Genetics, Genomics and Control of Rice Blast Disease*, eds G.-L. Wang and B. Valent (Dordrecht: Springer), 59–72.

Emons, A. M. C., and Mulder, B. M. (2000). How the deposition of cellulose microfibrils builds cell wall architecture. *Trends Plant Sci.* 5, 35–40.

Fernandez-Garcia, N., Hernandez, M., Casado-Vela, J., Bru, R., Elortza, F., Hedden, P., et al. (2011). Changes to the proteome and targeted metabolites of xylem sap in *Brassica oleracea* in response to salt stress. *Plant Cell Environ.* 34, 821–836.

Fradin, E. F., Abd-El-Haliem, A., Masini, L., van den Berg, G. C. M., Joosten, M. H. A. J., and Thomma, B. P. H. J. (2011). Interfamily transfer of tomato ve1 mediates *Verticillium* resistance in *Arabidopsis*. *Plant Physiol.* 156, 2255–2265.

Fradin, E. F., and Thomma, B. P. H. J. (2006). Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Mol. Plant Pathol.* 7, 71–86.

Fradin, E. F., Zhang, Z., Ayala, J. C. J., Castroverde, C. D. M., Nazar, R. N., Robb, J., et al. (2009). Genetic dissection of *Verticillium* wilt resistance mediated by tomato ve1. *Plant Physiol.* 150, 320–332.

Fukuda, H. (1996). Xylogenesis: initiation, progression, and cell death. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 299–325.

Fukuda, H. (1997). Programmed cell death during vascular system formation. *Cell Death Differ.* 4, 684–688.

Fukuda, H. (2004). Signals that control plant vascular cell differentiation. *Nat. Rev. Mol. Cell Biol.* 5, 379–391.

Gartemann, K.-H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flugel, M., et al. (2008). The genome sequence of the tomato-pathogenic *Actinomycete Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. *J. Bacteriol.* 190, 2138–2149.

Gayoso, C., Pomar, F., Novo-Uzal, E., Merino, F., and Martinez de Ilarduya, O. (2010). The Ve-mediated resistance response of the tomato to *Verticillium dahliae* involves H2O2, peroxidase and lignins and drives PAL gene expression. *BMC Plant Biol.* 10:232. doi: 10.1186/1471-2229-10-232

Genin, S. (2010). Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum*. *New Phytol.* 187, 920–928.

Gómez-Gómez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in *Arabidopsis*. *Plant Cell* 13, 1155–1163.

Grimault, V., Gélie, B., Lemattre, M., Prior, P., and Schmit, J. (1994). Comparative histology of resistant and susceptible tomato cultivars infected by *Pseudomonas solanacearum*. *Physiol. Mol. Plant Pathol.* 44, 105–123.

Harwood, T. D., Tomlinson, I., Potter, C. A., and Knight, J. D. (2011). Dutch elm disease revisited: past, present and future management in Great Britain. *Plant Pathol.* 60, 545–555.

Hayslett, M., Juzwik, J., and Moltzan, B. (2008). Three *Colopterus* beetle species carry the oak wilt fungus to fresh wounds on red oak in Missouri. *Plant Dis.* 92, 270–275.

Hayward, A. C. (1991). Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29, 65–87.

Hilaire, E., Young, S. A., Willard, L. H., McGee, J. D., Sweat, T., Chittoor, J. M., et al. (2001). Vascular defense responses in rice: peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. *Mol. Plant Microbe Interact.* 14, 1411–1419.

Houterman, P. M., Ma, L., Van Ooijen, G., De Vroomen, M. J., Cornelissen, B. J. C., Takken, F. L. W., et al. (2009). The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J.* 58, 970–978.

Houterman, P. M., Speijer, D., Dekker, H. L., De Koster, C. G., Cornelissen, B. J. C., and Rep, M. (2007). The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants: short communication. *Mol. Plant Pathol.* 8, 215–221.

Huang, C.-C., and Lindhout, P. (1997). Screening for resistance in wild *Lycopersicon* species to *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2. *Euphytica* 93, 145–153.

Janse, J. D., and Obradovic, A. (2010). *Xylella fastidiosa*: its biology, diagnosis, control and risks. *J. Plant Pathol.* 92, S1.35–S31.47.

Jansen, S., Baas, P., Gasson, P., Lens, F., and Smets, E. (2004). Variation in xylem structure from tropics to tundra: evidence from vestured pits. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8833–8837.

Jha, G., Rajeshwari, R., and Sonti, R. V. (2005). Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. *Mol. Plant Microbe Interact.* 18, 891–898.

Ji, X., Lu, G., Gai, Y., Zheng, C., and Mu, Z. (2008). Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. *FEMS Microbiol. Ecol.* 65, 565–573.

Jiang, Y., Chen, X., Ding, X., Wang, Y., Chen, Q., and Song, W.-Y. (2012). The XA21 binding protein XB25 is required for maintaining XA21-mediated disease resistance. *Plant J.* 73, 814–823.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Juzwik, J., Harrington, T. C., MacDonald, W. L., and Appel, D. N. (2008). The origin of *Ceratocystis fagacearum*, the oak wilt fungus. *Annu. Rev. Phytopathol.* 46, 13–26.

Kawchuk, L. M., Hachey, J., Lynch, D. R., Kulcsar, F., Van Rooijen, G., Waterer, D. R., et al. (2001). Tomato Ve disease resistance genes encode cell surface-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 6511–6515.

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. (2009). Diversity, pathogenicity, and management of *Verticillium* species. *Annu. Rev. Phytopathol.* 47, 39–61.

Klosterman, S. J., Subbarao, K. V., Kang, S., Veronese, P., Gold, S. E., Thomma, B. P. H. J., et al. (2011). Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. *PLoS Pathol.* 7:e1002137. doi: 10.1371/journal.ppat.1002137

Krishnan, H. B., Natarajan, S. S., Bennett, J. O., and Sicher, R. C. (2011). Protein and metabolite composition of xylem sap from field-grown soybeans (*Glycine max*). *Planta* 233, 921–931.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16, 3496–3507.

Lee, S.-W., Han, S.-W., Sririyanum, M., Park, C.-J., Seo, Y.-S., and Ronald, P. C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. *Science* 326, 850–853.

Lee, B. M., Park, Y. J., Park, D. S., Kang, H. W., Kim, J. G., Song, E. S., et al. (2005). The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res.* 33, 577–586.

Leubner-Metzger, G., and Meins, F. (1999). "Functions and regulation of plant beta-1,3-glucanases (PR-2)," in *Pathogenesis-Related Proteins in Plants*, eds S. K. Datta, and S. Muthukrishnan (Boca Raton: CRC Press), 49–76.

Lévesque, C. A., Brouwer, H., Cano, L., Hamilton, J., Holt, C., Huitema, E., et al. (2010). Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. *Genome Biol.* 11, R73.

Lievens, B., Rep, M., and Thomma, B. P. (2008). Recent developments in the molecular discrimination of formae speciales of *Fusarium oxysporum*. *Pest Manag. Sci.* 64, 781–788.

López-Escudero, F., and Mercado-Blanco, J. (2011). *Verticillium* wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 344, 1–50.

Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., et al. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. *Nature* 464, 367–373.

Markakis, E. A., Tjamos, S. E., Chatzipavlidis, I., Antoniou, P. P., and Paplomatas, E. J. (2008). Evaluation of compost amendments for control of vascular wilt diseases. *J. Phytopathol.* 156, 622–627.

Martin, F. N., and Loper, J. E. (1999). Soilborne plant diseases caused by *Pythium* spp.: ecology, epidemiology, and prospects for biological control. *Crit. Rev. Plant Sci.* 18, 111–181.

Martín, J. A., Solla, A., Domingues, M. R., Coimbra, M. A., and Gil, L. (2008). Exogenous phenol increase resistance of *Ulmus minor* to Dutch elm disease through formation of suberin-like compounds on xylem tissues. *Environ. Exp. Bot.* 64, 97–104.

Marzluf, G. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. Rev.* 61, 17–32.

McCully, M. E. (2001). Niches for bacterial endophytes in crop plants: a plant biologist's view. *Funct. Plant Biol.* 28, 983–990.

Michielse, C. B., and Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. *Mol. Plant Pathol.* 10, 311–324.

Möbius, N., and Hertweck, C. (2009). Fungal phytotoxins as mediators of

virulence. *Curr. Opin. Plant Biol.* 12, 390–398.

Mollenhauer, H. H., and Hopkins, D. L. (1974). Ultrastructural study of Pierce's disease bacterium in grape xylem tissue. *J. Bacteriol.* 119, 612–618.

Moser, J. C., Konrad, H., Blomquist, S. R., and Kirisits, T. (2010). Do mites phoretic on elm bark beetles contribute to the transmission of Dutch elm disease? *Naturwissenschaften* 97, 219–227.

Nadarasah, G., and Stavrinides, J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* 35, 555–575.

Nakamura, S.-I., Akiyama, C., Sasaki, T., Hattori, H., and Chino, M. (2008). Effect of cadmium on the chemical composition of xylem exudate from oilseed rape plants (*Brassica napus* L.). *Soil Sci. Plant Nutr.* 54, 118–127.

Newcombe, G., and Robb, J. (1988). The function and relative importance of the vascular coating response in highly resistant, moderately resistant and susceptible alfalfa infected by *Verticillium albo-atrum*. *Physiol. Mol. Plant Pathol.* 33, 47–58.

Nieminen, K. M., Kauppinen, L., and Helariutta, Y. (2004). A weed for wood? *Arabidopsis* as a genetic model for xylem development. *Plant Physiol.* 135, 653–659.

Niño-Liu, D. O., Ronald, P. C., and Bogdanove, A. J. (2006). *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Mol. Plant Pathol.* 7, 303–324.

Oliver, J. P., Castro, A., Gaggero, C., Cascón, T., Schmelz, E. A., Castresana, C., et al. (2009). Pythium infection activates conserved plant defense responses in mosses. *Planta* 230, 569–579.

Palmer, C. S., Saleeba, J. A., and Lyon, B. R. (2005). Phytotoxicity on cotton ex-plants of an 18.5 kDa protein from culture filtrates of *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 67, 308–318.

Park, C.-J., Bart, R., Chern, M., Canlas, P. E., Bai, W., and Ronald, P. C. (2010a). Overexpression of the endoplasmic reticulum chaperone BiP3 regulates XA21-mediated innate immunity in rice. *PLoS ONE* 5:e9262. doi: 10.1371/journal.pone.0009262

Park, C. J., Han, S. W., Chen, X., and Ronald, P. C. (2010b). Elucidation of XA21-mediated innate immunity. *Cell. Microbiol.* 12, 1017–1025.

Passardi, F., Cosio, C., Penel, C., and Dunand, C. (2005). Peroxidases have more functions than a Swiss army knife. *Plant Cell Rep.* 24, 255–265.

Pérez-Donoso, A. G., Greve, L. C., Walton, J. H., Shackel, K. A., and Labavitch, J. M. (2007). *Xylella fastidiosa* infection and ethylene exposure result in xylem and water movement disruption in grapevine shoots. *Plant Physiol.* 143, 1024–1036.

Pérez-Donoso, A. G., Sun, Q., Roper, M. C., Greve, L. C., Kirkpatrick, B., and Labavitch, J. M. (2010). Cell wall-degrading enzymes enlarge the pore size of intervessel pit membranes in healthy and *Xylella fastidiosa*-infected grapevines. *Plant Physiol.* 152, 1748–1759.

Powney, R., Smits, T. H. M., Sawbridge, T., Frey, B., Blom, J., Frey, J. E., et al. (2011). Genome sequence of an *Erwinia amylovora* strain with pathogenicity restricted to Rubus plants. *J. Bacteriol.* 193, 785–786.

Purcell, A. H., and Hopkins, D. L. (1996). Fastidious xylem-limited bacterial plant pathogens. *Annu. Rev. Phytopathol.* 34, 131–151.

Qian, W., Jia, Y., Ren, S.-X., He, Y.-Q., Feng, J.-X., Lu, L.-F., et al. (2005). Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Res.* 15, 757–767.

Qin, G. M., Vallad, G. E., and Subbarao, K. V. (2008). Characterization of *Verticillium dahliae* and *V. tricorpus* isolates from lettuce and artichoke. *Plant Disease* 92, 69–77.

Rahman, M. A., Abdullah, H., and Vanhaecke, M. (1999). Histopathology of susceptible and resistant *Capsicum annuum* cultivars infected with *Ralstonia solanacearum*. *J. Phytopathol.* 147, 129–140.

Rep, M., Dekker, H. L., Vossen, J. H., De Boer, A. D., Houterman, P. M., De Koster, C. G., et al. (2003). A tomato xylem sap protein represents a new family of small cysteine-rich proteins with structural similarity to lipid transfer proteins. *FEBS Lett.* 534, 82–86.

Rep, M., Dekker, H. L., Vossen, J. H., De Boer, A. D., Houterman, P. M., Speijer, D., et al. (2002). Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiol.* 130, 904–917.

Reusche, M., Thole, K., Janz, D., Truskina, J., Rindfleisch, S., Drübert, C., et al. (2012). *Verticillium* infection triggers VASCULAR-RELATED NAC DOMAIN7-dependent de novo xylem formation and enhances drought tolerance in *Arabidopsis*. *Plant Cell* 24, 3823–3837.

Rey, P., Benhamou, N., and Tirilly, Y. (1998). Ultrastructural and cytochemical investigation of asymptomatic infection by *Pythium* spp. *Phytopathology* 88, 234–244.

Roncero, M. I. G., Hera, C., Ruiz-Rubio, M., García Maceira, F. I., Madrid, M. P., Caracuel, Z., et al. (2003). *Fusarium* as a model for studying virulence in soilborne plant pathogens. *Physiol. Mol. Plant Pathol.* 62, 87–98.

Roper, M. C. (2011). Pantoea stewartii subsp. stewartii: lessons learned from a xylem-dwelling pathogen of sweet corn. *Mol. Plant Pathol.* 12, 628–637.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., et al. (2002). Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* 415, 497–502.

Santhanam, P., van Esse, H. P., Albert, I., Faino, L., Nürnberger, T., and Thomma, B. P. H. J. (2013). Evidence for functional diversification within a fungal NEP1-like protein family. *Mol. Plant Microbe Interact.* 26, 278–286.

Scheffer, R. J., Voeten, J. G. W. F., and Guries, R. P. (2008). Biological control of Dutch elm disease. *Plant Dis.* 92, 192–200.

Schumann, G. L., and D'Arcy, C. J. (2010). *Essential Plant Pathology*. St. Paul, MN: APS Press.

Sebaihia, M., Bocsanczy, A. M., Biehl, B. S., Quail, M. A., Perna, N. T., Glasner, J. D., et al. (2010). Complete genome sequence of the plant pathogen *Erwinia amylovora* strain ATCC 49946. *J. Bacteriol.* 192, 2020–2021.

Siebrecht, S., Herdel, K., Schurr, U., and Tischner, R. (2003). Nutrient translocation in the xylem of poplar – diurnal variations and spatial distribution along the shoot axis. *Planta* 217, 783–793.

Simpson, A. J., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., et al. (2000). The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406, 151–159.

Smits, T. H., Rezzonico, F., Kamber, T., Blom, J., Goesmann, A., Frey, J. E., et al. (2010). Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Mol. Plant Microbe Interact.* 23, 384–393.

Song, W. Y., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., et al. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270, 1804–1806.

Spadaro, D., and Gullino, M. L. (2005). Improving the efficacy of biocontrol agents against soilborne pathogens. *Crop Prot.* 24, 601–613.

Stipanovic, R. D., Puckhaber, L. S., Liu, J., and Bell, A. A. (2011). Phytotoxicity of fusaric acid and analogs to cotton. *Toxicon* 57, 176–178.

Street, P. F. S., Robb, J., and Ellis, B. E. (1986). Secretion of vascular coating components by xylem parenchyma cells of tomatoes infected with *Verticillium albo-atrum*. *Protoplasma* 132, 1–11.

Suárez-Estrella, F., Vargas-García, C., López, M. J., Capel, C., and Moreno, J. (2007). Antagonistic activity of bacteria and fungi from horticultural compost against *Fusarium oxysporum* f. sp. *melonis*. *Crop Prot.* 26, 46–53.

Sun, Q. H., Hu, J., Huang, G. X., Ge, C., Fang, R. X., and He, C. Z. (2005). Type-II secretion pathway structural gene xpsE, xylanase- and cellulase secretion and virulence in *Xanthomonas oryzae* pv. *oryzae*. *Plant Pathol.* 54, 15–21.

Takken, F., and Rep, M. (2010). The arms race between tomato and *Fusarium oxysporum*. *Mol. Plant Pathol.* 11, 309–314.

Talboys, P. W. (1972). Resistance to vascular wilt fungi. *Proc. R. Soc. Lond. B Biol. Sci.* 181, 319–332.

Temple, B., and Horgen, P. A. (2000). Biological roles for cerato-ulmin, a hydrophobin secreted by the elm pathogens, *Ophiostoma ulmi* and *O. novo-ulmi*. *Mycologia* 92, 1–9.

Tjamos, E. C., and Beckman, C. H. (1989). *Vascular Wilt Diseases of Plants: Basic Studies and Control (NATO ASI Series H: Cell Biology)*. Berlin: Springer-Verlag.

Tsuda, K., Kosaka, Y., Tsuge, S., Kubo, Y., and Horino, O. (2001). Evaluation of the endophyte *Enterobacter cloacae* SM10 isolated from spinach roots for biological control against *Fusarium* wilt of spinach. *J. Gen. Plant Pathol.* 67, 78–84.

Tyree, M. T., and Zimmermann, M. H. (2002). *Xylem Structure and the Ascent of Sap*. Berlin: Springer.

van Esse, H. P., Fradin, E. F., de Groot, P. J., de Wit, P. J. G. M., and Thomma, B. P. H. J. (2009). Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct. *Mol. Plant Microbe Interact.* 22, 245–258.

van Loon, L. C., Rep, M., and Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44, 135–162.

Vicente, J. G., Conway, J., Roberts, S. J., and Taylor, J. D. (2001). Identification and origin of *Xanthomonas campestris* pv. *campestris* races and

related pathovars. *Phytopathology* 91, 492–499.

Wang, G., Ellendorff, U., Kemp, B., Mansfield, J. W., Forsyth, A., Mitchell, K., et al. (2008). A genome-wide functional investigation into the roles of receptor-like proteins in *Arabidopsis*. *Plant Physiol.* 147, 503–517.

Wang, J. Y., Cai, Y., Gou, J. Y., Mao, Y. B., Xu, Y. H., Jiang, W. H., et al. (2004). VdNEP, an elicitor from *Verticillium dahliae*, induces cotton plant wilting. *Appl. Environ. Microbiol.* 70, 4989–4995.

Williams, J. S., Hall, S. A., Hawkesford, M. J., Beale, M. H., and Cooper, R. M. (2002). Elemental sulfur and Thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiol.* 128, 150–159.

Xu, R.-Q., Blanvillain, S., Feng, J.-X., Jiang, B.-L., Li, X.-Z., Wei, H.-Y., et al. (2008). AvrACXcc8004, a type III effector with a leucine-rich repeat domain from *Xanthomonas campestris* pathovar *campestris* confers avirulence in vascular tissues of *Arabidopsis thaliana* ecotype Col-0. *J. Bacteriol.* 190, 343–355.

Yadeta, K. A., Hanemian, M., Smit, P., Hiemstra, J. A., Pereira, A., Marco, Y., et al. (2011). The *Arabidopsis thaliana* DNA-binding protein AHL19 mediates *Verticillium* wilt resistance. *Mol. Plant Microbe Interact.* 24, 1582–1591.

Ye, Z. H. (2002). Vascular tissue differentiation and pattern formation in plants. *Annu. Rev. Plant Biol.* 53, 183–202.

Yokoyama, R., and Nishitani, K. (2006). Identification and characterization of *Arabidopsis thaliana* genes involved in xylem secondary cell walls. *J. Plant Res.* 119, 189–194.

Zhang, J., Elo, A., and Helariutta, Y. (2011). *Arabidopsis* as a model for wood formation. *Curr. Opin. Biotechnol.* 22, 293–299.

Zhang, Z., Fradin, E., Jonge, R., van Esse, H. P., Smit, P., Liu, C.-M., et al. (2013). Optimized agroinfiltration and virus-induced gene silencing to study Ve1-mediated *Verticillium* resistance in tobacco. *Mol. Plant Microbe Interact.* 26, 182–190.

Zhou, B.-J., Jia, P.-S., Gao, F., and Guo, H.-S. (2012). Molecular characterization and functional analysis of a necrosis- and ethylene-inducing, protein-encoding gene family from *Verticillium dahliae*. *Mol. Plant Microbe Interact.* 25, 964–975.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T., et al. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 December 2012; accepted: 28 March 2013; published online: 23 April 2013.

Citation: Yadeta KA and Thomma BPHJ (2013) The xylem as battleground for plant hosts and vascular wilt pathogens. Front. Plant Sci. ***4****:97. doi: 10.3389/fpls.2013.00097*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Yadeta and Thomma. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 11 June 2013
doi: 10.3389/fpls.2013.00191

Pathogenicity of and plant immunity to soft rot pectobacteria

Pär R. Davidsson, Tarja Kariola†, Outi Niemi† and E. Tapio Palva*

Division of Genetics, Department of Biosciences, University of Helsinki, Helsinki, Finland

Edited by:
Saskia C. M. Van Wees, Utrecht University, Netherlands

Reviewed by:
Giulia De Lorenzo, Sapienza Università di Roma, Italy
Dominik Klauser, University of Basel, Switzerland

***Correspondence:**
E. Tapio Palva, Division of Genetics, Department of Biosciences, University of Helsinki, PO Box 56, Helsinki, FI-00014, Finland
e-mail: tapio.palva@helsinki.fi

†Tarja Kariola and Outi Niemi have contributed equally to this work.

Soft rot pectobacteria are broad host range enterobacterial pathogens that cause disease on a variety of plant species including the major crop potato. Pectobacteria are aggressive necrotrophs that harbor a large arsenal of plant cell wall-degrading enzymes as their primary virulence determinants. These enzymes together with additional virulence factors are employed to macerate the host tissue and promote host cell death to provide nutrients for the pathogens. In contrast to (hemi)biotrophs such as *Pseudomonas*, type III secretion systems (T3SS) and T3 effectors do not appear central to pathogenesis of pectobacteria. Indeed, recent genomic analysis of several *Pectobacterium* species including the emerging pathogen *Pectobacterium wasabiae* has shown that many strains lack the entire T3SS as well as the T3 effectors. Instead, this analysis has indicated the presence of novel virulence determinants. Resistance to broad host range pectobacteria is complex and does not appear to involve single resistance genes. Instead, activation of plant innate immunity systems including both SA (salicylic acid) and JA (jasmonic acid)/ET (ethylene)-mediated defenses appears to play a central role in attenuation of *Pectobacterium* virulence. These defenses are triggered by detection of pathogen-associated molecular patterns (PAMPs) or recognition of modified-self such as damage-associated molecular patterns (DAMPs) and result in enhancement of basal immunity (PAMP/DAMP-triggered immunity or pattern-triggered immunity, PTI). In particular plant cell wall fragments released by the action of the degradative enzymes secreted by pectobacteria are major players in enhanced immunity toward these pathogens. Most notably bacterial pectin-degrading enzymes release oligogalacturonide (OG) fragments recognized as DAMPs activating innate immune responses. Recent progress in understanding OG recognition and signaling allows novel genetic screens for OG-insensitive mutants and will provide new insights into plant defense strategies against necrotrophs such as pectobacteria.

Keywords: Pectobacterium, oligogalacturonides, necrotrophs, plant hormones, cell wall-degrading enzymes, genomics, genetics, DAMPs and PAMPs

INTRODUCTION

Plant pathogens including pathogenic bacteria use a variety of strategies ranging from stealth to brute force to colonize plants and derive nutrients from their hosts. The stealth strategy is employed by biotrophs and hemibiotrophs such as *Pseudomonas syringae* and *Xanthomonas* spp. that rely on living plant cells for nutrient acquisition at least until later stages of infection. Their lifestyle is largely dependent on their ability to avoid and suppress plant defense responses most notably by secretion of effector proteins enabling them to obtain nutrients and multiply within living plant tissue (Göhre and Robatzek, 2008; Collmer et al., 2009; Kay and Bonas, 2009). Bacterial effectors are secreted mainly through the type III secretion system (T3SS) which is a multi-protein injection machinery capable of translocating proteins directly from the bacterial cytosol into the host cell (Alfano and Collmer, 2004). Different effector proteins target specific components of plant defense and are effective only against a particular plant species or cultivar. Therefore, strains of (hemi)biotrophic bacteria often show a high degree of host specificity (Niks and Marcel, 2009; Lindeberg et al., 2012). Although essential to pathogenicity of (hemi)biotrophs, T3SS and effectors play a much less central role in the virulent lifestyle of necrotrophs. Instead, necrotrophs use a brute force strategy employing plant cell wall-degrading enzymes (PCWDEs), necrosis-inducing proteins and toxins to actively kill plant tissue and feed on the nutrients released. For example, the broad host range necrotrophic fungus *Botrytis cinerea* uses enzymes to break down the host cell walls to access the host tissue and causes host cell death by production of non-specific fungal toxins and reactive oxygen species (ROS; van Kan, 2006; Choquer et al., 2007). Similarly, bacterial necrotrophs such as soft rot pectobacteria are broad host range pathogens that are particularly effective in macerating the host tissues and obtaining nutrients from the dead cells. Recent progress in genomic analysis of several species of pectobacteria has provided new insights into the necrotrophic lifestyle of these pathogens and has also made them excellent models for elucidating the strategies and immune responses plants employ to combat bacterial necrotrophs.

The invasion of a phytopathogen triggers immune responses in the host plant. While lacking the somatic, adaptive immune system as well as mobile defender cells present in animals, plants are still capable of defending themselves in various ways. The recognition of the invader usually occurs via pathogen-associated molecular patterns (PAMPs), conserved structures such as the bacterial flagellin essential for the microbial lifestyle (Gómez-Gómez and Boller, 2000; Boller and Felix, 2009). The resulting pattern-triggered immunity (PTI) response as the first line of defense is sufficient to fend off many but not all potential pathogens. Successful pathogens can bypass PTI for example by secreting the above mentioned effector proteins that interfere with the PTI responses and hence, benefit pathogen virulence by causing effector-triggered susceptibility (ETS). More severe defense responses triggered upon effector recognition including hypersensitive response (HR) and programmed cell death (PCD) result in effector-triggered immunity (ETI) particularly effective against strains of (hemi)biotrophs (Jones and Dangl, 2006). Plants can also sense danger via recognition of so called danger or damage-associated molecular patterns (DAMPs) that report of "damage to self" and induce a variety of host defense responses in many aspects similar to those triggered by PAMPs (Ridley et al., 2001; Galletti et al., 2009). These can be fragments of plant cell wall released by the action of chewing insects but also by PCWDEs secreted by necrotrophic pathogens as an essential part of their virulence strategy. Central in mediating the innate immunity responses are phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Dodds and Rathjen, 2010). The focus of this review will be on the virulence strategies of pectobacteria and the corresponding immune responses of plants addressing both similarities and differences of immune responses of pectobacteria to those of biotrophs/hemibiotrophs such as *Pseudomonas*.

SOFT ROT PECTOBACTERIA

Soft rot enterobacteria of genera *Pectobacterium* and *Dickeya* are classical and well-studied examples of necrotrophic plant pathogenic bacteria. Their taxonomical classification has been revised several times in recent years. They were first characterized in the early 20th century (Jones, 1901) and for decades were known as members of the genus *Erwinia* (Winslow et al., 1920). In 1998, the genus was divided into three phylogenetic groups (Hauben et al., 1998). Soft rot pathogens were moved out of the genus *Erwinia* which now contains plant pathogens such as the hemibiotroph *Erwinia amylovora*. Two new genera were created: *Brenneria* and *Pectobacterium*, the latter of which harbors the soft rot species. Later on, subspecies of *P. carotovorum* were raised at species level giving rise to for example *P. wasabiae* and *P. atrosepticum* (Gardan et al., 2003). Further, *P. aroidearum* was described as a novel species consisting of soft rot pathogens mainly infecting monocotyledonous plants (Nabhan et al., 2012a). Finally, *P. chrysanthemi* was separated from *Pectobacterium* and the new genus *Dickeya* was formed (Samson et al., 2005).

Soft rot enterobacteria are the most important causative agents of the economically significant soft rot disease which results in significant crop losses during the growth season as well as during storage (Perombelon and Kelman, 1980; Pérombelon, 2002; Czajkowski et al., 2011). The most distinctive feature of soft rot pathogenesis is the co-ordinate production of a large arsenal of PCWDEs such as pectinases, cellulases, hemicellulases, and proteinases which makes pectobacteria very effective in decaying plant tissue. PCWDEs are secreted mainly through type II secretion system (T2SS; Pérombelon, 2002; Charkowski et al., 2012). In addition to PCWDEs, soft rot bacteria secrete proteins that promote plant cell death such as the necrosis-inducing protein (Nip) and the effector protein DspE (Mattinen et al., 2004; Kim et al., 2011). Typical for necrotrophs, soft rot bacteria generally display a broad host range. The disease affects several important crop and ornamental species across the world. Bacteria of the genus *Dickeya* cause disease especially in tropical and subtropical climates and the host plants include maize, banana, and increasingly potato among many other crop species (Pérombelon, 2002; Toth et al., 2003, Toth et al., 2011; Samson et al., 2005). The host range of *P. carotovorum* is considered to be the widest of all the soft rot bacteria, potato being the most important crop affected in temperate regions (Pérombelon, 2002; Toth et al., 2003). Common soft rot of potato tubers caused by *P. carotovorum* can result in extensive crop losses also post-harvest during storage (Perombelon and Kelman, 1980; Pérombelon, 2002). *P. atrosepticum*, unlike *P. carotovorum*, appears more host-specific. This pathogen causes a stem disease called blackleg on potato in temperate climates (Perombelon and Kelman, 1980; Pérombelon, 2002). The reason for the narrow host range remains to be elucidated. The third economically important *Pectobacterium* species, *P. wasabiae*, was originally characterized as a pathogen of Japanese horseradish (*Wasabia japonica*), i.e., wasabi (Goto and Matsumoto, 1987). However, recently *P. wasabiae* has received attention as a potato pathogen in several countries around the world (Ma et al., 2007; Pitman et al., 2008; Baghaee-Ravari et al., 2010). Also, strains previously characterized as *P. carotovorum* have recently been re-identified as *P. wasabiae* (Nabhan et al., 2012b) including a well-studied Finnish model strain SCC3193 (Nykyri et al., 2012). At this point, it is not known if *P. wasabiae* represents an emerging potato pathogen currently spreading around the world or if the species has long been present on potato fields but only recently sequence based methods have enabled the differentiation of *P. wasabiae* from *P. carotovorum*.

Although soft rot enterobacteria have been studied for decades, very little still is known of their survival strategies between growing seasons. Soft rot pathogens have been shown to be able to persist in soil only for weeks or months depending on environmental conditions and overwintering in soil is not considered likely. However, survival on decomposing plant material in soil is known to happen (Perombelon and Kelman, 1980; Czajkowski et al., 2011). Introduction of the soft rot pathogens via contaminated planting material such as seed tubers is considered to be the most common way for the disease to spread and considerable effort is taken to ensure disease free planting material. However, due to the ability of soft rot bacteria to colonize plants latently without symptoms, the level of control achieved varies and is highly dependent on environmental conditions (Czajkowski et al., 2011). Further, dispersal of the bacteria could also happen via usage of surface water for irrigation, via aerosols generated by rain, via movement of the bacteria in soil water or mechanically via contaminated agricultural equipment (Perombelon and Kelman, 1980; Czajkowski

et al., 2011). Moreover, insects can act as vectors for many plant pathogenic bacteria (Nadarasah and Stavrinides, 2011). Soft rot enterobacteria have also been found associated with insects and transmission via insects has been suggested for decades (Perombelon and Kelman, 1980; Charkowski et al., 2012). Certain strains of *P. carotovorum* have indeed been shown to interact with *Drosophila* and activate an immune response in the fly (Basset et al., 2000). The interaction has been shown to be promoted by the bacterial gene *evf* which improves the persistence of the bacteria in the gut of the fly host (Basset et al., 2003). The existence of bacterial genes promoting interactions with insects suggests that adaptation to insects as vectors or as alternative hosts may have played an important role in the evolution of these plant pathogenic bacteria.

GENOME ANALYSIS AND VIRULENCE FACTORS OF PECTOBACTERIA

Genome sequencing has provided new insights into the lifestyle of *Pectobacterium*. *P. atrosepticum* SCRI1043 was sequenced in 2004 as the first soft rot pathogen (Bell et al., 2004, accession: BX950851). By December 2012, seven more *Pectobacterium* genome sequences have become publicly available: *P. carotovorum* WPP14 (Glasner et al., 2008, accession: PRJNA31123), *P. brasiliensis* PBR1692 (Glasner et al., 2008, accession: PRJNA31121), *P. aroidearum* PC1 (accession: CP001657.1), *P. wasabiae* WPP163 (accession: CP001790.1), *P. wasabiae* SCC3193 (Koskinen et al., 2012, accession: CP003415), *P. wasabiae* CFBP 3304^T (Nykyri et al., 2012, accession: AKVS00000000), and *P. carotovorum* subsp. *carotovorum* PCC21 (Park et al., 2012, accession: CP003776).

The necrotrophic nature of the symptomatic stage of *Pectobacterium* infection and the role of PCWDEs has long been appreciated. Genomic approaches have indeed shown that different *Pectobacterium* species share a similar collection of PCWDEs instrumental for host tissue maceration (Glasner et al., 2008; Nykyri et al., 2012). The genes encoding PCWDEs are scattered around the *Pectobacterium* genomes and are mainly found from the core genome (Toth et al., 2006; Glasner et al., 2008; Nykyri et al., 2012). Apart from the similarities in the enzyme arsenal, limited strain specific differences exist for example in the composition of putative proteinases and in the lack of the pectate lyase HrpW and the polygalacturonase (PG) PehK in *P. wasabiae* strains (Nykyri et al., 2012). Consequently, differences in host specificity or disease type and severity between different *Pectobacterium* species and strains is not explained by the arsenal of PCWDEs but is likely to rely on other factors yet to be characterized.

Although the necrotrophic lifestyle of *Pectobacterium* is a hallmark of the symptomatic phase of infection, in recent years the view of *Pectobacterium* has shifted from a simple necrotroph toward a more sophisticated pathogen whose action at the early stages of infection can be better described as biotrophic (Toth and Birch, 2005; Liu et al., 2008). The initiation of soft rot disease is highly dependent on environmental conditions and the soft rot bacteria are indeed considered as opportunistic pathogens (Pérombelon, 2002; Toth and Birch, 2005). They are capable of living within the plant tissue without causing symptoms but this asymptomatic stage ends when high moisture and low oxygen concentration lower plant resistance favoring bacterial growth (Perombelon and Kelman, 1980; Pérombelon, 2002). PCWDEs are produced, and therefore the symptoms appear, only when the cell density of the bacteria is high (Perombelon and Kelman, 1980; Pérombelon, 2002; Toth et al., 2003). The production of PCWDEs is strictly controlled in a population density-dependent manner through quorum sensing (QS) regulation (Jones et al., 1993; Pirhonen et al., 1993; Liu et al., 2008). This is suggested to prevent premature activation of plant defenses as the action of PCWDEs releases cell wall fragments which trigger defense responses in the host plant (Palva et al., 1993; Salmond et al., 1995; Mäe et al., 2001). QS is proposed to function as the master switch controlling various virulence determinants to achieve a successful transition from the asymptomatic biotrophic phase to necrotrophy (Liu et al., 2008). In addition to QS, the production of PCWDEs and other virulence determinants is further controlled by a number of two component systems and other regulators which sense physiological or environmental cues such as plant derived organic acids and pectin derivatives indicative of presence of the host plant. The effect of different cellular and environmental signals is then integrated by global regulators to assure an appropriate response (reviewed in Toth et al., 2006; Charkowski et al., 2012).

The role of horizontal gene transfer in acquisition of determinants related to interaction with host plants has been highlighted in *Pectobacterium* genome studies (Bell et al., 2004; Toth et al., 2006; Glasner et al., 2008; Nykyri et al., 2012). Many of these traits could benefit the bacterium at the early stages of infection. Bell et al. (2004) identified putative virulence determinants within horizontally acquired islands in the genome of *P. atrosepticum* and experimentally verified the contribution of a putative *virB*-type type IV secretion system (T4SS) and a polyketide phytotoxin (encoded by the *cfa* cluster, see below) to virulence of the bacterium. T4SS machineries translocate DNA and/or proteins across the bacterial cell wall into bacterial or eukaryotic cells (Christie et al., 2005). *virB*-T4SS was first described in *Agrobacterium tumefaciens* where it is used to deliver the tumorigenic Ti-plasmid into the plant cell (Gelvin, 2009). The nature of the material translocated through the T4SS in *P. atrosepticum* remains unknown and the role of T4SS in other *Pectobacterium* species, where sporadically present, has not been characterized. *P. wasabiae* SCC3193 and CFBP 3304^T as well as *P. carotovorum* subsp. *brasiliensis* PBR1692 harbor T4SS, whereas it is absent from *P. wasabiae* WPP163, *P. carotovorum* WPP14, and *P. aroidearum* PC1 (Glasner et al., 2008; Nykyri et al., 2012). The *cfa* cluster in *P. atrosepticum* encodes enzymes for the synthesis of coronafacic acid part of the coronatine phytotoxin characterized in *P. syringae* as an important virulence determinant which acts by mimicking JA (Bender et al., 1999; Bell et al., 2004). However, coronatine is not produced by *P. atrosepticum* as enzymes for synthesis of coronamic acid, which is also required for biosynthesis of coronatine, are missing (Bell et al., 2004). Bell et al. (2004) speculate that the effect of the *cfa* cluster on virulence of *P. atrosepticum* could be through the production of an alternative polyketide phytotoxin.

The collection of horizontally acquired islands differs between *Pectobacterium* species and strains (Glasner et al., 2008; Nykyri et al., 2012). Some islands are present in all genomes studied, whereas many islands can only be identified in one strain or species. It remains to be seen if the varying collection of horizontally acquired islands is responsible for the differences in virulence

and host specificity between *Pectobacterium* species. For example, the *cfa* cluster present on an island in *P. atrosepticum* is missing from the genomes of other *Pectobacterium* (Glasner et al., 2008; Nykyri et al., 2012). Further, Nykyri et al. (2012) reported the finding of several *P. wasabiae* specific islands present in all three *P. wasabiae* strains (WPP163, SCC3193, and CFBP 3304^T) but absent from genomes of other *Pectobacterium* species. These islands contain uncharacterized genes but also genes encoding for example a second type VI secretion (T6SS) machinery and a bacterial microcompartment of unknown function. T6SS is a common protein secretion system in Gram-negative bacteria and it has been reported to function in interactions with animals, plants and other bacteria (Schwarz et al., 2010; Records, 2011; Russell et al., 2011; Zheng et al., 2011). T6SS was first shown to contribute to virulence on potato in *P. atrosepticum* (Liu et al., 2008). *P. atrosepticum* and *P. carotovorum* genomes contain only one T6SS machinery whereas *P. wasabiae* harbors two machineries of which one is similar to the T6SS in other *Pectobacterium* species and the other rather resembles the machinery in *Pantoae* and *Erwinia* (Nykyri et al., 2012). In *P. wasabiae* SCC3193, the two T6SS machineries were experimentally shown to have at least partially overlapping functions during potato infection (Nykyri et al., 2012). Effectors secreted through the T6SS have not yet been identified in *Pectobacterium* and the exact role of T6SS during infection remains to be elucidated.

MODULATION OF HOST RESPONSES BY PECTOBACTERIA

Very little is known of the mechanisms used by *Pectobacterium* to avoid being destroyed by host defense responses during the asymptomatic phase. Most hemibiotrophic bacterial plant pathogens rely on T3SS and T3 effector proteins to manipulate their hosts in order to achieve suppression of plant defenses and mutants in T3SS are consequently non-pathogenic (Grant et al., 2006; Collmer et al., 2009). T3SS has been shown to contribute to virulence of *P. atrosepticum* and *P. carotovorum* (Rantakari et al., 2001; Holeva et al., 2004; Kim et al., 2011). Indeed, inactivation of T3SS in *P. carotovorum* resulted in delayed growth of the bacteria at the early stages of infection (Rantakari et al., 2001), suggesting that T3SS could be used to suppress plant defense responses also in *Pectobacterium*. However, the number of T3 effectors encoded by *Pectobacterium* genomes is apparently much smaller than that of hemibiotroph genomes (Glasner et al., 2008; Kim et al., 2009) and no T3 effectors suppressing plant defense responses have been described. In contrast, the only characterized T3 effector in *Pectobacterium*, DspE, elicits plant cell death which in turn promotes disease progression and maceration of plant tissue by *Pectobacterium* at the nectrotrophic stage of infection (Kim et al., 2011). It was concluded that *P. carotovorum* may not at all use T3SS to suppress plant defense responses as the gene expression profile of *Nicotiana benthamiana* after *P. carotovorum* infection is similar to that of *P. syringae* T3SS mutant rather than wild type *P. syringae*. Moreover, *P. carotovorum* was unable to suppress a typical basal defense response, callose deposition in leaves (Kim et al., 2011). This is contrary to *P. syringae* DC3000 where the corresponding T3 effector AvrE inactivates SA-dependent basal defenses (DebRoy et al., 2004). The limited role of T3SS in *Pectobacterium* virulence is further supported by studies showing that when the virulence of T3SS harboring and naturally T3SS-deficient *P. carotovorum* strains was compared, no obvious differences were observed (Kim et al., 2009). Furthermore, *P. wasabiae* seems to entirely lack T3SS (Ma et al., 2007; Kim et al., 2009; Pitman et al., 2009; Nykyri et al., 2012). Although the machinery and associated effectors may contribute to virulence, *Pectobacterium* clearly does not rely on T3SS to establish a successful infection. Other subtle mechanisms to manipulate the host at the early stages of infection must exist. For example, T6SS is hypothesized to manipulate host defense responses (Liu et al., 2008), but no T6 effectors with this ability have so far been described. It remains an open question, whether all *Pectobacterium* species use the same strategies or if each species or strain possesses its own collection of mechanisms enabling a successful interaction with the host.

One putative strategy for an effector protein or a virulence determinant to function early on in infection is to manipulate the hormonal balance of the host plant. Plant hormones are central mediators of plant growth, development, and responses to abiotic stress as well as plant defenses. Furthermore, hormonal crosstalk plays a key role in determining plant response priorities to environmental cues influencing the outcome of plant–pathogen interactions (Dong, 1998; Robert-Seilaniantz et al., 2011). Consequently, many plant pathogenic, as well as plant growth-promoting, bacteria have the ability to manipulate hormonal signaling in plants by producing plant hormones and hormone mimics or by influencing the crosstalk between hormonal pathways (Costacurta and Vanderleyden, 1995; Robert-Seilaniantz et al., 2011). Bacterial synthesis of auxin (indole-3-acetic acid), cytokinins, ET, and abscisic acid (ABA) has been reported (Robert-Seilaniantz et al., 2011). Among soft rot bacteria, only *Dickeya dadantii* 3937 has been shown to produce auxin (Yang et al., 2007). However, auxin was described to have a regulatory role in bacterial virulence gene expression and it remains to be shown whether the production also affects plant hormone signaling. The coronatine toxin produced by *P. syringae* has been shown to act as a JA mimic activating JA-dependent defenses and suppressing antagonistic SA-dependent defenses (Laurie-Berry et al., 2006; Uppalapati et al., 2007; Zheng et al., 2012). In the case of *Pectobacterium*, no direct evidence for virulence determinants affecting plant hormones exists. The *P. atrosepticum* polyketide phytotoxin encoded by the *cfa* cluster can be thought of as a potential candidate. Furthermore, Nykyri et al. (2012) reported the interesting finding of a gene encoding a putative *S*-adenosyl-L-methionine:benzoic acid/SA carboxyl methyltransferase on a horizontally acquired island in the genome of *P. wasabiae* SCC3193. This gene is also present in the genome of *P. wasabiae* WPP163 but it is absent from other *Pectobacterium* strains. In fact, the putative protein resembles plant enzymes and was concluded to be of probable eukaryotic origin. The corresponding methyltransferases in plants are involved in production of the mobile signal methyl salicylate by methylation of SA in response to pathogen attack (Ross et al., 1999; Chen et al., 2003; Park et al., 2007). The *P. wasabiae* benzoic acid/SA methyltransferase could represent a novel direct way to manipulate SA-mediated defenses instead of indirect effect through antagonistic hormonal pathways. However, this hypothesis still needs to be experimentally verified.

PLANT INNATE IMMUNITY

Inducible plant innate immunity responses are comprised of two separate lines of defense that are distinguished by the type of pathogen-derived molecules (elicitors) recognized. The first has an equivalent in animals and is triggered after the perception of a group of conserved, or general, pathogen-derived molecules, called PAMPs/MAMPs that can be present in both pathogenic and non-pathogenic microorganisms (Parker, 2003; Jones and Dangl, 2006). Well-characterized PAMPs include bacterial flagellin, lipopolysaccharides (LPSs) and elongation factor Tu (EF-Tu) as well as chitin, a component of fungal cell walls (Boller, 1995; Gómez-Gómez and Boller, 2000; Robatzek et al., 2006) and are central for pathogen fitness (Parker, 2003; Chisholm et al., 2006; Dodds and Rathjen, 2010; Segonzac and Zipfel, 2011). Plants recognize PAMPs via specific pattern recognition receptors, PRRs. Of several known PRRs, best characterized are the *Arabidopsis* receptor kinase FLAGELLIN SENSING 2 FLS2) and EF-Tu receptor (EFR), that recognizes one of the most abundant and conserved proteins of bacteria, Ef-Tu (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004; Boller and Felix, 2009). Recognition of PAMPs ultimately leads to PTI and hence, improved resistance. Independent of their lifestyle, different types of pathogens trigger plant defenses via PAMP recognition. For example both hemibiotrophic *Pseudomonas* and necrotrophic *Pectobacterium* trigger PTI responses through the recognition of flagellin (Desender et al., 2007; Dodds and Rathjen, 2010). Furthermore, similar to animals, plant immunity also relies on the ability to sense invading microbes by means of endogenous molecular patterns that are present only when plant tissue is infected or damaged (i.e., damage to self). The defense response elicited by recognition of these DAMPs, shares similar elements to that triggered by PAMPs (Boller and Felix, 2009; Zipfel and Robatzek, 2010).

The second line of inducible plant defense is activated in response to pathogen-secreted effectors that aim to suppress the PTI response triggered by PAMP/DAMP recognition (Jones and Dangl, 2006; Dodds and Rathjen, 2010). In contrast to PAMPs, effectors are characteristically variable and dispensable. The difference between effectors and PAMPs is the specificity of effector action to certain pathogen strains, mainly those with biotrophic and hemibiotrophic lifestyles (Tao et al., 2003; Dodds and Rathjen, 2010). For example individual strains of the hemibiotroph *Pseudomonas syringae* usually express 15–30 effectors depending on the strain (Lindeberg et al., 2012). Effectors target many processes in the plant cell. Examples of effector action in dampening the PTI are *P. syringae* effectors AvrPto and AvrPtoB that directly target PAMP receptors FLS2 and EFR (Lindeberg et al., 2012). Effectors can be recognized by corresponding resistance (R) proteins of the plant: either directly or through their action on host targets of the effectors (Jones and Dangl, 2006). The recognition events trigger defense responses, ETI including a local PCD, the HR that is efficient in limiting the infection of biotrophs that require living cells for nutrition (Glazebrook, 2005).

In contrast to biotrophs, necrotrophic pathogens have more aggressive and wide-ranging virulence strategies aiming for host cell death and hence, acquisition of nutrients from dead plant tissue. Some, like *Pectobacterium* secrete an extensive array of PCWDEs and others, like the fungal necrotroph *B. cinerea* rely on the secretion of toxins as main virulence factors (Mengiste, 2012). As a result of their lifestyle also plant immune response to necrotrophs is to some extent contrasting to that triggered by biotrophic pathogens. For example, HR resulting from effector-R-protein interaction would rather benefit than stop necrotrophic pathogens, since the success of their virulence relies on the capability to kill plant cells (Glazebrook, 2005; Mengiste, 2012). Thus, in contrast to biotrophs host cell death can actually be promoted by pathogens with necrotrophic lifestyle to facilitate their infection (Lai et al., 2011). Indeed, necrotrophic fungi such as *B. cinerea* has been shown to trigger dell death by producing ROS and non-specific toxins (Govrin and Levine, 2000) while other fungal necrotrophs employ host selective toxins to subvert ETI to ETS (Mengiste, 2012). Similarly, bacterial necrotrophs like *Pectobacterium* secrete necrosis-inducing proteins like Nip and putative effectors like HrpN proteins to promote cell death (Kariola et al., 2003; Mattinen et al., 2004). Consequently, PTI can be considered as the main plant defense strategy against necrotrophs like *Pectobacterium*.

PHYTOHORMONE SIGNALING IN PLANT IMMUNITY

Interestingly, according to current knowledge, the perception of all the defense elicitors, PAMPs, DAMPs, and effectors appears to trigger similar immediate defense signaling. The difference between PTI and ETI is rather in strength and durability of the response than in quality and of these ETI is stronger and longer lasting (Tao et al., 2003; Espinosa and Alfano, 2004; Kim et al., 2005; Jones and Dangl, 2006). The defense responses are typically mediated by and dependent on the action of different phytohormones and indeed, depending on the lifestyle of the attacking pathogen, plant synthesizes one or more phytohormones to achieve the best possible defense response. The roles of SA, JA, and ET in orchestrating the main defense pathways triggered in response to different pathogens are well-established (Kunkel and Brooks, 2002; Glazebrook, 2005; Pieterse et al., 2009). SA has traditionally been thought to activate defense signaling targeted against biotrophic and hemibiotrophic pathogens while JA and ET defenses are associated with defense responses against necrotrophs (Kunkel and Brooks, 2002; Glazebrook, 2005). Although this remains broadly true, the signaling network triggered by many pathogens appears more complex; for example the combination of JA and ET signaling is efficient against the necrotroph *B. cinerea*, yet also SA appears to have a role in local immunity against this fungus (Ferrari et al., 2007). Interestingly, resistance against *Pectobacterium* can be enhanced by the induction of either JA/ET-mediated (Vidal et al., 1997; Norman-Setterblad et al., 2000) or SA-mediated (Palva et al., 1994; Li et al., 2004) defenses. This apparent controversy could be explained partly by the overlapping defenses triggered and partly by the different efficacies of the defenses induced by the two pathways at the different stages of the infection. Thus, SA-mediated defenses appear to be more efficient during the latent phase of infection, i.e., when PTI is triggered via PAMP recognition (Li et al., 2004, 2006; Kariola et al., 2005). During the necrotrophic phase of *Pectobacterium* infection secretion of massive amounts of PCWDEs results in prominent tissue maceration and release of

DAMPs activating JA/ET-dependent defenses (Palva et al., 1993; Vidal et al., 1997; Norman-Setterblad et al., 2000; Brader et al., 2001).

Defense pathways influence each other through a network of regulatory interactions, and thus, plant responses to pathogens are a result of this complex hormonal crosstalk (Kunkel and Brooks, 2002; Bostock, 2005; Robert-Seilaniantz et al., 2011). Crosstalk (both synergistic and antagonistic) between the hormonal pathways is indeed central to defense signaling and in defining the response priorities. For example, SA and JA signaling interact on many levels, and this relationship is in many cases mutually antagonistic (Kunkel and Brooks, 2002; Spoel et al., 2003; Li et al., 2004; Glazebrook, 2005; Spoel and Loake, 2011). For example, synthesis of JA as well as accumulation of proteinase inhibitors in response to wounding and oligosaccharides can be inhibited by SA (Pena-Cortés et al., 1993; Doares et al., 1995). Conversely, overexpression of transcription factor *WRKY70*, a central component in SA signaling in *Arabidopsis* was followed by increased SA and decreased JA signaling resulting in enhanced resistance to the hemibiotroph *Pseudomonas* but susceptibility to the fungal necrotroph *A. brassicicola* suggesting that WRKY70 is a node of interaction between these hormonal pathways (Li et al., 2004, 2006).

While the roles of phytohormones SA, JA, and ET in orchestrating the main defense pathways are well-established, other phytohormones can modulate and influence the outcome of pathogen triggered defense signaling and there is even crosstalk between biotic and abiotic signaling (Fujita et al., 2006). ABA mediates adaptive responses to various abiotic stresses and is also central to many developmental processes (Finkelstein et al., 2002; Verslues and Zhu, 2005). The role of ABA in plant–pathogen interaction is multifaceted (Ton et al., 2009; Cao et al., 2011). Activation of ABA biosynthetic and signaling pathways promotes disease susceptibility to several plant pathogens. Many studies have demonstrated antagonism between ABA and SA signaling. Endogenous ABA accumulation induced by drought stress or ABA treatment prior to infection with a virulent *P. syringae pv tomato* resulted in necrosis and chlorosis in *Arabidopsis*, symptoms similar to susceptible infection (Mohr and Cahill, 2003). Moreover, overexpression of a negative regulator of ABA-responses, *ERD15* resulted in enhanced SA signaling and improved tolerance to *Pectobacterium* (Kariola et al., 2006; Aalto et al., 2012).

Both synergistic and antagonistic effects have been described for the interaction of ABA with JA/ET signaling. (Anderson et al., 2004; Mauch-Mani and Mauch, 2005; Adie et al., 2007). For example, disruption of AtMYC2, a positive regulator of ABA signaling, resulted in up-regulation of JA/ET-dependent gene expression. Additionally, ABA-deficient mutants were shown to have improved resistance against necrotrophic fungal pathogen *Fusarium oxysporum* in *Arabidopsis* (Anderson et al., 2004). At the same time, disruption of BOS1 (*Botrytis susceptible 1*), that controls several ABA- and JA-regulated genes resulted in decreased tolerance to necrotrophic pathogens but also to water deficit and salt stress (Mengiste et al., 2003).

The control of stomatal aperture and hence plant water relations is one of the processes under strict hormonal control mainly by ABA. Interestingly, *Arabidopsis* stomata also close in response to bacteria or bacterial PAMPs such as Flg22 and LPS, altering their role from being plain passive pathogen entry portals into actual components of plant innate immunity (Melotto et al., 2006). PAMP-triggered stomatal closure requires SA and ABA, and thus, the response is impaired for example in ABA biosynthesis mutants (Melotto et al., 2006). Furthermore, Melotto and colleagues demonstrated that *P. syringae* strains producing the JA-Ile mimic coronatine were able to induce stomatal re-opening (Melotto et al., 2006; Zeng and He, 2010). Intriguingly, similarly to the hemibiotroph *P. syringae,* even the necrotroph *Pectobacterium* is capable of inducing stomatal re-opening after the initial, PAMP-triggered stomatal closure in *Arabidopsis* (Po-Wen et al., 2013). Furthermore, priming of PTI response with the non-protein amino acid BABA (β-aminobutyric acid) was shown to enhance SA-dependent defenses, inhibit stomatal re-opening and hence, increase the plant tolerance to *Pectobacterium* (Po-Wen et al., 2013).

DAMAGE-ASSOCIATED MOLECULAR PATTERNS – OLIGOGALACTURONIDES

Besides rapid recognition of PAMPS, both plants and animals need to sense endogenous molecular patterns that are released upon tissue damage. Such damage can result from wounding caused by chewing insects or herbivores, or degradation of plant cell walls by microbial enzymes (Boller and Felix, 2009). The released cell wall fragments act as DAMPs and trigger PTI. Secretion of PCWDEs is central to the virulence of many necrotrophic fungi and bacteria. The fragments of plant cell wall, cutin monomers and peptides released by the action of these enzymes act as DAMPs (Boller and Felix, 2009; Galletti et al., 2009). The released peptides include systemin found in the *Solanaceae* family that triggers a response similar to that induced by mechanical wounding (Hind et al., 2010). AtPeps of *Arabidopsis* resembles systemin and are believed to be released and bind their apoplastic receptors upon cell damage (Huffaker et al., 2006). Moreover, homologues of AtPeps have now been found in most higher plants (Huffaker et al., 2013) and have been shown to induce defense against necrotrophic pathogens (Liu et al., 2013). Similar to these peptides, oligogalacturonides (OGs) seem to act as DAMPs throughout the plant kingdom and hence, operate in an evolutionary old danger sensing system resulting in PTI even in monocots (Baker et al., 1990; Côté and Hahn, 1994; Randoux et al., 2009, 2010). OGs are biologically active carbohydrates (oligosaccharins) that are breakdown products of homogalacturonan, a major component of pectin (Côté and Hahn, 1994; Ridley et al., 2001). OGs of varying chain length with a degree of polymerization (DP) ranging from 2 to over 20 are released by PGs of both bacterial and fungal pathogens. These enzymes typically break down the non-methylated polygalacturonan component of pectin and play an important role in the infection by necrotrophs. In *P. carotovorum* the endo-polygalacturonase PehA is one of the major players carrying out this function, whereas *Dickeya dadantii* only has exo cleaving PGs (Kotoujansky, 1987; Saarilahti et al., 1990; Hugouvieux-Cotte-Pattat et al., 2001). The OGs released by the action of PCWDEs secreted by necrotrophs like *Pectobacterium* trigger typical PTI responses (OG-PTI) overlapping at least partly with those induced by PAMPs including oxidative burst, cell wall strengthening, production of phytoalexins and proteinase

inhibitors as well as hormone biosynthesis (Ridley et al., 2001). PCWDs are not only secreted by necrotrophs, but also play a critical role during the colonization of plant roots by symbiotic rhizobia and it has recently been proposed that OGs play a role in *Rhizobium*-legume communications (Moscatiello et al., 2012).

OLIGOGALACTURONIDE PERCEPTION

Although OGs were the first oligosaccharins characterized (Bishop et al., 1981; Hahn et al., 1981), the signaling pathways still largely remain to be elucidated. Indeed, it was not until quite recently that the first receptor for OGs was identified (Brutus et al., 2010). The elucidation of OG signaling has been hampered by the complexity of OG responses (Côté and Hahn, 1994): OGs are involved in control of plant growth and development as well as in plant response to pathogens. The interconnected nature of OG-PTI and plant growth and development through phytohormone regulation adds another layer of complexity to this analysis. Thus, when focusing on the role of OGs in plant–pathogen interactions it is not possible, nor even desirable, to ignore the developmental role of OGs. As an example, the first observed development-related effect of exogenously applied OGs was an inhibition of auxin-induced stem elongation in peas (Branca et al., 1988), and since then further studies have solidified the role of OGs as having an antagonistic effect on auxin signaling and enhancing cytokinin-induced vegetative shoot formation (Falasca et al., 2008). Clarifying the mechanistics behind this antagonistic role to auxin could provide a fruitful approach in elucidating the detailed role of OGs in plant–pathogen interactions (Savatin et al., 2011; Qi et al., 2012).

It has long been suspected that wall-associated kinases (WAKs) are involved in OG sensing. WAK1 and WAK2 have been shown to bind to pectin *in vitro* (Kohorn et al., 2009) and WAK1 has been shown to bind specifically to OGs (Morris et al., 1982; Decreux and Messiaen, 2005; Cabrera et al., 2008). The *in vitro* binding of OGs to WAK1 required OGs with a DP over nine subunits and more particularly it seems to require formation of a calcium-induced conformational state known as egg box dimers (Morris et al., 1982; Decreux and Messiaen, 2005; Cabrera et al., 2008). The egg box form progressively, and there seems to be two different forms of perception systems in which WAK1 can recognize these dimers (Cabrera et al., 2008). Even shorter chains OGs can form calcium-induced egg box dimers. However, unlike the dimers formed by longer chains, these are unstable and easily disrupted by competing monovalent ions.

Additional indication for the role of WAKs in OG signaling came from gene expression studies demonstrating that *WAK1* is up-regulated by OGs (Denoux et al., 2008). However, silencing of the WAK gene family resulted in lethality, probably due to their involvement in regulation of growth and development. Furthermore, redundancy between different WAKs complicated the study of these potential receptors (Brutus et al., 2010). Following up on the leads, Brutus et al recently employed a domain swap approach to verify that WAK1 indeed does act as a receptor of OGs. They created chimeric receptors of EFR and WAK1 and showed that the WAK1 ectodomain could be triggered by long chain OGs to activate the EFR kinase domain, and vice versa the EFR ectodomain could be triggered by elf18 peptide to activate the WAK1 kinase domain, resulting in a defense response mimicking a normal OG response. Furthermore, WAK1 overexpressing plants were seen to be more resistant to *B. cinerea* (Brutus et al., 2010). These studies indeed suggest that WAKs could be PRRs that are involved in OG perception but do not rule out the presence of other receptor-like kinases (RLKs) involved in monitoring the cell wall integrity. Such potential candidates include for example the potato RLKs responsive to short OGs and *Pectobacterium* (Montesano et al., 2001).

OLIGOGALACTURONIDE SIGNALING

Oligogalacturonides have been shown to rapidly stimulate an increase in cytosolic calcium (Messiaen et al., 1993; Hu et al., 2004) and production of ROS, in a wide array of different plant species (Ridley et al., 2001). Other OG-PTI responses include; expression of proteinase inhibitors, induction of phenylalanine ammonia-lyase (PAL) leading to production of phytoalexins, peroxidases, glucosinolates, lignin, production of chitinase and β-1,3-glucanase, as well as increased expression of PG-inhibiting proteins (PGIPs; reviewed in Côté and Hahn, 1994; Ridley et al., 2001). OGs induce a very strong AtRBOHD-dependent apoplastic ROS burst in *Arabidopsis* (Galletti et al., 2008). However, this oxidative burst appears not to be required for OG-induced resistance against *B. cinerea* nor expression of several OG marker genes; PAD3, AtPGIP1, RetOx, CYP 81F2, and AtWRKY40. Also it would seem that induced callose deposition does not play a major role in basal or elicitor-induced resistance to *B. cinerea* (Galletti et al., 2008).

Binding of OGs to WAK1 and most biological responses appear to require longer chain OGs (Brutus et al., 2010). However, there are a number of studies that indicate plant responses to short chain OGs. Such responses include induction of genes involved in JA biosynthesis (Norman et al., 1999) in *Arabidopsis*, induction of ET production (O'Donnell et al., 1996; Simpson et al., 1998) and production of proteinase inhibitors (Thain et al., 1990; Moloshok et al., 1992; O'Donnell et al., 1996) and depolarization of leaf mesophyll cells (Thain et al., 1990), induction of RLKs (Montesano et al., 2001) and induction of resistance against *P. carotovorum* (Weber et al., 1996; Wegener et al., 1996) in potato. Also short OGs have been seen to have a developmental effect by increasing the shoot and leaf number in strawberry plants (Miranda et al., 2007). In summary, although recent studies indicate a requirement of longer chain OGs, the specific role of shorter OGs as elicitors of PTI and developmental responses remains to be clarified. One could speculate that shorter OGs play a larger role in resistance against bacterial necrotrophs and herbivores whereas longer OGs play a more significant role against necrotrophic fungi.

Polygalacturonase-inhibiting proteins are among the OG-PTI-induced proteins produced in response to fungal necrotrophs and act directly as a defense protein by reducing the hydrolytic activity of fungal PGs, but also by delaying the breakdown by PGs they increase the formation of longer chain OGs thought to be more biologically active (De Lorenzo et al., 2001; Decreux and Messiaen, 2005).The role of PGIPs in defense against pathogens has mainly been studied using fungi, such as for example *B. cinerea* (De Lorenzo et al., 2001). However, recent studies have identified PGIP as a potentially important player also in plant defense against bacteria: PGIPs were seen to play a

role in resistance of Chinese cabbage against *P. carotovorum ssp. carotovorum* (Hwang et al., 2010) and PGIPS from tomato where shown to inhibit PGs from *Ralstonia solanacearum* (Schacht et al., 2011).

Several studies have characterized the reprogramming of the transcriptome in response to OGs using microarrays of *Arabidopsis* exposed to exogenous long chain (DP 10–15) OGs and also compared the expression changes between OG-PTI and Flg22-PTI (Moscatiello et al., 2006; Ferrari et al., 2007; Denoux et al., 2008). The first genome wide transcriptome analysis OG responses employed mesophyll cell suspension cultures and focused on elucidating calcium-dependent and independent signaling pathways (Moscatiello et al., 2006). The study showed that OG-induced activation of genes involved in ET signaling required both pathways, whereas activation of JA-responsive genes appeared mainly calcium-dependent, in agreement with an earlier study (Hu et al., 2003). Further it would seem that protein kinase-dependent phosphorylation is involved in the early stages of OG signaling (Moscatiello et al., 2006). Taking a slightly different approach Ferrari et al. (2007) compared OG responses with responses to infection with *B. cinerea*. The results indicated that approximately 50% of all genes were similarly regulated upon both treatments. OG-induced resistance to *B. cinerea* was found to be independent of JA, ET, and SA signaling and dependent on PAD3. Further it was shown that both Flg22 and OGs induced resistance to *B. cinerea*. As seen previously for AtPGIP1 (Ferrari et al., 2003), PAD3 was induced independently of JA, ET, and SA. This approach was further expanded trying to elucidate the similarities and dissimilarities in response to exogenous OGs and Flg22 (Denoux et al., 2008). In general, the defense response triggered by the DAMP (OG) and the PAMP (Flg22) were quite similar. Both responses were seen to be fast and transient, with a high degree of overlap especially at shorter time points. Responses to Flg22 were generally stronger, both in number of genes and expression levels. Both Flg22 and OGs were found to activate multiple components of ET, JA, and SA pathways. Noticeably several SA-dependent genes in general, and *PR1* in particular, were found to be significantly induced only by Flg22 but not with long chain

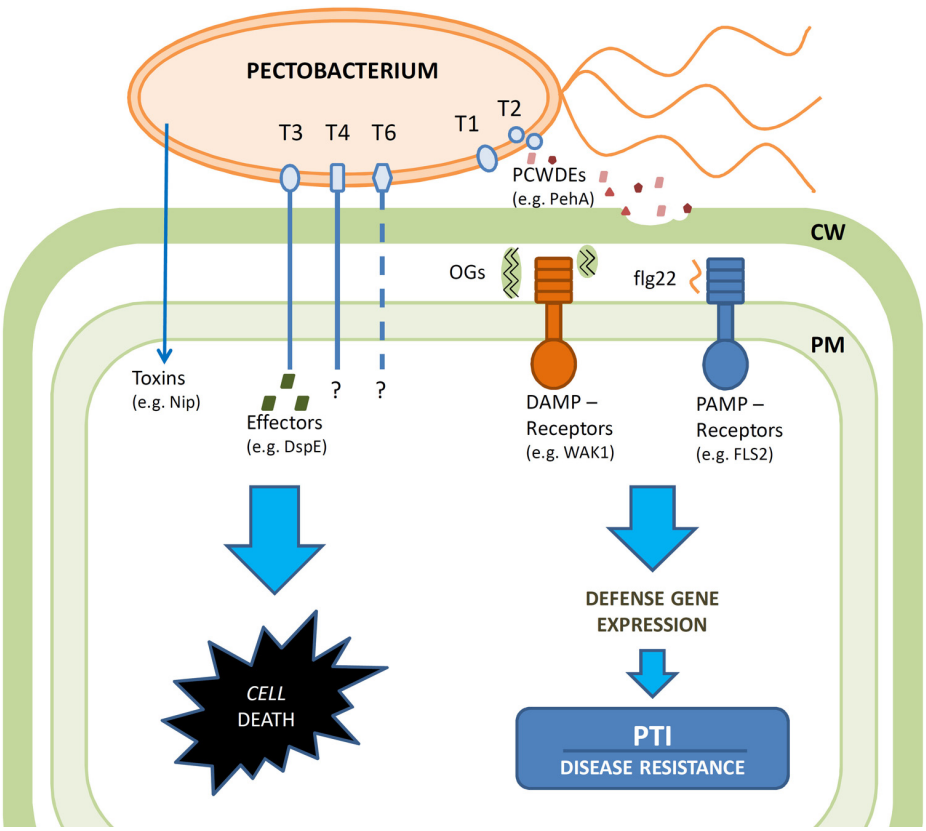


FIGURE 1 | Schematic diagram of the interactions between the bacterial necrotroph *Pectobacterium* and its host plants. *Pectobacterium* virulence relies on macerating plant tissue through the action of PCWDEs secreted by the type I (T1) and type II (T2) secretion systems. Plant cell death is promoted by the action of toxins such as Nip and the effector DspE, which is secreted though the type III (T3) secretion system. Type IV (T4) secretion system and type VI (T6) secretion system may contribute to virulence. Plant immune responses are triggered by recognition of conserved pathogen-associated molecular patterns (PAMPs) such as flagella or damage-associated molecular patterns (DAMPs) such as OGs released by the action of PCWDEs by respective pattern recognition receptors. These recognition events in turn trigger partly overlapping defense responses including induction of defense gene expression and synthesis of various defensive compounds such as phytoalexins, defensins and PR-proteins – resulting in PTI. Bacteria can attenuate PTI particularly in the early phase of infection by tight control of PCWDE production minimizing DAMP generation. The defenses are overwhelmed at later stages by promotion of cell death and massive PCWDE production at high bacterial cell densities.

OGs, even after extended exposure. This is in contrast to an earlier study characterizing *Arabidopsis* response to mixed length OGs and showing calcium and H_2O_2-dependent induction of several defense-related marker genes; *CHS*, *GST*, *PAL*, and noticeably *PR1* (Hu et al., 2004). In conclusion the comparison of OG- and Flg22-triggered responses suggest that DAMP-PTI might rely more on the JA/ET-dependent signaling, in agreement with several studies of *Pectobacterium* (Palva et al., 1993; Doares et al., 1995; Vidal et al., 1997; Norman et al., 1999; Norman-Setterblad et al., 2000). This is logical, since jasmonates and other oxylipins have central role in defense responses following tissue damage and have been proposed to mediate the induction of defense in response to OG signals generated by pathogen or herbivore attacks (Farmer and Ryan, 1992).

Interestingly, recent studies indicate participation of NO in OG-PTI (Rasul et al., 2012). It was demonstrated that exogenous OGs trigger calcium and nitrate reductase-dependent NO production in *Arabidopsis*. Further, NO was found to adjust AtRBOHD-mediated ROS production, as well as regulation of OG responsive genes (*PER4* and a β-1,3-glucanase). Furthermore, NO was found to contribute to OG-induced immunity against *B. cinerea*. Whether this applies to *Pectobacterium* remains to be demonstrated.

SUMMARY

In summary, see **Figure 1**, PTI appears central to plant defense against broad host range bacterial necrotrophs like *Pectobacterium*. ETI, which is highly efficient against (hemi)biotrophs such as *Pseudomonas* is not an effective strategy to combat necrotroph infection, as ETI relies on localized cell death to trigger the downstream defense responses. Necrotrophs like *Pectobacterium* employ induction of cell death as part of their virulence strategy, thus ETI would rather enhance than prevent the infection. Indeed *Pectobacterium* species have a very limited arsenal of T3 effectors and those few that have been studied (e.g., DspE) appear to promote infection by causing cell death. Consequently, plant immune responses are triggered by recognition of conserved pathogen-associated molecular patterns (PAMPs) such as flagella or damage-associated molecular patterns (DAMPs) such as OGs released by the action of PCWDEs by respective pattern recognition receptors. While the PTI induced by PAMP recognition is a common response to both biotrophs and necrotrophs, DAMPs are more typical to necrotrophic interactions. Consequently, *Pectobacterium* strives to attenuate PTI particularly in the early phase of infection by tight control of PCWDE production minimizing DAMP generation. PAMP and DAMP recognition events trigger partly overlapping defense responses including induction of defense gene expression and synthesis of various defensive compounds such as phytoalexins, defensins, and PR-proteins – resulting in PTI. Indeed prior induction of either response will enhance plant resistance to *Pectobacterium*. Elucidating the molecular details of these two partially redundant signal networks is essential for our understanding of the plant-necrotroph interactions and can take advantage of the rapidly developing genomic techniques including transcriptional profiling and RNA sequencing combined with the powerful genetic screens available in *Arabidopsis* for mutants altered in their PTI responses. In particular elucidation of the less well-characterized OG-induced PTI deserves further studies including correlation of the chain length of the OG elicitor to a particular response at specific stages of infection and defining the downstream components that are most significant for bacterial resistance. Such studies would be crucial for providing new insights into plant defense strategies against necrotrophs. Further genome level analysis would also help to elucidate the interactions of *Pectobacterium* with other plant associated microbes, as well as their insect vectors.

REFERENCES

Aalto, M. K., Helenius, E., Kariola, T., Pennanen, V., Heino, P., Hõrak, H., et al. (2012). ERD15–an attenuator of plant ABA responses and stomatal aperture. *Plant Sci.* 182, 19–28. doi: 10.1016/j.plantsci.2011.08.009

Adie, B. A. T., Pérez-Pérez, J., Pérez-Pérez, M. M., Godoy, M., Sánchez-Serrano, J.-J., Schmelz, E. A., et al. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* 19, 1665–1681. doi: 10.1105/tpc.106.048041

Alfano, J. R., and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42, 385–414. doi: 10.1146/annurev.phyto.42.040103.110731

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16, 3460–3479. doi: 10.1105/tpc.104.025833

Baghaee-Ravari, S., Rahimian, H., Shams-Bakhsh, M., Lopez-Solanilla, E., Antúnez-Lamas, M., and Rodríguez-Palenzuela, P. (2010). Characterization of *Pectobacterium* species from Iran using biochemical and molecular methods. *Eur. J. Plant Pathol.* 129, 413–425. doi: 10.1007/s10658-010-9704-z

Baker, C. J., Mock, N., Atkinson, M. M., and Hutcheson, S. (1990). Inhibition of the hypersensitive response in tobacco by pectate lyase digests of cell wall and of polygalacturonic acid. *Physiol. Mol. Plant Pathol.* 37, 155–167. doi: 10.1016/0885-5765(90)90008-L

Basset, A., Khush, R. S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J. A., et al. (2000). The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3376–3381. doi: 10.1073/pnas.97.7.3376

Basset, A., Tzou, P., Lemaitre, B., and Boccard, F. (2003). A single gene that promotes interaction of a phytopathogenic bacterium with its insect vector, *Drosophila* melanogaster. E*MBO Rep.* 4, 205–209. doi: 10.1038/sj.embor.embor730

Bell, K. S., Sebaihia, M., Pritchard, L., Holden, M. T. G., Hyman, L. J., Holeva, M. C., et al. (2004). Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. atroseptica and characterization of virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11105–11110. doi: 10.1073/pnas.0402424101

Bender, C. L., Alarcón-Chaidez, F., and Gross, D. C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* 63, 266–292.

Bishop, P. D., Makus, D. J., Pearce, G., and Ryan, C. A. (1981). Proteinase inhibitor-inducing factor activity in tomato leaves resides in oligosaccharides enzymically released from cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 78, 3536–3540. doi: 10.1073/pnas.78.6.3536

Boller, T. (1995). Chemoperception of microbial signals in plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46, 189–214. doi: 10.1146/annurev.pp.46.060195.001201

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bostock, R. M. (2005). Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* 43, 545–580. doi: 10.1146/annurev.phyto.41.052002.095505

Brader, G., Tas, É., and Palva, E. T. (2001). Jasmonate-dependent induction of indole glucosinolates in *Arabidopsis* by culture filtrates of the nonspecific pathogen *Erwinia*

carotovora. *Plant Physiol.* 126, 849–860. doi: 10.1104/pp.126.2.849

Branca, C., De Lorenzo, G., and Cervone, F. (1988). Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. *Physiol. Plant.* 72, 499–504. doi: 10.1111/j.1399-3054.1988.tb09157.x

Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9452–9457. doi: 10.1073/pnas.1000675107

Cabrera, J. C., Boland, A., Messiaen, J., Cambier, P., and Van Cutsem, P. (2008). Egg box conformation of oligogalacturonides: the time-dependent stabilization of the elicitor-active conformation increases its biological activity. *Glycobiology* 18, 473–482. doi: 10.1093/glycob/cwn027

Cao, F. Y., Yoshioka, K., and Desveaux, D. (2011). The roles of ABA in plant–pathogen interactions. *J. Plant Res.* 124, 489–499. doi: 10.1007/s10265-011-0409-y

Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. (2012). The role of secretion systems and small molecules in soft-rot enterobacteriaceae pathogenicity. *Annu. Rev. Phytopathol.* 50, 425–449. doi: 10.1146/annurev-phyto-081211-173013

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., et al. (2003). An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.* 36, 577–588. doi: 10.1046/j.1365-313X.2003.01902.x

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host–microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814. doi: 10.1016/j.cell.2006.02.008

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.-M., Simon, A., et al. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiol. Lett.* 277, 1–10. doi: 10.1111/j.1574-6968.2007.00930.x

Christie, P. J., Atmakuri, K., Krishnamoorthy, V., Jakubowski, S., and Cascales, E. (2005). Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu. Rev. Microbiol.* 59, 451–485. doi: 10.1146/annurev.micro.58.030603.123630

Collmer, A., Schneider, D. J., and Lindeberg, M. (2009). Lifestyles of the effector rich: genome-enabled characterization of bacterial plant pathogens. *Plant Physiol.* 150, 1623–1630. doi: 10.1104/pp.109.140327

Costacurta, A., and Vanderleyden, J. (1995). Synthesis of phytohormones by plant-associated bacteria. *Crit. Rev. Microbiol.* 21, 1–18. doi: 10.3109/10408419509113531

Côté, F., and Hahn, M. G. (1994). Oligosaccharins: structures and signal transduction. *Plant Mol. Biol.* 26, 1379–1411. doi: 10.1007/BF00016481

Czajkowski, R., Pérombelon, M. C. M., van Veen, J. A., and van der Wolf, J. M. (2011). Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. *Plant Pathology* 60, 999–1013. doi: 10.1111/j.1365-3059.2011.02470.x

DebRoy, S., Thilmony, R., Kwack, Y.-B., Nomura, K., and He, S. Y. (2004). A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9927–9932. doi: 10.1073/pnas.0401601101

Decreux, A., and Messiaen, J. (2005). Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. *Plant Cell Physiol.* 46, 268–278. doi: 10.1093/pcp/pci026

De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* 39, 313–335. doi: 10.1146/annurev.phyto.39.1.313

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., et al. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. *Mol. Plant* 1, 423–445. doi: 10.1093/mp/ssn019

Desender, S., Andrivon, D., and Val, F. (2007). Activation of defence reactions in *Solanaceae*: where is the specificity? *Cell. Microbiol.* 9, 21–30. doi: 10.1111/j.1462-5822.2006.00831.x

Doares, S. H., Syrovets, T., Weiler, E. W., and Ryan, C. A. (1995). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4095–4098. doi: 10.1073/pnas.92.10.4095

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Dong, X. (1998). SA, JA, ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* 1, 316–323. doi: 10.1016/1369-5266(88)80053-0

Espinosa, A., and Alfano, J. R. (2004). Disabling surveillance: bacterial type III secretion system effectors that suppress innate immunity. *Cell. Microbiol.* 6, 1027–1040. doi: 10.1111/j.1462-5822.2004.00452.x

Falasca, G., Capitani, F., Della Rovere, F., Zaghi, D., Franchin, C., Biondi, S., et al. (2008). Oligogalacturonides enhance cytokinin-induced vegetative shoot formation in tobacco explants, inhibit polyamine biosynthetic gene expression, and promote long-term remobilisation of cell calcium. *Planta* 227, 835–852. doi: 10.1007/s00425-007-0660-6

Farmer, E. E., and Ryan, C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4, 129–134. doi: 10.1105/tpc.4.2.129

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M., and Dewdney, J. (2007). Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol.* 144, 367–379. doi: 10.1104/pp.107.095596

Ferrari, S., Vairo, D., Ausubel, F. M., Cervone, F., and De Lorenzo, G. (2003). Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell* 15, 93–106. doi: 10.1105/tpc.005165

Finkelstein, R. R., Gampala, S. S. L., and Rock, C. D. (2002). Abscisic acid signaling in seeds and seedlings. *Plant Cell* 14, S15–S45. doi: 10.1105/tpc.010441

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., et al. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.* 9, 436–442. doi: 10.1016/j.pbi.2006.05.014

Galletti, R., De Lorenzo, G., and Ferrari, S. (2009). Host-derived signals activate plant innate immunity. *Plant Signal. Behav.* 4, 33–34. doi: 10.4161/psb.4.1.7224

Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F. M., De Lorenzo, G., et al. (2008). The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in *Arabidopsis* is dispensable for the activation of defense responses effective against *Botrytis cinerea*. *Plant Physiol.* 148, 1695–1706. doi: 10.1104/pp.108.127845

Gardan, L., Gouy, C., Christen, R., and Samson, R. (2003). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 53, 381–391. doi: 10.1099/ijs.0.02423-0

Gelvin, S. B. (2009). *Agrobacterium* in the genomics age. *Plant Physiol.* 150, 1665–1676. doi: 10.1104/pp.109.139873

Glasner, J. D., Marquez-Villavicencio, M., Kim, H.-S., Jahn, C. E., Ma, B., Biehl, B. S., et al. (2008). Niche-specificity and the variable fraction of the *Pectobacterium* pan-genome. *Mol. Plant Microbe Interact.* 21, 1549–1560. doi: 10.1094/MPMI-21-12-1549

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Gómez-Gómez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* 5, 1003–1011. doi: 10.1016/S1097-2765(00)80265-8

Goto, M., and Matsumoto, K. (1987). *Erwinia carotovora* subsp. *wasabiae* subsp. nov. isolated from diseased rhizomes and fibrous roots of Japanese horseradish (*Eutrema wasabi* Maxim.). *Int. J. Syst. Bacteriol.* 37, 130–135. doi: 10.1099/00207713-37-2-130

Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1

Göhre, V., and Robatzek, S. (2008). Breaking the barriers: microbial effector molecules subvert plant immunity. *Annu. Rev. Phytopathol.* 46, 189–215. doi: 10.1146/annurev.phyto.46.120407.110050

Grant, S. R., Fisher, E. J., Chang, J. H., Mole, B. M., and Dangl, J. L.

(2006). Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annu. Rev. Microbiol.* 60, 425–449. doi: 10.1146/annurev.micro.60.080805.142251

Hahn, M. G., Darvill, A. G., and Albersheim, P. (1981). Host–pathogen interactions 1. *Plant Physiol.* 68, 1161–1169. doi: 10.1104/pp.68.5.1161

Hauben, L., Moore, E. R., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., et al. (1998). Phylogenetic position of phytopathogens within the Enterobacteriaceae. *Syst. Appl. Microbiol.* 21, 384–397. doi: 10.1016/S0723-2020(98)80048-9

Hind, S. R., Malinowski, R., Yalamanchili, R., and Stratmann, J. W. (2010). Tissue-type specific systemin perception and the elusive systemin receptor. *Plant Signal. Behav.* 5, 42–44. doi: 10.4161/psb.5.1.10119

Holeva, M. C., Bell, K. S., Hyman, L. J., Avrova, A. O., Whisson, S. C., Birch, P. R. J., et al. (2004). Use of a pooled transposon mutation grid to demonstrate roles in disease development for *Erwinia carotovora* subsp. atroseptica putative type III secreted effector (DspE/A) and helper (HrpN) proteins. *Mol. Plant Microbe Interact.* 17, 943–950. doi: 10.1094/MPMI.2004.17.9.943

Hu, X., Neill, S., Cai, W., and Tang, Z. (2003). Hydrogen peroxide and jasmonic acid mediate oligogalacturonic acid-induced saponin accumulation in suspension-cultured cells of *Panax ginseng*. *Physiol. Plant.* 118, 414–421. doi: 10.1034/j.1399-3054.2003.00124.x

Hu, X. Y., Neill, S. J., Cai, W. M., and Tang, Z. C. (2004). Induction of defence gene expression by oligogalacturonic acid requires increases in both cytosolic calcium and hydrogen peroxide in *Arabidopsis thaliana*. *Cell Res.* 14, 234–240. doi: 10.1038/sj.cr.7290224

Huffaker, A., Pearce, G., and Ryan, C. A. (2006). An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10098–10103. doi: 10.1073/pnas.0603727103

Huffaker, A., Pearce, G., Veyrat, N., Erb, M., Turlings, T. C. J., Sartor, R., et al. (2013). Plant elicitor peptides are conserved signals regulating direct and indirect antiherbivore defense. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5707–5712. doi: 10.1073/pnas.1214668110

Hugouvieux-Cotte-Pattat, N., Blot, N., and Reverchon, S. (2001). Identification of TogMNAB, an ABC transporter which mediates the uptake of pectic oligomers in *Erwinia chrysanthemi* 3937. *Mol. Microbiol.* 41, 1113–1123. doi: 10.1046/j.1365-2958.2001.02564.x

Hwang, B. H., Bae, H., Lim, H.-S., Kim, K. B., Kim, S. J., Im, M.-H., et al. (2010). Overexpression of polygalacturonase-inhibiting protein 2 (PGIP2) of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) increased resistance to the bacterial pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. *Plant Cell Tissue Organ Cult.* 103, 293–305. doi: 10.1007/s11240-010-9779-4

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jones, L. R. (1901). Bacillus carotovorus n. sp., die Ursache einer weichen Fäulnis der Möhre. *Centralblatt Bakteriol. Parasitenkd. Infekt Hyg.* 2, 12–21.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., et al. (1993). The lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J.* 12, 2477–2482.

Kariola, T., Brader, G., Helenius, E., Li, J., Heino, P., and Palva, E. T. (2006). EARLY RESPONSIVE TO DEHYDRATION 15, a negative regulator of abscisic acid responses in *Arabidopsis*. *Plant Physiol.* 142, 1559–1573. doi: 10.1104/pp.106.086223

Kariola, T., Brader, G., Li, J., and Palva, E. T. (2005). Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *Plant Cell* 17, 282–294. doi: 10.1105/tpc.104.025817

Kariola, T., Palomäki, T. A., Brader, G., and Palva, E. T. (2003). *Erwinia carotovora* subsp. *carotovora* and *Erwinia*-derived elicitors HrpN and PehA trigger distinct but interacting defense responses and cell death in *Arabidopsis*. *Mol. Plant Microbe Interact.* 16, 179–187. doi: 10.1094/MPMI.2003.16.3.179

Kay, S., and Bonas, U. (2009). How *Xanthomonas* type III effectors manipulate the host plant. *Curr. Opin. Microbiol.* 12, 37–43. doi: 10.1016/j.mib.2008.12.006

Kim, H.-S., Ma, B., Perna, N. T., and Charkowski, A. O. (2009). Phylogeny and virulence of naturally occurring type III secretion system-deficient Pectobacterium strains. *Appl. Environ. Microbiol.* 75, 4539–4549. doi: 10.1128/AEM.01336-08

Kim, H.-S., Thammarat, P., Lommel, S. A., Hogan, C. S., and Charkowski, A. O. (2011). Pectobacterium carotovorum elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant–microbe interactions. *Mol. Plant Microbe Interact.* 24, 773–786. doi: 10.1094/MPMI-06-10-0143

Kim, M. G., da Cunha, L., McFall, A. J., Belkhadir, Y., DebRoy, S., Dangl, J. L., et al. (2005). Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*. *Cell* 121, 749–759. doi: 10.1016/j.cell.2005.03.025

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., et al. (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60, 974–982. doi: 10.1111/j.1365-313X.2009.04016.x

Koskinen, J. P., Laine, P., Niemi, O., Nykyri, J., Harjunpää, H., Auvinen, P., et al. (2012). Genome sequence of *Pectobacterium* sp. Strain SCC3193. *J. Bacteriol.* 194, 6004. doi: 10.1128/JB.00681-12

Kotoujansky, A. (1987). Molecular genetics of pathogenesis by soft-rot Erwinias. *Annu. Rev. Phytopathol.* 25, 405–430. doi: 10.1146/annurev.py.25.090187.002201

Kunkel, B. N., and Brooks, D. M. (2002).Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* 5, 325–331. doi: 10.1016/S1369-5266(02)00275-3

Lai, Z., Wang, F., Zheng, Z., Fan, B., and Chen, Z. (2011). A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. *Plant J.* 66, 953–968. doi: 10.1111/j.1365-313X.2011.04553.x

Laurie-Berry, N., Joardar, V., Street, I. H., and Kunkel, B. N. (2006). The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. *Mol. Plant Microbe Interact.* 19, 789–800. doi: 10.1094/MPMI-19-0789

Li, J., Brader, G., Kariola, T., and Palva, E. T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* 46, 477–491. doi: 10.1111/j.1365-313X.2006.02712.x

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331. doi: 10.1105/tpc.016980

Lindeberg, M., Cunnac, S., and Collmer, A. (2012). *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. *Trends Microbiol.* 20, 199–208. doi: 10.1016/j.tim.2012.01.003

Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., et al. (2008). Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen Pectobacterium atrosepticum. *PLoS Pathog.* 4:e1000093. doi: 10.1371/journal.ppat.1000093

Liu, Z., Wu, Y., Yang, F., Zhang, Y., Chen, S., Xie, Q., et al. (2013). BIK1 interacts with PEPRs to mediate ethylene-induced immunity. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6205–6210. doi: 10.1073/pnas.1215543110

Ma, B., Hibbing, M. E., Kim, H.-S., Reedy, R. M., Yedidia, I., Breuer, J., et al. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytopathology* 97, 1150–1163. doi: 10.1094/PHYTO-97-9-1150

Mäe, A., Montesano, M., Koiv, V., and Palva, E. T. (2001). Transgenic plants producing the bacterial pheromone *N*-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. *Mol. Plant Microbe Interact.* 14, 1035–1042. doi: 10.1094/MPMI.2001.14.9.1035

Mattinen, L., Tshuikina, M., Mäe, A., and Pirhonen, M. (2004).Identification and characterization of Nip, necrosis-inducing virulence protein of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant Microbe Interact.* 17, 1366–1375. doi: 10.1094/MPMI.2004.17.12.1366

Mauch-Mani, B., and Mauch, F. (2005).The role of abscisic acid in plant–pathogen interactions. *Curr. Opin. Plant Biol.* 8, 409–414. doi: 10.1016/j.pbi.2005.05.015

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980. doi: 10.1016/j.cell.2006.06.054

Mengiste, T. (2012).Plant immunity to necrotrophs. *Annu. Rev. Phytopathol.* 50, 267–294. doi: 10.1146/annurev-phyto-081211-172955

Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R. (2003). The BOTRYTIS SUSCEPTIBLE1 gene encodes an

R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. *Plant Cell* 15, 2551–2565. doi: 10.1105/tpc.014167

Messiaen, J., Read, N. D. V., Cutsem, P., and Trewavas, A. J. (1993). Cell wall oligogalacturonides increase cytosolic free calcium in carrot protoplasts. *J. Cell Sci.* 104, 365–371.

Miranda, J. H., Williams, R. W., and Kerven, G. (2007). Galacturonic acid-induced changes in strawberry plant development in vitro. *In Vitro Cell. Dev. Biol. Plant* 43, 639–643. doi: 10.1007/s11627-007-9052-7

Mohr, P. G., and Cahill, D. M. (2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. *Funct. Plant Biol.* 30, 461–469. doi: 10.1071/FP02231

Moloshok, T., Pearce, G., and Ryan, C. A. (1992). Oligouronide signaling of proteinase inhibitor genes in plants: structure–activity relationships of Di- and trigalacturonic acids and their derivatives. *Arch. Biochem. Biophys.* 294, 731–734. doi: 10.1016/0003-9861(92)90748-L

Montesano, M., Kõiv, V., Mäe, A., and Palva, E. T. (2001). Novel receptor-like protein kinases induced by *Erwinia carotovora* and short oligogalacturonides in potato. *Mol. Plant Pathol.* 2, 339–346. doi: 10.1046/j.1464-6722.2001.00083.x

Morris, E. R., Powell, D. A., Gidley, M. J., and Rees, D. A. (1982).Conformations and interactions of pectins. I. Polymorphism between gel and solid states of calcium polygalacturonate. *J. Mol. Biol.* 155, 507–516. doi: 10.1016/0022-2836(82)90484-3

Moscatiello, R., Baldan, B., Squartini, A., Mariani, P., and Navazio, L. (2012). Oligogalacturonides: novel signaling molecules in Rhizobium-legume communications. *Mol. Plant Microbe Interact.* 25, 1387–1395. doi: 10.1094/MPMI-03-12-0066-R

Moscatiello, R., Mariani, P., Sanders, D., and Maathuis, F. J. M. (2006). Transcriptional analysis of calcium-dependent and calcium-independent signalling pathways induced by oligogalacturonides. *J. Exp. Bot.* 57, 2847–2865. doi: 10.1093/jxb/erl043

Nabhan, S., De Boer, S. H., Maiss, E., and Wydra, K. (2012a). *Pectobacterium aroidearum* sp. nov., a soft rot pathogen with preference for monocotyledonous plants. *Int. J. Syst. Evol. Microbiol.* doi: 10.1099/ijs.0.046011-0 [Epub ahead of print].

Nabhan, S., Wydra, K., Linde, M., and Debener, T. (2012b). The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. *Plant Pathol.* 61, 498–508.. doi: 10.1111/j.1365-3059.2011.02546.x

Nadarasah, G., and Stavrinides, J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* 35, 555–575. doi: 10.1111/j.1574-6976.2011.00264.x

Niks, R. E., and Marcel, T. C. (2009). Nonhost and basal resistance: how to explain specificity? *New Phytol.* 182, 817–828. doi: 10.1111/j.1469-8137.2009.02849.x

Norman, C., Vidal, S., and Palva, E. T. (1999). Oligogalacturonide-mediated induction of a gene involved in jasmonic acid synthesis in response to the cell-wall-degrading enzymes of the plant pathogen *Erwinia carotovora*. *Mol. Plant Microbe Interact.* 12, 640–644. doi: 10.1094/MPMI.1999.12.7.640

Norman-Setterblad, C., Vidal, S., and Palva, E. T. (2000). Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Mol. Plant Microbe Interact.* 13, 430–438. doi: 10.1094/MPMI.2000.13.4.430

Nykyri, J., Niemi, O., Koskinen, P., Nokso-Koivisto, J., Pasanen, M., Broberg, M., et al. (2012). Revised phylogeny and novel horizontally acquired virulence determinants of the model soft rot phytopathogen *Pectobacterium wasabiae* SCC3193. *PLoS Pathog.* 8:e1003013. doi: 10.1371/journal.ppat.1003013

O'Donnell, P. J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H. M. O., and Bowles, D. J. (1996). Ethylene as a signal mediating the wound response of tomato plants. *Science* 274, 1914–1917. doi: 10.1126/science.274.5294.1914

Palva, T. K., Holmström, K. O., Heino, P., and Palva, E. T. (1993). Induction of plant defense response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant Microbe Interact.* 6, 190–196. doi: 10.1094/MPMI-6-190

Palva, T. K., Hurtig, M., Saindrenan, P., and Palva, E. T. (1994). Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. *Mol. Plant Microbe Interact.* 7, 356–363. doi: 10.1094/MPMI-7-0356

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Park, T.-H., Choi, B.-S., Choi, A.-Y., Choi, I.-Y., Heu, S., and Park, B.-S. (2012). Genome sequence of *Pectobacterium carotovorum* subsp. *carotovorum* strain PCC21, a pathogen causing soft rot in Chinese cabbage. *J. Bacteriol.* 194, 6345–6346. doi: 10.1128/JB.01583-12

Parker, J. E. (2003). Plant recognition of microbial patterns. *Trends Plant Sci.* 8, 245–247. doi: 10.1016/S1360-1385(03)00105-5

Pena-Cortés, H., Albrecht, T., Prat, S., Weiler, E. W., and Willmitzer, L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191, 123–128. doi: 10.1007/BF00240903

Pérombelon, M. C. M. (2002). Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathol.* 51, 1–12. doi: 10.1046/j.0032-0862.2001.Short title.doc.x

Perombelon, M. C. M., and Kelman, A. (1980). Ecology of the soft rot erwinias. *Annu. Rev. Phytopathol.* 18, 361–387. doi: 10.1146/annurev.py.18.090180.002045

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. (1993). A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* 12, 2467–2476.

Pitman, A. R., Harrow, S. A., and Visnovsky, S. B. (2009).Genetic characterisation of Pectobacterium wasabiae causing soft rot disease of potato in New Zealand. *Eur. J. Plant Pathol.* 126, 423–435. doi: 10.1007/s10658-009-9551-y

Pitman, A. R., Wright, P. J., Galbraith, M. D., and Harrow, S. A. (2008). Biochemical and genetic diversity of pectolytic enterobacteria causing soft rot disease of potatoes in New Zealand. *Australas. Plant Pathol* 37, 559. doi: 10.1071/AP08056

Po-Wen, C., Singh, P., and Zimmerli, L. (2013).Priming of the *Arabidopsis* pattern-triggered immunity response upon infection by necrotrophic *Pectobacterium carotovorum* bacteria. *Mol. Plant Pathol.* 14, 58–70. doi: 10.1111/j.1364-3703.2012.00827.x

Qi, L., Yan, J., Li, Y., Jiang, H., Sun, J., Chen, Q., et al. (2012). *Arabidopsis thaliana* plants differentially modulate auxin biosynthesis and transport during defense responses to the necrotrophic pathogen Alternaria brassicicola. *New Phytol.* 195, 872–882. doi: 10.1111/j.1469-8137.2012.04208.x

Randoux, B., Renard-Merlier, D., Duyme, F., Sanssené, J., Courtois, J., Durand, R., et al. (2009). Oligogalacturonides induce resistance in wheat against powdery mildew. *Commun. Agric. Appl. Biol. Sci.* 74, 681–685.

Randoux, B., Renard-Merlier, D., Mulard, G., Rossard, S., Duyme, F., Sanssené, J., et al. (2010). Distinct defenses induced in wheat against powdery mildew by acetylated and nonacetylated oligogalacturonides. *Phytopathology* 100, 1352–1363. doi: 10.1094/PHYTO-03-10-0086

Rantakari, A., Virtaharju, O., Vähämiko, S., Taira, S., Palva, E. T., Saarilahti, H. T., et al. (2001). Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: partial characterization of the hrp gene cluster. *Mol. Plant Microbe Interact.* 14, 962–968. doi: 10.1094/MPMI.2001.14.8.962

Rasul, S., Dubreuil-Maurizi, C., Lamotte, O., Koen, E., Poinssot, B., Alcaraz, G., et al. (2012). Nitric oxide production mediates oligogalacturonide-triggered immunity and resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Plant Cell Environ.* 35, 1483–1499. doi: 10.1111/j.1365-3040.2012.02505.x

Records, A. R. (2011). The type VI secretion system: a multipurpose delivery system with a phage-like machinery. *Mol. Plant Microbe Interact.* 24, 751–757. doi: 10.1094/MPMI-11-10-0262

Ridley, B. L., O'Neill, M. A., and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57, 929–967. doi: 10.1016/S0031-9422(01)00113-3

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20, 537–542. doi: 10.1101/gad.366506

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Ross, J. R., Nam, K. H., D'Auria, J. C., and Pichersky, E. (1999). *S*-Adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents

a new class of plant methyltransferases. *Arch. Biochem. Biophys.* 367, 9–16. doi: 10.1006/abbi.1999.1255

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., and Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature* 475, 343–347. doi: 10.1038/nature10244

Saarilahti, H. T., Heino, P., Pakkanen, R., Kalkkinen, N., Palva, I., and Palva, E. T. (1990).Structural analysis of the pehA gene and characterization of its protein product, endopolygalacturonase, of *Erwinia carotovora* subspecies *carotovora*. *Mol. Microbiol.* 4, 1037–1044. doi: 10.1111/j.1365-2958.1990.tb00676.x

Salmond, G. P., Bycroft, B. W., Stewart, G. S., and Williams, P. (1995). The bacterial "enigma": cracking the code of cell-cell communication. *Mol. Microbiol.* 16, 615–624. doi: 10.1111/j.1365-2958.1995.tb02424.x

Samson, R., Legendre, J. B., Christen, R., Fischer-Le Saux, M., Achouak, W., and Gardan, L. (2005). Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 55, 1415–1427. doi: 10.1099/ijs.0.02791-0

Savatin, D. V., Ferrari, S., Sicilia, F., and De Lorenzo, G. (2011). Oligogalacturonide-auxin antagonism does not require post-transcriptional gene silencing or stabilization of auxin response repressors in *Arabidopsis*. *Plant Physiol.* 157, 1163–1174. doi: 10.1104/pp.111.184663

Schacht, T., Unger, C., Pich, A., and Wydra, K. (2011). Endo- and exopolygalacturonases of *Ralstonia solanacearum* are inhibited by polygalacturonase-inhibiting protein (PGIP) activity in tomato stem extracts. *Plant Physiol. Biochem.* 49, 377–387. doi: 10.1016/j.plaphy.2011.02.001

Schwarz, S., West, T. E., Boyer, F., Chiang, W.-C., Carl, M. A., Hood, R. D., et al. (2010). Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS Pathog.* 6:e1001068. doi: 10.1371/journal.ppat.1001068

Segonzac, C., and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* 14, 54–61. doi: 10.1016/j.mib.2010.12.005

Simpson, S. D., Ashford, D. A., Harvey, D. J., and Bowles, D. J. (1998). Short chain oligogalacturonides induce ethylene production and expression of the gene encoding aminocyclopropane 1-carboxylic acid oxidase in tomato plants. *Glycobiology* 8, 579–583. doi: 10.1093/glycob/8.6.579

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770. doi: 10.1105/tpc.009159

Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Curr. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.-S., Han, B., et al. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* 15, 317–330. doi: 10.1105/tpc.007591

Thain, J. F., Doherty, H. M., Bowles, D. J., and Wildon, D. C. (1990). Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarization of tomato leaf cells. *Plant Cell Environ.* 13, 569–574. doi: 10.1111/j.1365-3040.1990.tb01074.x

Ton, J., Flors, V., and Mauch-Mani, B. (2009).The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* 14, 310–317. doi: 10.1016/j.tplants.2009.03.006

Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. (2003). Soft rot erwiniae: from genes to genomes. *Mol. Plant Pathol.* 4, 17–30. doi: 10.1046/j.1364-3703.2003.00149.x

Toth, I. K., and Birch, P. R. J. (2005).Rotting softly and stealthily. *Curr. Opin. Plant Biol.* 8, 424–429. doi: 10.1016/j.pbi.2005.04.001

Toth, I. K., Pritchard, L., and Birch, P. R. J. (2006). Comparative genomics reveals what makes an enterobacterial plant pathogen. *Annu. Rev. Phytopathol.* 44, 305–336. doi: 10.1146/annurev.phyto.44.070505.143444

Toth, I. K., van der Wolf, J. M., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., et al. (2011). Dickeya species: an emerging problem for potato production in Europe. *Plant Pathol.* 60, 385–399. doi: 10.1111/j.1365-3059.2011.02427.x

Uppalapati, S. R., Ishiga, Y., Wangdi, T., Kunkel, B. N., Anand, A., Mysore, K. S., et al. (2007). The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant Microbe Interact.* 20, 955–965. doi: 10.1094/MPMI-20-8-0955

van Kan, J. A. L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11, 247–253. doi: 10.1016/j.tplants.2006.03.005

Verslues, P. E., and Zhu, J.-K. (2005). Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. *Biochem. Soc. Trans.* 33, 375–379. doi: 10.1042/BST0330375

Vidal, S., Ponce de Leon, I., Denecke, J., and Palva, E. T. (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce plant defense genes by antagonistic pathways. *Plant J.* 115–123. doi: 10.1046/j.1365-313X.1997.11010115.x

Weber, J., Olsen, O., Wegener, C., and von Wettstein, D. (1996). Digalacturonates from pectin degradation induce tissue responses against potato soft rot. *Physiol. Mol. Plant Pathol.* 48, 389–401. doi: 10.1006/pmpp.1996.0031

Wegener, C., Bartling, S., Olsen, O., Weber, J., and von Wettstein, D. (1996). Pectate lyase in transgenic potatoes confers pre-activation of defence against *Erwinia carotovora*. *Physiol. Mol. Plant Pathol.* 49, 359–376. doi: 10.1006/pmpp.1996.9998

Winslow, C. E., Broadhurst, J., Buchanan, R. E., Krumwiede, C., Rogers, L. A., and Smith, G. H. (1920). The families and genera of the bacteria: final report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types. *J. Bacteriol.* 5, 191–229.

Yang, S., Zhang, Q., Guo, J., Charkowski, A. O., Glick, B. R., Ibekwe, A. M., et al. (2007). Global effect of indole-3-acetic acid biosynthesis on multiple virulence factors of *Erwinia chrysanthemi* 3937. *Appl. Environ. Microbiol.* 73, 1079–1088. doi: 10.1128/AEM.01770-06

Zeng, W., and He, S. Y. (2010). A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv tomato DC3000 in *Arabidopsis*. *Plant Physiol.* 153, 1188–1198. doi: 10.1104/pp.110.157016

Zheng, J., Ho, B., and Mekalanos, J. J. (2011). Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PLoS ONE* 6:e23876. doi: 10.1371/journal.pone.0023876

Zheng, X.-Y., Spivey, N. W., Zeng, W., Liu, P.-P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596. doi: 10.1016/j.chom.2012.04.014

Zipfel, C., and Robatzek, S. (2010). Pathogen-associated molecular pattern-triggered immunity: Veni, Vidi. . .? *Plant Physiol.* 154, 551–554. doi: 10.1104/pp.110.161547

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D. G., Felix, G., et al. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428, 764–767. doi: 10.1038/nature02485

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2013; accepted: 23 May 2013; published online: 11 June 2013.

*Citation: Davidsson PR, Kariola T, Niemi O and Palva ET (2013) Pathogenicity of and plant immunity to soft rot pectobacteria. Front. Plant Sci. **4**:191. doi: 10.3389/fpls.2013.00191*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Davidsson, Kariola, Niemi and Palva. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 01 May 2013
doi: 10.3389/fpls.2013.00117

Prospects for advancing defense to cereal rusts through genetical genomics

Elsa Ballini*†, *Nick Lauter* and *Roger Wise*

Corn Insects and Crop Genetics Research, Department of Plant Pathology and Microbiology, US Department of Agriculture - Agricultural Research Service, Center for Plant Responses to Environmental Stresses, Iowa State University, Ames, IA, USA

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Nils Rostoks, University of Latvia, Latvia
Brande B. Wulff, The Sainsbury Laboratory, UK

****Correspondence:***
Roger Wise, Corn Insects and Crop Genetics Research, Department of Plant Pathology and Microbiology, US Department of Agriculture - Agricultural Research Service, Center for Plant Responses to Environmental Stresses, Iowa State University, 351 Bessey Hall, Ames, IA 50011-1020, USA.
e-mail: rpwise@iastate.edu

†Present address:
Elsa Ballini, UMR BGPI - TA A-54/K - Equipe riz, SupAgro Montpellier, Département Biologie et Ecologie, Campus International de Baillarguet, 34 398 Montpellier Cedex 5, France.

Rusts are one of the most severe threats to cereal crops because new pathogen races emerge regularly, resulting in infestations that lead to large yield losses. In 1999, a new race of stem rust, *Puccinia graminis* f. sp. *tritici* (*Pgt* TTKSK or Ug99), was discovered in Uganda. Most of the wheat and barley cultivars grown currently worldwide are susceptible to this new race. *Pgt* TTKSK has already spread northward into Iran and will likely spread eastward throughout the Indian subcontinent in the near future. This scenario is not unique to stem rust; new races of leaf rust (*Puccinia triticina*) and stripe rust (*Puccinia striiformis*) have also emerged recently. One strategy for countering the persistent adaptability of these pathogens is to stack complete- and partial-resistance genes, which requires significant breeding efforts in order to reduce deleterious effects of linkage drag. These varied resistance combinations are typically more difficult for the pathogen to defeat, since they would be predicted to apply lower selection pressure. Genetical genomics or expression Quantitative Trait Locus (eQTL) analysis enables the identification of regulatory loci that control the expression of many to hundreds of genes. Integrated deployment of these technologies coupled with efficient phenotyping offers significant potential to elucidate the regulatory nodes in genetic networks that orchestrate host defense responses. The focus of this review will be to present advances in genetical genomic experimental designs and analysis, particularly as they apply to the prospects for discovering partial disease resistance alleles in cereals.

Keywords: eQTL, parallel expression, wheat, barley, *Puccinia*, *Triticeae*, cereal rusts

INTRODUCTION

The heteroecious rust fungi are some of the most important pathogens of cereal crops. These comprise 3000 *Puccinia* species (Van Der Merwe et al., 2007), including stem rust [*Puccinia graminis* f. sp. *tritici* (*Pgt*)], wheat leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis*), barley leaf rust (*Puccinia hordei*), and oat crown rust (*Puccinia coronata*). All of these species can infect a large range of cereal hosts: 365 grass species for *Puccinia graminis* alone.

Rust fungi pose a serious threat to cereal production because new races continue to emerge, and because infestation almost invariably leads to dramatic yield losses across large geographic areas (Leonard and Szabo, 2005; Bolton et al., 2008b). Breeding for genetic resistance to rusts reduces negative environmental impacts to agrosystems. In practice however, this approach is not infallible; the adaptability of rusts allows them to routinely overcome resistance gene (*R*) alleles bred into elite varieties (Singh et al., 2004a). Moreover, breeding efforts in response to new rust races does not always prevent crop loss, since wind dispersal of urediniospores can cause additional infections across continents in a short period of time (Hovmoller et al., 2008). Indeed, new races of leaf rust (Singh et al., 2004a), stripe rust (Milus et al., 2009), and stem rust (Stokstad, 2007) became widespread well before genetic resistance could be delivered in elite cultivars. Thus, the central challenge to the small grains industry is to reduce the periodicity of such outbreaks through renewed breeding efforts and continued management of epidemiological parameters that affect the evolution of pathogen virulence.

Stem rust, caused by the obligate fungal biotroph *Pgt*, has been a periodic, but serious problem wherever wheat and barley are grown (Roelfs, 1985; Leonard and Szabo, 2005). In North America, severe epidemics of stem rust have occurred from the late 1800's through the 1950's (http://www.globalrust.org). A new threat to wheat and barley production is the discovery of a novel race (*Pgt* TTKSK) of wheat stem rust in East Africa (Wanyera et al., 2006; Stokstad, 2007). This race, commonly called Ug99, is virulent to the majority of wheat varieties grown as well as advanced lines in current breeding programs (Jin and Singh, 2006; Bonman et al., 2007; Jin et al., 2007). *Pgt* TTKSK, and races of this lineage, infect barley as well as wheat, and has the potential to spread throughout the Middle East and to the Indian subcontinent in the near future (Singh et al., 2008). To overcome these new threats, additional resistances are needed in the short term, but more importantly, substantial new research efforts will be

Abbreviations: eQTL, (gene) expression quantitative trait locus; PAMP, pathogen-associated-molecular-pattern; ETI, effector triggered immunity; PTI, PAMP triggered immunity; DH, doubled haploid; RIL, recombinant inbred line, TDM, transcript-derived marker.

required in order to identify durable resistance to rusts over the long term (Ayliffe et al., 2008).

Genetic and/or molecular identification of novel sources of rust resistance in small grains will be greatly facilitated by recent gains in our basic knowledge of plant defense mechanisms. Plants detect the presence of the pathogen by two interconnected mechanisms (Jones and Dangl, 2006). One mechanism takes advantage of a specific response of the plant host induced by pathogen effectors; historically, these have has been referred to as gene-for-gene interactions (Flor, 1971), or in current terms, effector triggered immunity (ETI) (Jones and Dangl, 2006). This mechanism depends on direct or indirect recognition between pathogen effector (avirulence) proteins and plant R proteins (Innes, 2004; Deyoung and Innes, 2006; Deyoung et al., 2012). Another mechanism, designated PAMP triggered immunity (PTI), is induced by general elicitors or PAMPs (pathogen-associated-molecular-patterns), and is characterized by basal defense responses (Chisholm et al., 2006; Bent and Mackey, 2007). Based on this general doctrine, different strategies have been used to implement disease resistance in crops.

GENETIC STRATEGIES FOR DEPLOYMENT OF HOST RESISTANCE IN CROPS

The two primary genetic strategies for identification of disease resistance alleles useful for breeding are to focus on complete resistance conferred by *R* genes, or to focus on partial resistance that can be identified using quantitative genetic approaches. Significant progress has been made in identifying *R* genes in wheat and barley. More than 40 *R* genes that activate defense in response to *Puccinia triticina* and 40 *R* genes against *Pgt* have been mapped in wheat, as well as 20 *R* genes against *Puccinia striiformis* that have been mapped in wheat or barley (McIntosh et al., 1995; Ayliffe et al., 2008). Despite the dramatic success of these longstanding efforts, only a few *R* genes conferring resistance to a cereal rust have been cloned and functionally characterized: *Rpg1* (Brueggeman et al., 2002) and the *rpg4*/*Rpg5* complex (Brueggeman et al., 2008, 2009; Kleinhofs et al., 2009; Wang et al., 2013) in barley, *Rp1* (Collins et al., 1999) and *Rp3* (Webb et al., 2002) in maize, as well as *Lr21* (Huang et al., 2003) and *Lr10* (Feuillet et al., 2003) in wheat. While *R* genes tend to confer quite strong resistance to rusts in these cereal hosts, their efficacy in agronomic systems has the potential to be overcome by dynamic and rapidly evolving pathogen populations.

As such, a good strategy for countering the persistent adaptability of rusts is to deploy a combination of quantitative and qualitative resistance alleles. This strategy is typically more complicated to implement, but has the advantage of being more difficult to defeat, given that the various combinations are effective against a broader spectrum of races and thus, are believed to apply lower selection pressures (Singh et al., 2004b). Consequently, an agronomic phenotype often sought by breeders is non-specific partial resistance, or "slow rusting."

Partial resistance loci are difficult to identify for three reasons. First, quantitative measurement of symptoms such as the length of latent period, pustule size and spore production require significant expertise and effort. Second, Quantitative trait locus (QTL) analyses aimed at identifying partial resistance loci require large population sizes in order to detect these less obvious effects (Singh et al., 2004b). Finally, specific combinations of alleles are often required in order for a partial resistance locus to display sufficient penetrance, which makes the parentage of the experimental populations critically important (Simmonds, 1988). Despite these challenges, two such partial resistance loci have recently been cloned, *Yr36* (Fu et al., 2009) and *Lr34* (Krattinger et al., 2009). These genes both define new classes of resistance genes, encoding a kinase with a putative START lipid-binding domain and an adenosine triphosphate-binding cassette transporter, respectively. Under field conditions, *Yr36* and *Lr34* confer quantitative levels of adult plant resistance and are expected to provide durable resistance to rusts in wheat. It should be noted that cloning these partial resistance genes required careful planning and large-scale execution of breeding strategies designed specifically for this type of effort (Simmonds, 1988).

MOLECULAR APPROACHES TO IDENTIFY HOST DEFENSE GENES AND REGULATORS

The identification of genes that have the capacity to confer quantitative levels of disease resistance to multiple pathogen races is an important step toward reliable crop protection over the long term (Poland et al., 2009). Although much of our understanding of PAMP- and ETI-mediated defense has been achieved through classic forward genetic approaches (Shirasu et al., 1999; Deyoung and Innes, 2006), there is great potential to combine these strategies with genome- or population-wide analysis of host transcriptomes during interactions with pathogens (Wise et al., 2007).

One of the exciting outcomes of these fundamental advances on host-pathogen interaction is the degree to which this basic knowledge is transferrable from one system to another. Regulators implicated in a specific interaction have been shown to play important roles in pathogen interactions across several species (Bent and Mackey, 2007; Shirasu, 2009). For example, *Rar1*, first identified in barley (Shirasu et al., 1999), has functional orthologs in Arabidopsis (Muskett et al., 2002), tobacco (Liu et al., 2002), rice (Thao et al., 2007), and wheat (Tai, 2008). Another example is the Arabidopsis PBS1 kinase, which is targeted by AvrPphB, a cysteine protease effector from *Pseudomonas syringae* pv. *phaseolicola* (Zhu et al., 2004). Cleavage of PBS1 by AvrPphB activates RPS5-specified resistance (Deyoung et al., 2012). PBS1 is widely conserved in monocots and dicots. Hence, a clear challenge is to accelerate discovery of such regulators, such that natural variants or transgenic alleles of these genes can be exploited (Innes, 2004; Deyoung and Innes, 2006; Shirasu, 2009; Deyoung et al., 2012).

Several cereal rust interactions have been investigated using parallel expression approaches (**Table 1**); not only to identify genes in particular defense pathways, but also to compare transcriptome reprogramming between mutants and their progenitors in order to clone defense regulators (Zhang et al., 2006) or genes involved in broad-spectrum resistance to stem rust (Zhang et al., 2008b). Upon comparison of the genes identified in these different experiments (**Table 1**), it was observed that the major classes of rust-responsive genes are similar with genes that are responsive during other biotic interactions. These include, but are not limited to genes that encode peroxidases,

Table 1 | Investigations of differentially expressed genes or proteins during interaction between rust and cereal crops.

Host	Pathogen[a]	Isolate	Target gene (s)	Time[b]	Type[c]	References
Barley	*Pgt*	TTKSK	*rpg4, Rpg5*	24	A	Moscou et al., 2011b
Barley	*Pgt*	MCC, QCC	*Rpg1, NecS1*	0, 6, 12, 18, 24, 36	A	Zhang et al., 2008a,b
Barley	*Pgt*	NI[d]	*Rpr1*	na	A	Zhang et al., 2006
Barley	*Ph*	1.2.1	*Rphq14, 11, 15,* and *8*	18	B	Chen et al., 2010a
Barley	*Ph*	1.2.1	*Rphq2, 3*	18	B	Chen et al., 2010b
Barley	*Ph*	Dg2	*Rdg2a*	168, 336	C	Haegi et al., 2008
Wheat	*Pt, Ps*	MFBL	*Lr34, Yr18*	72	A	Hulbert et al., 2007
Wheat	*Pt*	BBB	*Lr1, Lr34, Tc*	72, 168	A	Bolton et al., 2008b
Wheat	*Ps*	PST-100 (06-194)	*Yr5*	6, 12, 24, 48	A	Coram et al., 2008a,c
Wheat	*Ps*	PST-78	*Yr39*	12, 24, 48	A	Coram et al., 2008b
Wheat	*Pt*	BBB, TJB	*Lr1*	3, 6, 12, 24	C	Fofana et al., 2007
Wheat	*Pt*	BBB	*Lr1*	24	D	Hu et al., 2007
Wheat	*Pt*	BBBD	*Lr1*	72, 144, 216	E	Rampitsch et al., 2006
Red fescue	*Puccinia* spp.	na	na	2, 8, 16, 24, 36, 48, 60	F	Ergen et al., 2007

[a] *Pathogen abbreviation: Pgt, P. graminis f. sp. tritici (Stem Rust); Pt, P. triticina (Wheat Leaf Rust); Ps, P. striiformis (Stripe Rust); Ph, P. hordei (Barley Leaf Rust).*

[b] *Time, hours after inoculation.*

[c] *Type of technique used for data obtaining: A, Affymetrix GeneChip; B, Agilent oligonucleotide array; C, cDNA microarray; D, cDNA library; E, Proteomic; F, mRNA differential display.*

[d] *NI designates non-inoculated.*

chitinases, pathogenesis related (PR) proteins, MAPK kinases, kinases, WRKY transcription factors, transport proteins, and proteins transported to chloroplasts. However, the regulation and the kinetics of expression for these genes may be vastly different, depending on the specific host-pathogen interaction that has been interrogated (Wise et al., 2007). A deeper comparative approach should facilitate discovery of common defense pathways between barley and wheat during interactions with rusts.

Approaches such as expression correlation and protein-protein interaction studies have facilitated the construction of defense pathways, but a clear understanding of the larger network of defense pathways and how they overlap remains elusive. The best documented defense network is based on work performed on *Arabidopsis thaliana* (Consortium, 2011; Mukhtar et al., 2011). Several reviews approach the defense response as a whole (Hammond-Kosack and Parker, 2003; Hofius et al., 2007), while others take a more focused approach (Mittler et al., 2004). Unfortunately, in cereals, graphical representations that efficiently foster a collective understanding are for the most part lacking, although several authors have reported pathway inferences from microarray experiments in rice (Cooper et al., 2003; Qiu et al., 2008). However, in these types of experiments, it is not possible to distinguish between cause and effect among correlated nodes. Several questions typically arise when co-expression networks are viewed: How can the key regulators of the pathway be identified? Are there connections between these regulators? Can the results of this network help a breeder make decisions? If so, what type of follow-up efforts should be pursued? Fortunately, a genetical genomic approach can provide the solution to these shortcomings. Surprisingly though, there has been little utilization of this approach in plant-pathogen interaction studies, despite overwhelming success when applied in human and animal disease research (de Koning and Haley, 2005; Mozhui et al., 2008).

GENETICAL GENOMICS OFFERS NEW HORIZONS TO INVESTIGATE PLANT DEFENSE MECHANISMS

QTL mapping finds statistical associations between genotypes and phenotypes, allowing regions of the genome harboring allelic differences that cause variation in the phenotype to be identified; these regions are called QTLs (reviewed by Mackay, 2001). Transcript abundance of a single gene is a quantitative trait and its regulation can be genetically interrogated. This is often called genetical genomics, or expression Quantitative Trait Locus (eQTL) mapping because the phenotypes in question are the expression of individual genes (Kendziorski and Wang, 2006; Rockman and Kruglyak, 2006). With the availability of high quality gene-expression platforms for barley (Close et al., 2004; Chen et al., 2010b), wheat (Schreiber et al., 2009), *Puccinia* spp. genome sequences (Cantu et al., 2011; Duplessis et al., 2011), as well as emerging next generation sequencing technologies (Li et al., 2010, 2013; Mayer et al., 2012), new strategies can be envisaged that interrogate both host and pathogen on a genome-wide, as well as a population-based scale.

The use of an eQTL strategy to identify and/or clone phenotypic QTL is well documented (Hansen et al., 2008). By profiling gene expression in each member of a segregating population, it is possible to use linkage analyses to identify key regulators of gene expression for a particular condition (Jansen and Nap, 2001; Rockman and Kruglyak, 2006; Williams et al., 2007; Kliebenstein, 2009; Li et al., 2013). For example, eQTL analysis of transcript abundance in embryo-derived tissues in barley has been combined with a QTL experiment on stem rust resistance in the same population (Druka et al., 2008). Three major QTL were detected in this population: two of them correspond to the known resistance genes *Rpg1* and *Rpg5/rpg4*, on chromosome 7H and 5H, respectively, and a third QTL was found on chromosome 2H. The cloned resistance gene *Rpg1* was detected as one of the best

candidate genes to underlie the QTL detected on chromosome 7H, thereby substantiating the eQTL strategy for the candidate gene approach. Other strong candidates were detected for the two other loci. In another study, Moscou et al. (2011b) used an eQTL approach to identify a master regulator on chromosome 2H that controls hundreds of genes in response to Ug99 stem rust. In collaboration with U.S. and Kenyan partners, they showed that *rpg4*/*Rpg5*-mediated adult plant resistance is enhanced by allelic variants of the regulator. Thus, eQTL analysis is a useful strategy to identify and clone genes whose allelic variation results in phenotypic variation.

The eQTL strategy can also be applied to characterize gene networks or to confirm biological pathways (Keurentjes et al., 2007; Sonderby et al., 2007). For example, the Arabidopsis gene *ERECTA* is known to act pleiotropically on several pathways, including flowering time and resistance to bacterial wilt (Godiard et al., 2003). The role played by *ERECTA* in flowering time was confirmed using an eQTL mapping approach; in addition, new connections and regulatory nodes in *ERECTA*-specified pathways were identified (Keurentjes et al., 2007). Another interesting example is the MYB transcription factor MYB28, which was confirmed as a regulator of aliphatic glucosinolate, a defense metabolite in Brassicales (Sonderby et al., 2007). Several regulators have now been identified in plant/pathogen interactions. Network analysis should confirm the role played by these regulators, thus, developing a more complete picture of plant defense pathways.

Genetical genomics can also be used to investigate the heritability of gene expression, as well as the basis for transgressive segregation, where progeny phenotypes are statistically outside the range that would be predicted by parental phenotypes (Keurentjes et al., 2007; West et al., 2007; Li et al., 2013). Transgressive segregation has been measured in two plant studies (Keurentjes et al., 2007; West et al., 2007). In both cases ∼50% of the genes show a significant difference in gene expression between parents and progeny in the population. This difference can be ascribed to the reassortment of additive genetic effects contributed by both parents, or to epistatic interactions among eQTL. Transgressive segregation is one of the reasons why some loci are difficult to use by breeders. Indeed, there are several cases of resistance genes that do not show the same phenotype in parental varieties as compared to their progenies. Understanding this phenomenon was required in order identify the suppressor of the *Lr34* leaf rust resistance gene in wheat (Vanegas et al., 2008). In another case, the *Lr13* leaf rust resistance gene is known to enhance resistance only in the presence of *Lr17* (Kolmer, 1992). The strength of eQTL analysis is that it provides the capacity to explain such complexities in a single experiment, rather than merely identifying the challenge.

CHALLENGES TO DESIGNING, EXECUTING, AND ANALYZING AN eQTL EXPERIMENT

Several recent reviews focus on the advances in genetical genomics (Doerge, 2002; Rockman and Kruglyak, 2006; Rosa et al., 2006; Williams et al., 2007; Wise et al., 2007; Gilad et al., 2008; Kliebenstein, 2009, 2010). To rapidly and efficiently discover partial or quantitative resistance alleles, one goal for future studies should be to understand how key regulators can modify gene expression, both temporally and spatially, during pathogen challenge and subsequent infection. In this section, we will outline parameters of experimental design to investigate this question, including factors that can influence the mapping process such as: population, experimental procedures and statistical analysis.

POPULATION TYPES

Several factors determine the utility of a population for any given QTL mapping application, all of which are related to pedigree and/or population size (Lauter et al., 2008). Pedigree determines which alleles are contrasted, how many alleles per locus are tested, which modes of action can be investigated, and the degree of genetic resolution that is achievable. Population size partially determines genetic resolution and largely controls the level of statistical power that is available to accurately determine modes of allele action, including the detection of epistasis.

Most QTL studies in plants investigate allelic contrasts between only two alleles, which at some point in the pedigree were present in a single F_1 plant. Common population types include F_2, recombinant inbred line (RIL), intermated recombinant inbred lines (iRIL), double haploids (DHs), and back cross (BC). Based on simulation studies, a well-developed RIL population appears to be the most efficient for accurate QTL mapping (Ferreira et al., 2006). This makes sense, because during development of the population, each generation produces an additional round of meiotic recombination: i.e., $R = 2r \times (1 + 2r)^{-1}$, where r is the recombination frequency in the corresponding F_2 (Burr and Burr, 1991). RIL populations, as well as DH and iRIL populations, also have the advantage of isogenicity, permitting experimental replication and testing of treatment effects without further genotyping.

A limitation of working with isogenic and true breeding lines is that the mode of allele action can not be determined, such that effects of recessive, dosage dependent, and dominant QTL alleles are indistinguishable without further analysis. This is a bigger limitation for breeding hybrid crops such as maize, where discovery of dominant QTL is preferred, than it is for inbred crops such as wheat or barley. Although limiting genotypic complexity is a drawback for allele characterization, it is experimentally beneficial in other regards. More statistical power to detect recessive and epistatic effects exists in a RIL population than in an F_2 population of equivalent size. Imagine an extreme phenotype conditioned only by recessive allele action at three independent loci: in an F_2 population, only one in 64 plants will have this genotype, compared to one in every eight plants in a RIL population. Detection of the main effects as well as the epistatic interdependence of these three hypothetical loci could only be revealed in an F_2 study if a very large number of plants were used.

POPULATION SIZE

Increasing the population size for a QTL experiment increases statistical power for both detection and localization of effects. Improved statistical power comes from larger numbers of lines or plants representing a particular genotype, while increased resolving power comes from additional recombination events that more closely flank the loci of interest. However, increased population size comes at a price. Thus, the challenge is to optimize

population size as a function of price per significant gain in understanding the trait. Unfortunately, *a priori* determination of a minimum population size required for a particular level of success is largely an intuitive exercise. In combination with the population type, the mode of inheritance for the trait in question plays a prominent role in determining the genetic tractability of a trait. For example, a polygenic trait (in the classical sense) is much more difficult to dissect than one whose inheritance architecture is oligogenic (Lauter et al., 2004). For this reason, it is helpful to know the phenotypic distribution of the trait in order to have some indication of the underlying genotypic cause of something other than a normal phenotypic distribution.

ARE THE GENES DETERMINING RESISTANCE QUALITATIVE OR QUANTITATIVE?

A common case in plant pathological investigations, the presence of an effective *R* gene, illustrates this point quite clearly. It is common for partial resistance alleles to be more easily detected when they enhance the function of a resistance gene that has a major effect. There are many possible explanations for this, but a simple and intuitive way to look at it is that it is easier to distinguish between completely disease free and slightly diseased than between "mostly dead" and "all dead." Suppose then that detection (for whatever reason) of a partial resistance allele epistatically depends on the presence of the *R* gene allele that confers resistance (Wise et al., 1996; Yu et al., 2001): in a RIL population, only half of the lines are useful for isolating the effect of this locus. The phenotypic distribution for such a trait should be strongly bimodal, which could be used to improve the experimental design prior to spending the money for genotyping. Further breeding and selection of a subset of lines for use are two of the simple solutions for this case.

OPTIMIZING POPULATIONS

There have been some efforts to empirically determine the point of diminishing returns for manipulation of population size. Several simulation studies have predicted that a population of 200 RILs is required for a statistically accurate analysis (Kim et al., 2005; Silva et al., 2007). However, most of the time, such populations only allow the detection of phenotypic QTL with major effects, a severe limitation when partial resistance alleles are the target for discovery (de Koning and Haley, 2005). There are several useful tricks for overcoming the population size limitation without breaking the bank. One use of a genetical genomics approach is to identify key transcriptional regulators that likely harbor the genetic variation underlying phenotypic QTL. Accomplishing this feat requires good resolution of both the QTL and eQTL effects. The best way to globally improve genetic resolution without increasing population size is to intermate progenies during population construction. This breaks up linkage blocks without introducing additional alleles by capitalizing on successive rounds of recombination. The addition of four generations of intermating to a maize population breeding effort has been shown to provide up to 50-fold gains in genetic resolution (Balint-Kurti et al., 2007; Lauter et al., 2008). Another way to enhance resolution is to capitalize on evolutionary recombination events, as has been done with the nested association mapping (NAM) population for maize (Yu et al., 2008). However, the per-line gain in resolution of this approach is not yet clear. Moreover, the simultaneous use of many alleles in a partial resistance search is not advisable unless the nesting parent (B73 in the case of maize NAM) harbors an allele for which suppressors and enhancers are sought.

Increasing global resolution and power is often not the focus of an investigator's effort. Many pathologists already have ideal allelic contrasts ready for eQTL dissection with appropriate genetic materials, but simply wish to limit the effective population size in favor of improving replication. There are tricks for this as well. Potokina and associates (2008b) show that when the focus is on a particular phenotypic QTL, subsets of lines can be selected based on known recombination events and allelic composition to improve efficiency. To some degree, this strategy is generalizable any time an excess of previously genotyped lines exists (Rosa et al., 2006). Such selective phenotyping approaches sample the population in a way that minimizes segregation distortion while maximizing global recombination rate, thereby increasing both power and precision on a per line basis (de Koning et al., 2007). Indeed, the power of eQTL detection can be increased if a subset of the population is chosen for its genetic dissimilarity without a commensurate decrease in mapping power (Yan et al., 2006). Another appealing approach is to select two different subsets of the population for two different treatments (Li et al., 2008). In this way, it's possible to divide the population into two subsets with similar genetic background. During subsequent statistical analysis, the subset of the population used in one treatment (mock inoculated) can be considered as a reference for the other subset of the population used in the other treatment (inoculated), effectively doubling the number of lines compared to a classical experimental design; significantly increasing mapping power, as has been shown recently by Moscou and colleagues (2011b).

HERITABILITY

Since statistical power depends in part on heritability, the delicate balance of population size vs. replication needs to be optimized based on experimental goals. In general, when *de novo* detection of minor QTL effects is a primary goal, replication is much more important than when the aim is to more finely map a known minor effect locus. In our view, increasing population size in an eQTL experiment is its own form of replication, similar to how replicated evaluations of F_3 families had previously been a norm for QTL mapping in plants (Cowen, 1988). The additional lines provide the benefit of new recombination events, while population measures such as mean and variance can be used to assess what proportion of total variance should be heritable. If a QTL allele cannot be reliably detected in a good experimental design, it is difficult to imagine how it will be of agronomic value.

Several wheat and barley populations have been used to map QTL for stem rust, leaf rust and stripe rust (Singh et al., 2004b; Druka et al., 2008; Chen et al., 2010a; Moscou et al., 2011b). However, small population sizes restrict the utility of these populations at present. A wider sampling of wheat and barley alleles would also provide stronger foundations for future research. Therefore, additional populations, preferably iRILs, should be created to allow discovery and fine mapping of new partial

resistance loci. Particular attention must be paid to the *R* gene and QTL alleles carried when parents for these populations are selected.

TREATMENT AND STATISTICAL DESIGN FOR AN eQTL EXPERIMENT

Considering the statistical design details of an eQTL experiment, two principle questions should be resolved early: what alleles will be contrasted and under what conditions, where conditions need to include treatment, the tissue to be dissected, as well as the selection of the desired timepoint.

The scientific objectives generally pursued in molecular plant pathology are to find genes that capacitate critical steps in the specific interaction between host and pathogen. There are many possible experimental approaches available, including contrasts of host vs. non-host interactions, virulent vs. avirulent isolates, inoculated vs. mock-inoculated treatments, mutant vs. wild-type genotypes. Treatment design strongly affects the selection of alleles to be contrasted for pathological studies, as virulence vs. avirulence is so often controlled by gene-for-gene interactions between the pathogen and the host. Control treatments can also be beneficial, but are not necessarily required if the goal is to identify allelic differences that affect the regulation of transcription, rather than to characterize under what conditions the observed regulation occurs. In pathological experiments, non-inoculated control treatment often allow the researchers to distinguish between consequences of inoculation vs. infection, which can be essential for limiting the number of candidate genes that appear to regulate whether or not infection occurs (Moscou et al., 2011a,b).

In pathological experiments, timepoint needs to be considered as a function of plant development as well as a function of infection kinetics following controlled inoculation. It is important to know the kinetics of the interaction prior to designing an eQTL experiment, particularly if reducing costs is essential. The timing of events after inoculation have been measured for stem rust (Zhang et al., 2008a,b), leaf rust (Bolton et al., 2008a; Chen et al., 2010a), and stripe rust (Coram et al., 2008c). Penetration begins 12 hours after inoculation (HAI), haustorium formation at 18 HAI, and intercellular hyphal growth at 24 HAI (Sellam and Wilcoxson, 1976; Lin et al., 1998). The choice of timepoint within these parameters affects the outcome of the experiment. If resources were not limiting, an attractive option for a complete linkage and network analysis of infection would be to measure the expression of each individual of the population at each time point. In this way, one could track the eQTL actions as a temporal function of the interaction.

The tissue taken for RNA extraction should also be chosen carefully. Indeed, because only a small portion of the cells interact directly with the pathogen, the transcriptomic approach generally considers a population of cells, and thus, of an RNA mixture between interacting and non-interacting cells. For practical reasons, most microarray studies have used a seedling leaf 1- or 2-weeks old, with the assumption that the use of replicates would increase statistical power of small differences in expression. Yet, separation of even different leaf parts can be enlightening; in one study involving the interactions among wheat and wheat leaf rust and/or wheat stripe rust, significant differential gene expression was observed between basal and apical sections of each leaf (Hulbert et al., 2007).

Similarly, but not surprisingly, Potokina et al. (2008a) demonstrated that different eQTLs could be identified in embryo vs. seedling leaf tissue in barley. Wherever possible, one should collect tissue that is as narrowly defined as possible while minimizing perturbations to homeostasis (Li et al., 2010). In barley and wheat, epidermal peels are quite easy to isolate from the rest of the leaf; one can do so quickly and without wounding them such that the gain in specificity potentially outweighs the potential error introduced by manipulation. While this approach has proven useful for powdery mildew investigations (Zierold et al., 2005), no simple approach exists for rust research. Laser capture micro-dissection would be an option, but it would seem that this would be more appropriate if the experiment was focused on how infection and defense signaling are propagated across cell types.

CHALLENGES IN STATISTICAL ANALYSIS

Several reviews have described methods for statistical analysis of microarray data and subsequent eQTL mapping procedures (Manly et al., 2004; Kendziorski and Wang, 2006; Williams et al., 2006; Jiang et al., 2008). First, microarray data are normalized in order to provide the phenotypic data for the expression level of each gene. If a linkage map already exists, eQTL mapping can commence immediately. Alternatively, microarray data can be used to create transcript-derived markers (TDMs) that can in turn be used to construct a genetic linkage map (Luo et al., 2007; Potokina et al., 2008b; Druka et al., 2010; Moscou et al., 2011b). This feature of eQTL mapping alone can often make the effort worthwhile; even with inexpensive genotyping, it is often cost effective in the long term to generate a TDM map of several thousand markers, which usually ensures that any two recombination events have a marker between them and that locations of genetic cross-overs are well-defined. TDMs can be integrated with Genotyping by Sequencing (GBS) approaches to anchor genetic maps to physical maps (Poland et al., 2012; Sonah et al., 2013).

Surprisingly, eQTL mapping is actually the simple portion of eQTL analysis work. The more difficult part is to figure out how to identify trends and meaningful patterns and in such a large volume of data, typically >10,000 regulatory relationships between an eQTL locus and the transcript it regulates. One of the most important things to establish is whether an eQTL acts in *cis* or *trans*, i.e., does the regulatory difference that leads to differential expression exist at the locus where the gene resides (*cis*) or elsewhere (*trans*). This level of characterization helps define the putative function of the eQTL, promoter difference vs. transcription factor difference, for example. Also, when searching for genes that act as capacitors of a significant process, it is helpful to know if many genes involved in a process are regulated by a locus where few or none of them reside. Such loci are termed eQTL hotspots and can regulate more than 1000 genes in some cases (West et al., 2007; Potokina et al., 2008a; Moscou et al., 2011b). Identification of eQTL hotspots is an effective way toward building gene networks, especially if one can identify a locus that regulates a cluster of genes associated with the biological phenomenon of interest, such as disease defense (Chen et al., 2010a; Moscou et al., 2011b). In

barley and wheat, determining *cis* vs. *trans* is becoming clearer, with the recent emergence of genome sequence resources with many genes tied to a genetic and/or physical position (Brenchley et al., 2012; Mayer et al., 2012). Synteny coordinates in rice or Brachypodium can assist in such analyses. In addition, such an approach to hotspot detection can be enhanced by seeding the analysis only with genes that have at least two eQTL, which ensures that at least one of them must be acting in *trans*.

Beyond the many possible methods to identify eQTL hotspots (Williams et al., 2007), the nature of their action remains unclear: could they be gene-dense regions where recombination is limited, or do they actually exist as the signature of a high-level regulatory gene (Breitling et al., 2008)? Notably, the statistical models described to date do not consider the influence of gene function, co-location patterns or co-expression. Plant resistance and defense genes are often clustered in the genome; the influence of such genomic organization on eQTL detection needs to be integrated into the statistical analysis. Indeed, simulations have shown that expression correlation can explain a large part of eQTL co-localization (Wang et al., 2007). Fortunately, permutation tests should be able to assess the reliability of putative eQTL hotspots (Breitling et al., 2008).

Some of the statistical challenges could also be viewed as opportunities. When it is not possible to determine which force is acting, it should be true that tracking of either phenomena can lead to the source. For example, co-location of several hotspots that regulate genes with correlated expression patterns could lead to more robust eQTL detection. Indeed, within an eQTL hotspot, one of the best candidates for the "master regulator" may be a gene whose expression correlates with the other genes whose eQTL have mapped to the same locus. This parsimonious inference has been applied to further test the predicted flowering time network in Arabidopsis (Keurentjes et al., 2007) and also between particular eQTL and transcription factors in yeast (Sun et al., 2007). For plant pathological applications, many transcription factors regulating defense are known, facilitating application of this approach.

EPISTASIS

Epistasis has been shown to have an impact on numerous major phenotypic QTL and will likely explain significant variance components of plant gene expression (Rowe and Kliebenstein, 2008). However, the epistatic interdependencies of gene expression have generally been neglected in plant eQTL analysis studies (Sun et al., 2008). While such tests require more statistical power than is usually available, future populations and experimental designs are sure to be more powerful, so a challenge to build the analysis infrastructure and to improve definition of the statistical underpinnings for large-scale tests for epistasis lie clearly before us. It will be necessary to define common criteria and performance measures for such analyses in order to permit the establishment of a collective intuition that is meaningful for evaluation of inferences. To this end, the routine sharing of data and analysis methods in a database such as PLEXdb (http://plexdb.org/) (Dash et al., 2012), WebQTL, or GeneNetwork (http://www.genenetwork.org/) facilitates these goals.

CONCLUDING REMARKS

Genetical genomics offers a new approach to the study of plant pathogen interactions. This systems biology approach leverages the complementary strengths of classical genetics and transcriptomics to connect loci that confer resistance with gene expression networks that are responsive to infection. Given these complementary strengths, it is incumbent upon the community to have the vision to perform definitive experiments to associate cause and effect. In this way, these experiments will facilitate the identification and cloning of new loci as well as known phenotypic QTL.

For the near term, the community has only some of these resources in hand, so the challenge is to carry out beneficial experiments with existing resources, while continuing to develop the next generation of tools to answer critical questions. These include: To what extent do polymorphisms in transcription factors and the promoter regions with which they interact govern the outcome of plant defense? What has the highest influence on gene regulation: polymorphism in regulators, polymorphism in downstream pathway, or variability in the environment? What is the evolution of defense regulators and how are they maintained in populations?

Suggested experiments to begin to answer these questions should connect kinetics of pathogen infection with responsive host genes and regulatory networks. In order to best track individual eQTL through the interaction, an optimal experimental plan would require: (1) high resolution population(s) that harbor genetic variation for resistance to the pathogen—intermated RILs should provide the resolution, while simultaneously providing a reasonable number of individuals for downstream molecular work; (2) all-genes expression-profiling platforms for the hosts and pathogens in question—with NextGen sequencing technologies, these are becoming possible at a reasonable cost; (3) high-throughput genotyping—several platforms offer the possibility of genotyping individuals with multiplex capability (Poland et al., 2012; Sonah et al., 2013), and (4) detailed infection phenotyping—with a reasonable number of intermated RILs, response to multiple pathogens or isolates could realistically be accomplished. In addition to these host parameters, one could dramatically increase the power of the investigation if equivalent resources (population, expression profiling, genotyping) were in place for the pathogen. In that case, exploration of "all by all" (segregating host by segregating pathogen) could be pursued.

ACKNOWLEDGMENTS

The authors thank Matthew Moscou for helpful advice and ideas on genetical genomics. This research was supported in part by National Science Foundation Plant Genome grants 05-00461 and 09-22746, and USDA-ARS CRIS project 3625-21000-057-00D. Elsa Ballini was supported by a USDA-ARS Postdoctoral Research Associateship. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

REFERENCES

Ayliffe, M., Singh, R., and Lagudah, E. (2008). Durable resistance to wheat stem rust needed. *Curr. Opin. Plant Biol.* 11, 187–192.

Balint-Kurti, P. J., Zwonitzer, J. C., Wisser, R. J., Carson, M. L., Oropeza-Rosas, M. A., Holland, J. B., et al. (2007). Precise mapping of quantitative trait loci for resistance to southern leaf blight, caused by *Cochliobolus heterostrophus* race O, and flowering time using advanced intercross maize lines. *Genetics* 176, 645–657.

Bent, A. F., and Mackey, D. (2007). Elicitors, effectors, and *R* genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45, 399–436.

Bolton, M. D., Kolmer, J. A., and Garvin, D. F. (2008a). Wheat leaf rust caused by *Puccinia triticina*. *Mol. Plant Pathol.* 9, 563–575.

Bolton, M. D., Kolmer, J. A., Xu, W. W., and Garvin, D. F. (2008b). *Lr34*-mediated leaf rust resistance in wheat: transcript profiling reveals a high energetic demand supported by transient recruitment of multiple metabolic pathways. *Mol. Plant Microbe Interact.* 21, 1515–1527.

Bonman, J. M., Bockelman, H. E., Jin, Y., Hijmans, R. J., and Gironella, A. I. N. (2007). Geographic distribution of stem rust resistance in wheat landraces. *Crop Sci.* 47, 1955–1963.

Breitling, R., Li, Y., Tesson, B. M., Fu, J., Wu, C., Wiltshire, T., et al. (2008). Genetical genomics: spotlight on QTL hotspots. *PLoS Genet.* 4:e1000232. doi: 10.1371/journal.pgen.1000232

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L. A., D'Amore, R., Allen, A. M., et al. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature* 491, 705–710.

Brueggeman, R., Druka, A., Nirmala, J., Cavileer, T., Drader, T., Rostoks, N., et al. (2008). The stem rust resistance gene *Rpg5* encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14970–14975.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., et al. (2002). The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9328–9333.

Brueggeman, R., Steffenson, B. J., and Kleinhofs, A. (2009). The *rpg4/Rpg5* stem rust resistance locus in barley: resistance genes and cytoskeleton dynamics. *Cell Cycle* 8, 977–981.

Burr, B., and Burr, F. A. (1991). Recombinant inbreds for molecular mapping in maize: theoretical and practical considerations. *Trends Genet.* 7, 55–60.

Cantu, D., Govindarajulu, M., Kozik, A., Wang, M., Chen, X., Kojima, K. K., et al. (2011). Next generation sequencing provides rapid access to the genome of *Puccinia striiformis* f. sp. tritici, the causal agent of wheat stripe rust. *PLoS ONE* 6:e24230. doi: 10.1371/journal.pone.0024230

Chen, X., Hackett, C., Niks, R., Hedley, P., Booth, C., Druka, A., et al. (2010a). An eQTL analysis of partial resistance to *Puccinia hordei* in barley. *PLoS ONE* 5:e8598. doi: 10.1371/journal.pone.0008598

Chen, X., Niks, R., Hedley, P., Morris, J., Druka, A., Marcel, T., et al. (2010b). Differential gene expression in nearly isogenic lines with QTL for partial resistance to *Puccinia hordei* in barley. *BMC Genomics* 11:629. doi: 10.1186/1471-2164-11-629

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814.

Close, T. J., Wanamaker, S. I., Caldo, R. A., Turner, S. M., Ashlock, D. A., Dickerson, J. A., et al. (2004). A new resource for cereal genomics: 22K barley GeneChip comes of age. *Plant Physiol.* 134, 960–968.

Collins, N., Drake, J., Ayliffe, M., Sun, Q., Ellis, J., Hulbert, S., et al. (1999). Molecular characterization of the maize *Rp1-D* rust resistance haplotype and its mutants. *Plant Cell* 11, 1365–1376.

Consortium, A. I. M. (2011). Evidence for network evolution in an *Arabidopsis* interactome map. *Science* 333, 601–607.

Cooper, B., Clarke, J. D., Budworth, P., Kreps, J., Hutchison, D., Park, S., et al. (2003). A network of rice genes associated with stress response and seed development. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4945–4950.

Coram, T., Settles, M., Wang, M., and Chen, X. (2008a). Surveying expression level polymorphism and single-feature polymorphism in near-isogenic wheat lines differing for the *Yr5* stripe rust resistance locus. *Theor. Appl. Genet.* 117, 401–411.

Coram, T. E., Settles, M. L., and Chen, X. (2008b). Transcriptome analysis of high-temperature adult-plant resistance conditioned by *Yr39* during the wheat *Puccinia striiformis* f. sp. tritici interaction. *Mol. Plant Pathol.* 9, 479–493.

Coram, T. E., Wang, M. N., and Chen, X. M. (2008c). Transcriptome analysis of the wheat - *Puccinia striiformis* f. sp tritici interaction. *Mol. Plant Pathol.* 9, 157–169.

Cowen, N. M. (1988). The use of replicated progenies in marker-based mapping of QTLs. *Theor. Appl. Genet.* 75, 857–862.

Dash, S., Van Hemert, J., Hong, L., Wise, R. P., and Dickerson, J. A. (2012). PLEXdb: gene expression resources for plants and plant pathogens. *Nucleic Acids Res.* 40, D1194–D1201.

de Koning, D. J., Cabrera, C. P., and Haley, C. S. (2007). Genetical genomics: combining gene expression with marker genotypes in poultry. *Poult. Sci.* 86, 1501–1509.

de Koning, D. J., and Haley, C. S. (2005). Genetical genomics in humans and model organisms. *Trends Genet.* 21, 377–381.

Deyoung, B. J., and Innes, R. W. (2006). Plant NBS-LRR proteins in pathogen sensing and host defense. *Nat. Immunol.* 7, 1243–1249.

Deyoung, B. J., Qi, D., Kim, S. H., Burke, T. P., and Innes, R. W. (2012). Activation of a plant nucleotide binding-leucine rich repeat disease resistance protein by a modified self protein. *Cell Microbiol.* 14, 1071–1084.

Doerge, R. W. (2002). Mapping and analysis of quantitative trait loci in experimental populations. *Nat. Rev. Genet.* 3, 43–52.

Druka, A., Potokina, E., Luo, Z., Bonar, N., Druka, I., Zhang, L., et al. (2008). Exploiting regulatory variation to identify genes underlying quantitative resistance to the wheat stem rust pathogen *Puccinia graminis* f. sp. tritici in barley. *Theor. Appl. Genet.* 117, 261–272.

Druka, A., Potokina, E., Luo, Z., Jiang, N., Chen, X., Kearsey, M., et al. (2010). Expression quantitative trait loci analysis in plants. *Plant Biotechnol. J.* 8, 10–27.

Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., et al. (2011). Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proc. Natl. Acad. Sci.* 108, 9166–9171.

Ergen, N. Z., Dinler, G., Shearman, R. C., and Budak, H. (2007). Identifying, cloning and structural analysis of differentially expressed genes upon *Puccinia* infection of *Festuca rubra* var. rubra. *Gene* 393, 145–152.

Ferreira, A., Silva, M. F. D., Silva, L. D. C. E., and Cruz, C. D. (2006). Estimating the effects of population size and type on the accuracy of genetic maps. *Genet. Mol. Biol.* 29, 187–192.

Feuillet, C., Travella, S., Stein, N., Albar, L., Nublat, A. L., and Keller, B. (2003). Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15253–15258.

Flor, H. H. (1971). Current Status of Gene-for-gene concept. *Annu. Rev. Phytopathol.* 9, 275.

Fofana, B., Banks, T. W., McCallum, B. D., Strelkov, S. E., and Cloutier, S. (2007). Temporal gene expression profiling of the wheat leaf rust pathosystem using cDNA microarray reveals differences in compatible and incompatible defence pathways. *Int. J. Plant Genomics,* 2007:17542. doi: 10.1155/2007/17542

Fu, D. L., Uauy, C., Distelfeld, A., Blechl, A., Epstein, L., Chen, X. M., et al. (2009). A Kinase-START gene confers temperature-dependent resistance to wheat stripe rust. *Science* 323, 1357–1360.

Gilad, Y., Rifkin, S. A., and Pritchard, J. K. (2008). Revealing the architecture of gene regulation: the promise of eQTL studies. *Trends Genet.* 24, 408–415.

Godiard, L., Sauviac, L., Torii, K. U., Grenon, O., Mangin, B., Grimsley, N. H., et al. (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. *Plant J.* 36, 353–365.

Haegi, A., Bonardi, V., Dall'aglio, E., Glissant, D., Tumino, G., Collins, N. C., et al. (2008). Histological and molecular analysis of *Rdg2a* barley resistance to leaf stripe. *Mol. Plant Pathol.* 9, 463–478.

Hammond-Kosack, K. E., and Parker, J. E. (2003). Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Curr. Opin. Biotechnol.* 14, 177–193.

Hansen, B. G., Halkier, B. A., and Kliebenstein, D. J. (2008). Identifying the molecular basis of QTLs: eQTLs add a new dimension. *Trends Plant Sci.* 13, 72–77.

Hofius, D., Tsitsigiannis, D. I., Jones, J. D. G., and Mundy, J. (2007). Inducible cell death in plant immunity. *Semin. Cancer Biol.* 17, 166–187.

Hovmoller, M. S., Yahyaoui, A. H., Milus, E. A., and Justesen, A. F. (2008). Rapid global spread of two aggressive strains of a wheat rust fungus. *Mol. Ecol.* 17, 3818–3826.

Hu, G., Linning, R., McCallum, B. D., Banks, T. W., Cloutier, S., Butterfield, Y., et al. (2007).

Generation of a wheat leaf rust, *Puccinia triticina*, EST database from stage-specific cDNA libraries. *Mol. Plant Pathol.* 8, 451–467.

Huang, L., Brooks, S. A., Li, W., Fellers, J. P., Trick, H. N., and Gill, B. S. (2003). Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. *Genetics* 164, 655–664.

Hulbert, S. H., Bai, J., Fellers, J. P., Pacheco, M. G., and Bowden, R. L. (2007). Gene expression patterns in near isogenic lines for wheat rust resistance gene *Lr34*/*Yr18*. *Phytopathology* 97, 1083–1093.

Innes, R. W. (2004). Guarding the goods. New insights into the central alarm system of plants. *Plant Physiol.* 135, 695–701.

Jansen, R. C., and Nap, J.-P. (2001). Genetical genomics: the added value from segregation. *Trends Genet.* 17, 388–391.

Jiang, N., Leach, L., Hu, X., Potokina, E., Jia, T., Druka, A., et al. (2008). Methods for evaluating gene expression from Affymetrix microarray datasets. *BMC Bioinformatics* 9:284. doi: 10.1186/1471-2105-9-284

Jin, Y., Pretorius, Z. A., and Singh, R. P. (2007). New virulence within race TTKS (Ug99) of the stem rust pathogen and effective resistance genes. *Phytopathology* 97, S137.

Jin, Y., and Singh, R. P. (2006). Resistance in U.S. wheat to recent eastern African isolates of Puccinia graminis f. sp. tritici with virulence to resistance gene Sr31. *Plant Dis.* 90, 476–480.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Kendziorski, C., and Wang, P. (2006). A review of statistical methods for expression quantitative trait loci mapping. *Mamm. Genome* 17, 509–517.

Keurentjes, J. J. B., Fu, J., Terpstra, I. R., Garcia, J. M., Van Den Ackerveken, G., Snoek, L. B., et al. (2007). Regulatory network construction in Arabidopsis by using genome-wide gene expression quantitative trait loci. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1708–1713.

Kim, K., West, M. A. L., Michelmore, R. W., Clair, D. A. S., and Doerge, R. W. (2005). "Old methods for new ideas: Genetic dissection of the determinants of gene expression levels," in *Genome exploitation: data mining the genome. The 23rd volume in the Stadler Symposia*, eds J. P. Gustafson, R. Shoemaker, and J. W. Snape (New York, NY: Springer), 89–105.

Kleinhofs, A., Brueggeman, R., Nirmala, J., Zhang, L., Mirlohi, A., Druka, A., et al. (2009). Barley stem rust resistance genes: structure and function. *Plant Genome* 2, 109–120.

Kliebenstein, D. (2009). Quantitative genomics: analyzing intraspecific variation using global gene expression polymorphisms or eQTLs. *Annu. Rev. Plant Biol.* 60, 93–114.

Kliebenstein, D. J. (2010). Systems biology uncovers the foundation of natural genetic diversity. *Plant Physiol.* 152, 480–486.

Kolmer, J. A. (1992). Enhanced leaf rust resistance in wheat conditioned by resistance gene pairs with *Lr13*. *Euphytica* 61, 123–130.

Krattinger, S. G., Lagudah, E. S., Spielmeyer, W., Singh, R. P., Huerta-Espino, J., McFadden, H., et al. (2009). A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323, 1360–1363.

Lauter, N., Gustus, C., Westerbergh, A., and Doebley, J. (2004). The inheritance and evolution of leaf pigmentation and pubescence in teosinte. *Genetics* 167, 1949–1959.

Lauter, N., Moscou, M. J., Habiger, J., and Moose, S. P. (2008). Quantitative genetic dissection of shoot architecture traits in maize: towards a functional genomics approach. *Plant Genome* 1, 99–110.

Leonard, K. J., and Szabo, L. J. (2005). Stem rust of small grains and grasses caused by *Puccinia graminis*. *Mol. Plant Pathol.* 6, 99–111.

Li, L., Petsch, K., Shimizu, R., Liu, S., Xu, W. W., Ying, K., et al. (2013). Mendelian and non-mendelian regulation of gene expression in maize. *PLoS Genet.* 9:e1003202. doi: 10.1371/journal.pgen.1003202

Li, P., Ponnala, L., Gandotra, N., Wang, L., Si, Y., Tausta, S. L., et al. (2010). The developmental dynamics of the maize leaf transcriptome. *Nat. Genet.* 42, 1060–1067.

Li, Y., Breitling, R., and Jansen, R. C. (2008). Generalizing genetical genomics: getting added value from environmental perturbation. *Trends Genet.* 24, 518–524.

Lin, K. C., Bushnell, W. R., Smith, A. G., and Szabo, L. J. (1998). Temporal accumulation patterns of defence response gene transcripts in relation to resistant reactions in oat inoculated with *Puccinia graminis*. *Physiol. Mol. Plant Pathol.* 52, 95–114.

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. P. (2002). Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. *Plant J.* 30, 415–429.

Luo, Z. W., Potokina, E., Druka, A., Wise, R., Waugh, R., and Kearsey, M. J. (2007). SFP genotyping from Affymetrix arrays is robust but largely detects *cis*-acting expression regulators. *Genetics* 176, 789–800.

Mackay, T. F. C. (2001). The genetic architecture of quantitative traits. *Annu. Rev. Genet.* 35, 303–339.

Manly, K. F., Nettleton, D., and Hwang, J. T. G. (2004). Genomics, prior probability, and statistical tests of multiple hypotheses. *Genome Res.* 14, 997–1001.

Mayer, K., Waugh, R., Langridge, P., Close, T. J., Wise, R. P., Graner, A., et al. (2012). A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491, 711–716.

McIntosh, R. A., Wellings, C. R., and Park, R. F. (1995). *Wheat rusts: an Atlas of Resistance Genes*. London: Kluwer Academic Publishers.

Milus, E. A., Kristensen, K., and Hovmoller, M. S. (2009). Evidence for increased aggressiveness in a recent widespread strain of *Puccinia striiformis* f. sp *tritici* causing stripe rust of wheat. *Phytopathology* 99, 89–94.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498.

Moscou, M. J., Lauter, N., Caldo, R. A., Nettleton, D., and Wise, R. P. (2011a). Quantitative and temporal definition of the *Mla* transcriptional regulon during barley-powdery mildew interactions. *Mol. Plant Microbe Interact.* 24, 694–705.

Moscou, M. J., Lauter, N., Steffenson, B., and Wise, R. P. (2011b). Quantitative and qualitative stem rust resistance factors in barley are associated with transcriptional suppression of defense regulons. *PLoS Genet.* 7:e1002208. doi: 10.1371/journal.pgen.1002208

Mozhui, K., Ciobanu, D. C., Schikorski, T., Wang, X., Lu, L., and Williams, R. W. (2008). Dissection of a QTL hotspot on mouse distal chromosome 1 that modulates neurobehavioral phenotypes and gene expression. *PLoS Genet.* 4:e1000260. doi: 10.1371/journal.pgen.1000260

Mukhtar, M. S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601.

Muskett, P. R., Kahn, K., Austin, M. J., Moisan, L. J., Sadanandom, A., Shirasu, K., et al. (2002). Arabidopsis *RAR1* exerts rate-limiting control of *R* gene-mediated defenses against multiple pathogens. *Plant Cell* 14, 979–992.

Parlevliet, J. E. (1978). Further evidence of polygenic inheritance of partial resistance in barley to leaf rust, *Puccinia hordei*. *Euphytica* 27, 369–379.

Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C., and Nelson, R. J. (2009). Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* 14, 21–29.

Poland, J. A., Brown, P. J., Sorrells, M. E., and Jannink, J.-L. (2012). Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. *PLoS ONE* 7:e32253. doi: 10.1371/journal.pone.0032253

Potokina, E., Druka, A., Luo, Z., Moscou, M., Wise, R., Waugh, R., et al. (2008a). Tissue-dependent limited pleiotropy affects gene expression in barley. *Plant J.* 56, 287–296.

Potokina, E., Druka, A., Luo, Z., Wise, R., Waugh, R., and Kearsey, M. (2008b). Gene expression quantitative trait locus analysis of 16 000 barley genes reveals a complex pattern of genome-wide transcriptional regulation. *Plant J.* 53, 90–101.

Qiu, D., Xiao, J., Xie, W., Liu, H., Li, X., Xiong, L., et al. (2008). Rice gene network inferred from expression profiling of plants overexpressing *OsWRKY13*, a positive regulator of disease resistance. *Mol. Plant* 1, 538–551.

Rampitsch, C., Bykova, N. V., McCallum, B., Beimcik, E., and Ens, W. (2006). Analysis of the wheat and *Puccinia triticina* (leaf rust) proteomes during a susceptible host-pathogen interaction. *Proteomics* 6, 1897–1907.

Rockman, M. V., and Kruglyak, L. (2006). Genetics of global gene expression. *Nat. Rev. Genet.* 7, 862–872.

Roelfs, A. P. (1985). "Wheat and rye stem rust," in *The Cereal Rusts*, eds A. P. Roelfs and W. R. Bushnell (Orlando, FL: Academic Press), 3–37.

Rosa, G. J., De Leon, N., and Rosa, A. J. (2006). Review of microarray experimental design strategies for genetical genomics studies. *Physiol. Genomics* 28, 15–23.

Rowe, H. C., and Kliebenstein, D. J. (2008). Complex genetics control natural variation in *Arabidopsis thaliana* resistance to *Botrytis cinerea*. *Genetics* 180, 2237–2250.

Schreiber, A. W., Sutton, T., Caldo, R. A., Kalashyan, E., Lovell, B., Mayo, G., et al. (2009). Comparative transcriptomics in the Triticeae. *BMC Genomics* 10:285. doi: 10.1186/1471-2164-10-285

Sellam, M. A., and Wilcoxson, R. D. (1976). Development of *Puccinia graminis* f. sp. tritici on resistant and susceptible barley cultivars. *Phytopathology* 66, 667–668.

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. *Ann. Rev. Plant Biol.* 60, 139–164.

Shirasu, K., Lahaye, T., Tan, M. W., Zhou, F., Azevedo, C., and Schulze-Lefert, P. (1999). A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. *Cell* 99, 355–366.

Silva, L. D. C. E., Cruz, C. D., Moreira, M. A., and Barros, E. G. D. (2007). Simulation of population size and genome saturation level for genetic mapping of recombinant inbred lines (RILs). *Genet. Mol. Biol.* 30, 1101–1108.

Simmonds, N. W. (1988). "Synthesis: the strategy of rust resistance breeding," in *Breeding Strategies for Resistance to the Rusts of Wheat*, eds N. W. Simmonds and S. Rajaram (Mexico: Cimmyt), 119–136.

Singh, R. P., Hodson, D. P., Huerta-Espino, J., Jin, Y., Njau, P., Wanyera, R., et al. (2008). Will stem rust destroy the world's wheat crop? *Adv. Agron.* 98, 271–309.

Singh, R. P., Huerta-Espino, J., Pfeiffer, W., and Figueroa-Lopez, P. (2004a). Occurrence and impact of a new leaf rust race on durum wheat in northwestern Mexico from 2001 to 2003. *Plant Dis.* 88, 703–708.

Singh, R. P., William, H. M., Huerta-Espino, J., and Rosewarne, G. (2004b). "Wheat rust in Asia: meeting the challenges with old and new technologies," in *New Directions for a Diverse Planet: Proceedings for the 4th International Crop Science Congress*, eds T. Fisher, N. Turner, J. Angus, L. McIntyre, M. Robertson, A. Borrell, and D. Lloyd (Gosford, NSW: The Regional Institute Ltd.), 13.

Sonah, H., Bastien, M., Iquira, E., Tardivel, A. L., Légaré, G., Boyle, B., et al. (2013). An improved genotyping by sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. *PLoS ONE* 8:e54603. doi: 10.1371/journal.pone.0054603

Sonderby, I. E., Hansen, B. G., Bjarnholt, N., Ticconi, C., Halkier, B. A., and Kliebenstein, D. J. (2007). A systems biology approach identifies a *R2R3 MYB* gene subfamily with distinct and overlapping functions in regulation of aliphatic glucosinolates. *PLoS ONE* 2:e1322. doi: 10.1371/journal.pone.0001322

Stokstad, E. (2007). Plant pathology: deadly wheat fungus threatens world's breadbaskets. *Science* 315, 1786–1787.

Sun, W., Yu, T., and Li, K.-C. (2007). Detection of eQTL modules mediated by activity levels of transcription factors. *Bioinformatics* 23, 2290–2297.

Sun, W., Yuan, S., and Li, K.-C. (2008). Trait-trait dynamic interaction: 2D-trait eQTL mapping for genetic variation study. *BMC Genomics* 9:242. doi: 10.1186/1471-2164-9-242

Tai, Y. S. (2008). Interactome of signaling networks in wheat: the protein-protein interaction between TaRAR1 and TaSGT1. *Mol. Biol. Rep.* 35, 337–343.

Thao, N. P., Chen, L., Nakashima, A., Hara, S. I., Umemura, K., Takahashi, A., et al. (2007). RAR1 and HSP90 form a complex with Rac/Rop GTPase and function in innate-immune responses in rice. *Plant Cell* 19, 4035–4045.

Van Der Merwe, M., Ericson, L., Walker, J., Thrall, P. H., and Burdon, J. J. (2007). Evolutionary relationships among species of *Puccinia* and *Uromyces* (*Pucciniaceae*, *Uredinales*) inferred from partial protein coding gene phylogenies. *Mycol. Res.* 111, 163–175.

Vanegas, C. D. G., Garvin, D. F., and Kolmer, J. A. (2008). Genetics of stem rust resistance in the spring wheat cultivar Thatcher and the enhancement of stem rust resistance by Lr34. *Euphytica* 159, 391–401.

Wang, S., Zheng, T., and Wang, Y. (2007). Transcription activity hot spot, is it real or an artifact? *BMC Proc.* 1:S94. Available online at: http://www.biomedcentral.com/1753-6561/1/S1/S94

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., et al. (2013). The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene, and an actin depolymerization factor. *Mol. Plant Microbe Interact.* 26, 407–418.

Wanyera, R., Kinyua, M. G., Jin, Y., and Singh, R. P. (2006). The spread of stem rust caused by *Puccinia graminis* f. sp. tritici, with virulence on Sr31 in wheat in eastern Africa. *Plant Dis.* 90, 113.

Webb, C. A., Richter, T. E., Collins, N. C., Nicolas, M., Trick, H. N., Pryor, T., et al. (2002). Genetic and molecular characterization of the maize *rp3* rust resistance locus. *Genetics* 162, 381–394.

West, M. A., Kim, K., Kliebenstein, D. J., Van Leeuwen, H., Michelmore, R. W., Doerge, R. W., et al. (2007). Global eQTL mapping reveals the complex genetic architecture of transcript-level variation in Arabidopsis. *Genetics* 175, 1441–1450.

Williams, R. B. H., Chan, E. K. F., Cowley, M. J., and Little, P. F. R. (2007). The influence of genetic variation on gene expression. *Genome Res.* 17, 1707–1716.

Williams, R. B. H., Cotsapas, C. J., Cowley, M. J., Chan, E., Nott, D. J., and Little, P. F. R. (2006). Normalization procedures and detection of linkage signal in genetical-genomics experiments. *Nat. Genet.* 38, 855–856.

Wise, R. P., Lee, M., and Rayapati, P. J. (1996). Recombination within a 5-centimorgan region in diploid Avena reveals multiple specificities conferring resistance to *Puccinia coronata*. *Phytopathology* 86, 340–346.

Wise, R. P., Moscou, M. J., Bogdanove, A. J., and Whitham, S. A. (2007). Transcript profiling in host-pathogen interactions. *Annu. Rev. Phytopathol.* 45, 329–369.

Yan, J.-B., Tang, J.-H., Meng, Y.-J., Ma, X.-Q., Teng, W.-T., Chander, S., et al. (2006). Improving QTL mapping resolution based on genotypic sampling–a case using a RIL population. *Acta Genetica Sinica* 33, 617–624.

Yu, G.-X., Braun, E., and Wise, R. P. (2001). *Rds* and *Rih* Mediate hypersensitive cell death independent of gene-for-gene resistance to the oat crown rust pathogen *Puccinia coronata* f. sp. avenae. *Mol. Plant Microbe Interact.* 14, 1376–1383.

Yu, J., Holland, J. B., McMullen, M. D., and Buckler, E. S. (2008). Genetic design and statistical power of Nested Association Mapping in maize. *Genetics* 178, 539–551.

Zhang, L., Castell-Miller, C., Dahl, S., Steffenson, B., and Kleinhofs, A. (2008a). Parallel expression profiling of barley-stem rust interactions. *Funct. Integr. Genomics* 8, 187–198.

Zhang, L., Lavery, L., Gill, U., Gill, K., Steffenson, B., Yan, G., et al. (2008b). A cation/proton-exchanging protein is a candidate for the barley *NecS1* gene controlling necrosis and enhanced defense response to stem rust. *Theor. Appl. Genet.* 118, 385–397.

Zhang, L., Fetch, T., Nirmala, J., Schmierer, D., Brueggeman, R., Steffenson, B., et al. (2006). *Rpr1*, a gene required for *Rpg1*-dependent resistance to stem rust in barley. *Theor. Appl. Genet.* 113, 847–855.

Zhu, M., Shao, F., Innes, R. W., Dixon, J. E., and Xu, Z. (2004). The crystal structure of Pseudomonas avirulence protein AvrPphB: a papain-like fold with a distinct substrate-binding site. *Proc. Natl. Acad. Sci. U.S.A.* 101, 302–307.

Zierold, U. W. E., Scholz, U. W. E., and Schweizer, P. (2005). Transcriptome analysis of *mlo*-mediated resistance in the epidermis of barley. *Mol. Plant Pathol.* 6, 139–151.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 March 2013; accepted: 15 April 2013; published online: 01 May 2013.

*Citation: Ballini E, Lauter N and Wise R (2013) Prospects for advancing defense to cereal rusts through genetical genomics. Front. Plant Sci. **4**:117. doi: 10.3389/fpls.2013.00117*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Ballini, Lauter and Wise. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

GLOSSARY OF DEFINITIONS

Partial resistance is a form of resistance, phenotypically characterized by a temporally and/or spatially reduced rate of pathogen development on the host (Parlevliet, 1978); this also may be referred to as Quantitative resistance (Poland et al., 2009). Genetical Genomics refers to the use of quantitative genetic mapping to dissect the regulatory underpinnings of molecular phenotypes collected *en masse* using high-throughput genomic technologies (Jansen and Nap, 2001). expression Quantitative Trait Locus (eQTL) mapping is the treatment of transcript abundance as a quantitative trait.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 23 April 2013
doi: 10.3389/fpls.2013.00082

Metabolomics of cereals under biotic stress: current knowledge and techniques

Dirk Balmer[1†], Victor Flors[2†], Gaetan Glauser[3†] and Brigitte Mauch-Mani[1*†]

[1] Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland
[2] Metabolic Integration and Cell Signaling Group, Plant Physiology Section, Departamento de Ciencias Agrarias y del Medio Natural, Universitat Jaume I, Castellón, Spain
[3] Chemical Analytical Service of the Swiss Plant Science Web, University of Neuchâtel, Neuchâtel, Switzerland

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Lisa Jayne Vaillancourt, University of Kentucky, USA
Carla Caruso, University of Tuscia, Italy

***Correspondence:**
Brigitte Mauch-Mani, University of Neuchâtel, Faculty of Sciences, Institute of Botany, Rue Emile Argand 11, 2000 Neuchâtel, Switzerland.
e-mail: brigitte.mauch@unine.ch

† Dirk Balmer, Victor Flors, Gaetan Glauser and Brigitte Mauch-Mani have contributed equally to this work.

Prone to attacks by pathogens and pests, plants employ intricate chemical defense mechanisms consisting of metabolic adaptations. However, many plant attackers are manipulating the host metabolism to counteract defense responses and to induce favorable nutritional conditions. Advances in analytical chemistry have allowed the generation of extensive metabolic profiles during plant-pathogen and pest interactions. Thereby, metabolic processes were found to be highly specific for given tissues, species, and plant-pathogen/pest interactions. The clusters of identified compounds not only serve as base in the quest of novel defense compounds, but also as markers for the characterization of the plants' defensive state. The latter is especially useful in agronomic applications where meaningful markers are essential for crop protection. Cereals such as maize make use of their metabolic arsenal during both local and systemic defense responses, and the chemical response is highly adapted to specific attackers. Here, we summarize highlights and recent findings of metabolic patterns of cereals under pathogen and pest attack.

Keywords: chemical analytical techniques, chemical defense, metabolic profile, monocots, phytoalexins, secondary metabolites

INTRODUCTION

The major part of the world's food supply depends on the production of cereal crops such as rice, maize, wheat, barley, sorghum, oat, and millet. These crops are constantly jeopardized by biotic stressors such as viruses, bacteria, fungi, or herbivores leading to severe yield losses and therefore to eminent economic problems. For instance, the hemibiotrophic fungus *Colletotrichum graminicola*, the causal agent of maize anthracnose, is responsible for annual losses of up to one billion dollars in the U.S. (Frey et al., 2011). Hence, understanding the defense mechanisms of cereals is crucial in developing sustainable crop enhancement programs. Intriguingly, despite the large variety of potential attackers, only few pathogens and pests are able to successfully parasitize a particular plant species. This corroborates the fact that plants employ a highly intricate defense system that is capable of fending off the majority of attackers. Plant immunity is multilayered and consists of pre-formed, constitutive as well as inducible defense mechanisms (Pieterse et al., 2009). Besides physical pre-formed barriers such as the cell wall, plants also possess highly effective pre-formed chemical defenses called phytoanticipins (González-Lamothe et al., 2009). Those are constitutively present products of secondary plant metabolism. They represent a first defense layer and are released and activated as antimicrobial compounds upon pathogen entry. A diverse family of phytoanticipins is composed of the so-called saponins, secondary metabolites that can be found in many plant species but particularly in dicots. Intriguingly, with the exception of oats, cereals are generally deficient in saponins (Osbourn, 2003). In addition to pre-formed chemical defenses, plants also employ antimicrobial compounds that are induced only upon pathogen or pest attack. These compounds are defined as phytoalexins (Hammerschmidt, 1999), antimicrobial compounds whose induction is mediated by a pathogen-triggered activation of enzymes involved in their synthesis. Usually, phytoalexins possess rather unspecific inhibitory effects on a wide range of different pathogens.

The compounds that constitute the chemical defense arsenal of plants stem from various metabolic pathways, and can be roughly categorized in three major groups, namely alkaloids (e.g., the indole alkaloid camalexin), isoprenoids (e.g., diterpenes), and shikimates (e.g., flavonoids; Großkinsky et al., 2012; **Figure 1**). Alkaloids are mainly synthesized via the citric acid cycle or shikimate pathway; isoprenoids are synthesized via the acetate-mevalonate or methylerythritol phosphate pathway, whereas phenylpropanoids are mainly built over the shikimate pathway (Großkinsky et al., 2012). The entire set of metabolites synthesized via these and various other pathways is defined as the plant's metabolome, which may be viewed as the biochemical phenotype of a given plant tissue. In metabolomic analysis, such biochemical phenotypes can be qualitatively and quantitatively profiled on a large scale. In recent years, metabolite profiling has become a standard research tool for high-throughput diagnostics in various plant science applications, such as phenotyping of different species and analysis of resistance traits or responses to herbicides (Schauer and Fernie, 2006). In concert with transcriptomics, metabolomics has become an indispensable tool in screening crop germplasm collections during crop breeding

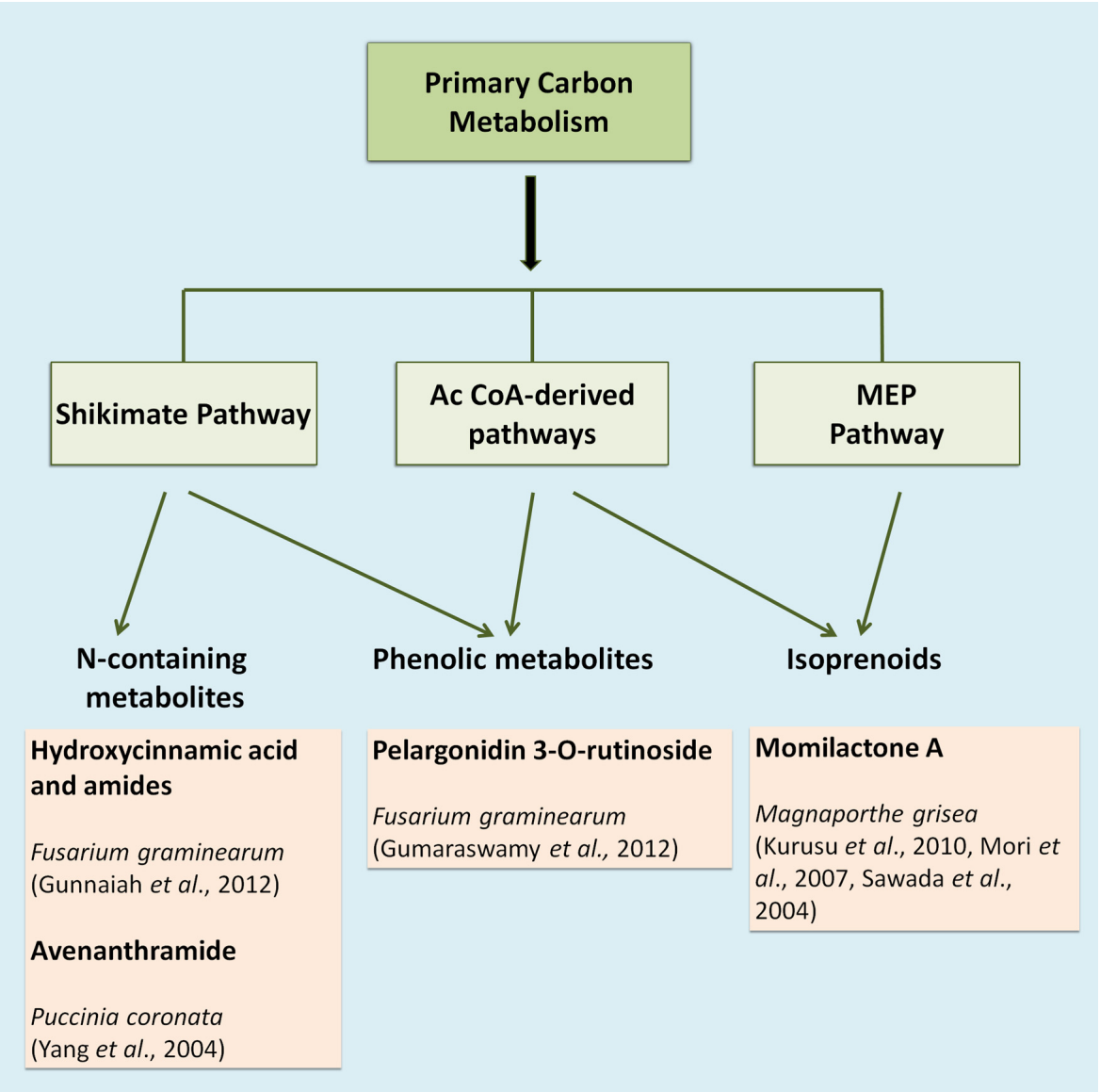


FIGURE 1 | Main metabolic pathways involved in cereal defense.

programs (Langridge and Fleury, 2011). A plant's metabolome plays an important role in a wide range of physiological processes, and current research on plant stress responses greatly benefits from recent advances in metabolite profiling methods (Großkinsky et al., 2012).

Apart from some recent articles (Allwood et al., 2010; Du Fall and Solomon, 2011) very few metabolomic studies on the interactions between biotic stressors and plants, especially cereals, have been reported. In this review, the role of metabolites in response to pathogens is elucidated, along with their role in herbivore defense. Moreover, recent advances in metabolite profiling and analysis techniques are summarized, giving a comprehensive overview of the current methods available for metabolomic analysis in cereals.

METABOLOMIC RESPONSES OF CEREALS TO NECROTROPHIC PATHOGENS

A model for a metabolomic study applied to fungal diseases must take into account several criteria such as: an accurate identification of the compounds or at least a putative identification of metabolites; a statistical significance within the studied variations; a strong change in concentration between resistant/susceptible plant-pathogen metabolome; and finally, if possible, assignation to a known plant defense pathway.

As an example, *Fusarium* head blight (FHB) is one of the most devastating diseases that affect several monocotyledonous plants such as barley, maize, wheat, and triticale (Choo, 2006). *Fusarium* is a necrotrophic pathogen and uses mycotoxins to kill the plant tissue before being able to feed on the host cells. Resistance to FHB

is associated with more than 100 quantitative trait loci (QTLs) distributed along the seven chromosomes of barley and wheat. However, only the function of the *Qfhs.ndsu-3BS* QTL has been clearly defined in resistance, since it is involved in the detoxification of the mycotoxin deoxynivalenol (DON) into its less toxic glucoside, DON-3-O-glucoside (Lemmens et al., 2005). In such a case, the study of resistance controlled by polygenes with low heritability that changes depending on environment, location and year, is time consuming and not very efficient. Fortunately, the existence of metabolomic resources is a very valuable tool to search for metabolites with resistance-related potential in such complicated landscapes. Using liquid chromatography coupled to mass spectrometry (LC-MS) Bollina et al. (2010) identified 496 metabolites in barley that were overrepresented in a metabolomic analysis of a resistant cultivar compared to a susceptible one. They assigned a putative identity based on the accurate mass, fragmentation pattern and the number of carbons in the formula to these metabolites. Interestingly, most of the metabolites from the resistance cluster (RR) were derived from the phenylpropanoid, flavonoid, fatty acid, and terpenoid metabolic pathways (**Figure 1**). Their putative role in resistance was further confirmed by *in vitro* bioassays for antifungal activity. Among the RR cluster several precursors of kaempferol were identified to play a relevant role in the enhanced defense capacity of the resistant cultivar (Bollina et al., 2010).

To study the role of metabolites participating in resistance identical genetic backgrounds should be used, since differences in the metabolites may derive from differences in the plant genotypes. Furthermore, it is also possible to find pathogen-derived metabolites. However, it is expected that resistance is also associated to lower levels of fungal growth and therefore, the selection criteria based on the higher abundance of metabolites in the resistant genotypes makes the selection of fungal compounds as resistance metabolites rather unlikely.

The range of resistance of barley spikelets to *F. graminearum* is classified as a type II resistance (Schroeder and Christensen, 1963). The *Fusarium* mutant trichothecene-non-producing (*tri5*-) fails to spread within inoculated spikes in wheat (Jansen et al., 2005). The combined system of resistant and susceptible barley, together with trichothecene-producing and non-producing *F. graminearum* strains, is a good model to study the metabolic responses that regulate resistance in barley to this fungal disease (Kumaraswamy et al., 2012). This research revealed the existence of constitutive resistance-related (RRC) and induced resistance-related (RRI) metabolites. Examples of specific RRC compounds with elevated levels found in resistant barley are coniferylaldehyde, pelargonidin 3-O-rutinoside, vitexin, and 8E-heptadecenoic acid (**Figure 2**; Kumaraswamy et al., 2012). Even more relevant was the finding that indole acetic acid, picolinic acid, and a glucoside of feruloyl alcohol showed higher concentrations in response to the trichothecene producing strain in the resistant barley.

In wheat, the Fhb1 (*Fusarium* head blight 1 resistance locus) QTL is believed to be responsible for resistance to the spread of *F. graminearum* within the spikes. This resistance is mainly attributed to the activation of the phenylpropanoid, terpenoid, and fatty acid metabolic pathways (**Figure 1**) in addition to the detoxification of DON to DON-3G. Non-targeted proteomics based on 2D gel electrophoresis combined with LC-MS/MS have been applied to this plant-pathogen system (Gunnaiah et al., 2012). Proteomic studies confirmed the implication of these pathways but also the relevant role of the oxidative burst and the accumulation of PR-1, 1,3-β-glucanases, chitinases, and PR-10 proteins. In addition, methionine synthase, S-adenosylmethionine synthase, 5,10-methylene-tetrahydrofolate reductase, and adenosylhomocysteine hydrolase, that increase the activity of the ethylene and phenylpropanoid pathway, were shown to be more active in the resistant lines of wheat. However, the participation of *Fhb1* in resistance is mainly due to its involvement in the regulation of the phenylpropanoid pathway. This is a good example demonstrating that proteo-metabolomic studies are not only restricted to the genetics of a given QTL (Gunnaiah et al., 2012). These studies also revealed that jasmonic acid isoleucine (JA-Ile and JA together with HCAAs (hydroxycinnamic acid amide, conjugates of phenol-polyamines) such as coumaroyl putrescine/agmatine and feruloyl putrescine/agmatine overaccumulate in resistant wheat cultivars (**Figure 2**; Gunnaiah et al., 2012).

Another model cereal studied in connection with interactions between plants and necrotrophic fungi is maize. Recently, a new function for benzoxazinones (BX) in the resistance against the necrotrophic fungus *Setosphaeria turcica* was elucidated (Ahmad et al., 2011). An accepted mode of action is attributed to the toxicity of the aglucones when the BX-glucosides are hydrolyzed by plastid-targeted β-glucosidases (Morant et al., 2008). The use of ultra-high pressure LC (UHPLC) coupled to QTOFMS is a valuable tool to determine the occurrence of these compounds under various experimental conditions (Ahmad et al., 2011; Glauser et al., 2011).

METABOLOMIC RESPONSES OF CEREALS TO BIOTROPHIC PATHOGENS

Magnaporthe oryzae shows a hemibiotrophic life style characterized by apparently unaffected host cells that retain the ability to plasmolyse (Koga et al., 2004). In contrast, during incompatible interactions of rice cells with the fungus, the cells lose membrane integrity and the ability to plasmolyse, showing granulation and other symptoms usually associated to a necrotrophic mechanism of infection. Therefore, the degree of incompatibility conditions the lifestyle of *Magnaporthe oryzae*, which behaves only as a pure biotroph in fully compatible interactions. *Magnaporthe oryzae* infects plant cells via germinating conidia at the leaf surface. The germtube produces an appressorium from which a penetration peg grows into the cell. The penetration peg gives rise to numerous invasive biotrophic hyphae that are separated from the host cytoplasm by a plant-generated membrane. Fungal progression to neighboring cells is likely taking place through plasmodesmata since plant cell wall integrity is not disturbed (Kankanala et al., 2007). In addition, the biotrophic strategy of rice blast is different when it invades the first layer of cells or subsequent cells. The metabolic interplay during such a finely controlled process is difficult to study.

In a detailed study of the metabolic interplay between rice and *Magnaporthe grisea*, two major findings that define the metabolic reprogramming were observed (**Figure 3**). Infected leaf

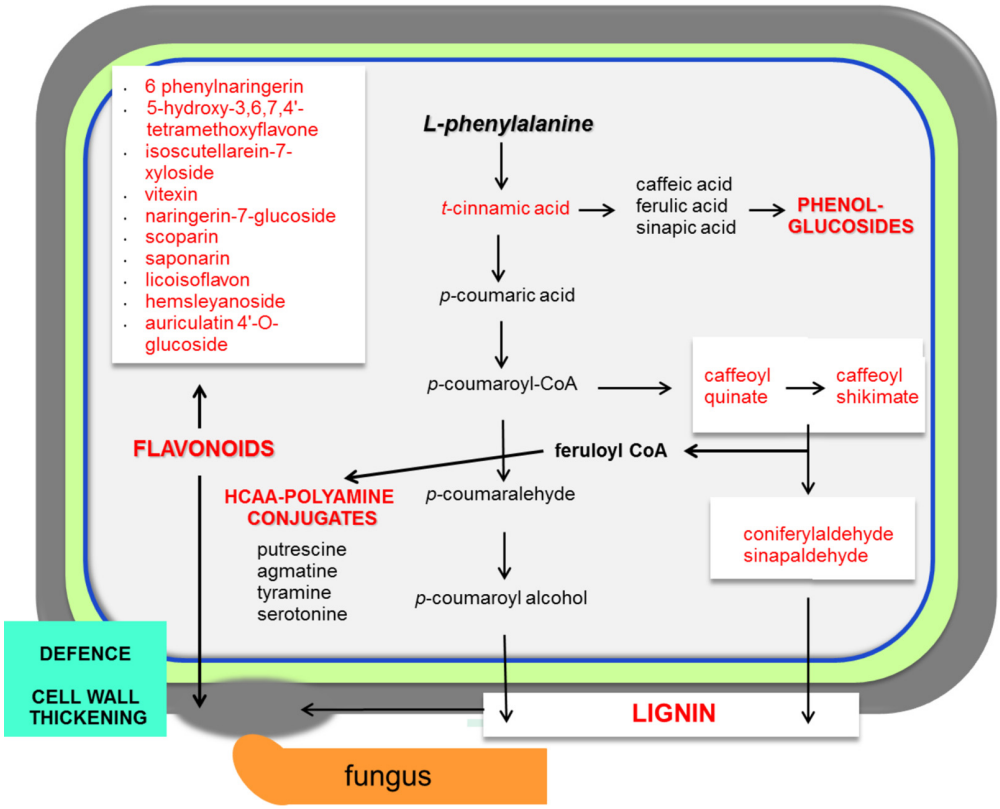


FIGURE 2 | Necrotrophs interacting with barley and wheat. The main responses of cereals during necrotrophic interactionsare focused in the activation of the phenylpropanoid pathway. The infected plant accumulates lignin, phenol-glucosides, hydroxycinnamic acid conjugated with polyamine derivatives (HCAA; Gunnaiah et al., 2012) and also flavonoids. Abundant metabolites in cereal–necrotoph interactions are represented in red. Pathways that are activated during the interaction with necrotrophs are represented in bold red. This model is based on interactions between barley/wheat and *Fusarium* sp. (Bollina et al., 2010)

tissues displaying lesions accumulated Ala (alanine), Pro (proline), His (histidine), Cys (cysteine), and Trp (tryptophan) among other amino acids, and sucrose, malate, fructose, and glucose (Parker et al., 2009; Jones et al., 2011). This has been observed in susceptible rice genotypes suggesting that infected leaves with visible lesions become metabolic photosynthetic sinks (Parker et al., 2009). This observation fits well with the biotrophic lifestyle of *Magnaporthe grisea*; however, there is also an accumulation of phenylpropanoid and phenolic compounds that resembles the plant-necrotroph responses described above. A very likely explanation is that rice is triggering cell wall reinforcements that are less pronounced in susceptible phenotypes due to the reduced generation of H_2O_2 (**Figure 3**). This causes a deficit in phenolic cross-linking in the cell compared to resistant phenotypes. Finally during the latter stages of infection, leakage of nutrients from dying cells might act as energy supply for the sporulation process of the fungus (Parker et al., 2009).

Again, the combination of compatible and incompatible strains of *Magnaporthe grisea* provides a perfect scenery to study metabolic reprogramming related to defense in rice. Jones et al. (2011) used MS and nuclear magnetic resonance (NMR)-based metabolomics to assess the response to the fungus at different time-points after infection. Among many other interesting compounds, they found that the major changes in each interaction involved malate, glutamine, Ala, Pro, cinnamate, and sugars. Interestingly, they proposed that fungus-triggered high levels of Ala may be responsible for cell death to facilitate *Magnaporthe grisea* invasion. These observations suggest that the negation of such responses may cause incompatibility in the interaction, thereby stopping the infection. Despite such attractive conclusions further studies are needed for a final demonstration of the roles of Ala in the establishment of compatibility (Jones et al., 2011).

METABOLOMICS IN DEFENSE AGAINST HERBIVORES AND NEMATODES

Plants also produce specific secondary metabolites to protect themselves against potential pest herbivores or nematodes. The importance of such metabolites is reflected in the extensive portion of the genome allocated to genes involved in primary or secondary metabolism, which has been estimated to be 25% of the rice (*Oryza sativa* L. ssp. *japonica*) genome as an example (Goff et al., 2002).

Metabolomics studies in the classical sense are scarce in cereals and even more so in the field of cereal–herbivore interactions. Although numerous QTLs linked to insect herbivore resistance have been identified, the genetic basis responsible for

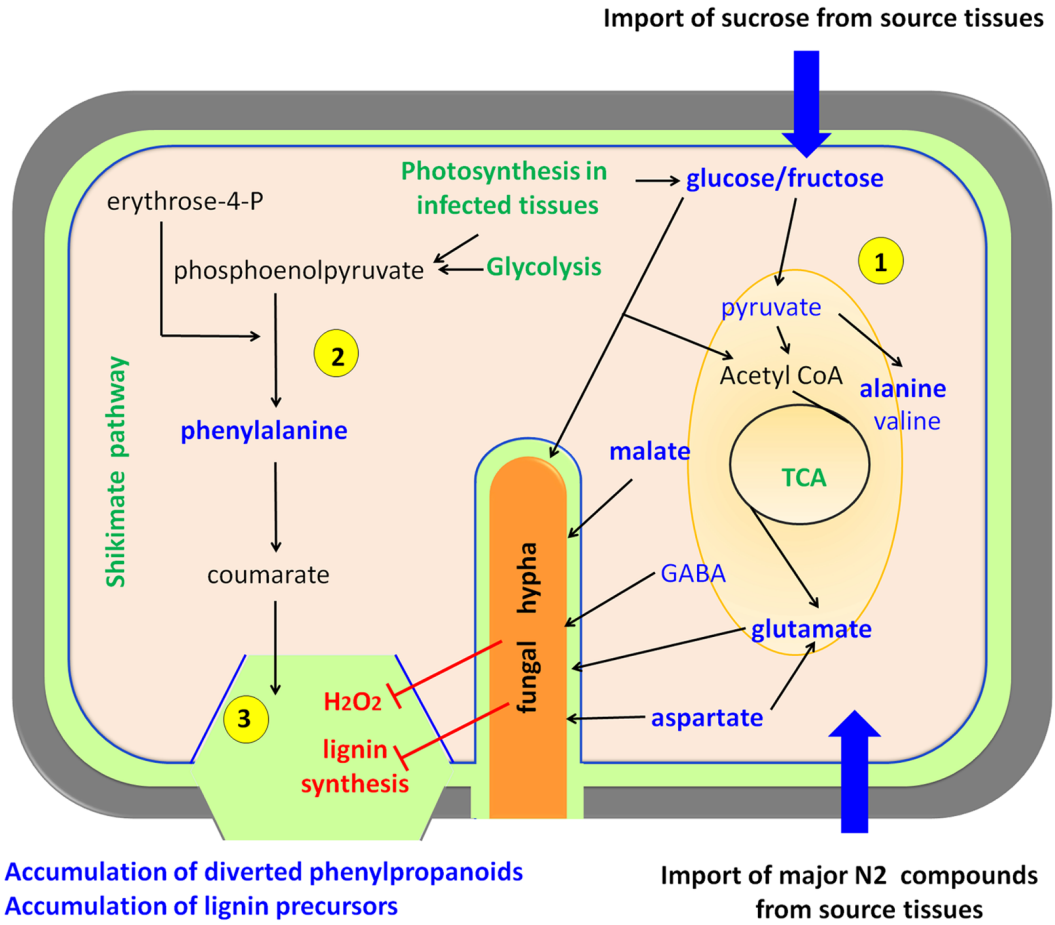


FIGURE 3 | Biotrophs interacting with rice. Biotrophic pathogens feed from living cells forcing the host to increase its primary metabolism. Plant cells over-compensate the carbon and nitrogen depletion by acting as a sink for organic compounds that are imported from plant source tissues, and also by increasing their photosynthesis, gluconeogenesis, and glycolysis **(1)** The main pathways activated upon fungal infection are the tricarboxylic acid cycle (TCA) and the glycolysis. On the other hand, plant defense attempts to stimulate the shikimate pathway and lignin biosynthesis **(2)**, but the fungus hijacks this processes with the help of effectors (Mentlak et al., 2012) and by inhibiting oxidative crosslinking of phenolics, thus leading to an over-accumulation of free phenylpropanoids and lignin precursors **(3)** (Parker et al., 2009). Upregulated metabolic pathways are depicted in green, compounds present in high abundance upon infection in blue, and processes inhibited by the pathogen in red. This model is essentially based on rice-*Magnaporthe grisea* interactions.

these traits is in most cases unknown. Most cereal metabolites with insecticidal and/or nematicidal properties have also been shown to inhibit the growth of pathogens and are derived from the same chemical classes as the ones active against microbes.

BENZOXAZINOIDS

The best-investigated anti-herbivore secondary metabolites in cereals are the benzoxazinoids, molecules with a 2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one skeleton. Among them, the hydroxamic acids are the most active ones (Niemeyer, 2009). In the plants, these molecules are usually glucosylated and their activity rises after enzymatic hydrolyses to an aglucone. The biosynthetic pathway leading to their generation is well known (Niemeyer, 1988; Sicker and Schulz, 2002).

Erb et al. (2009) investigated the reaction of maize (*Zea mays*) to belowground attack by the western corn rootworm *Diabrotica virgifera virgifera* on the defensive capacity of the aboveground organs against another herbivore insect pest, *Spodoptera littoralis* and also monitored the accumulation of defensive metabolites following challenge of the leaves with *Spodoptera*. Quantification of metabolites in the leaves by HPLC-DAD (high-performance LC with diode-array detection) revealed a direct induction of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) following root attack and an additional increase upon challenge with *Spodoptera*. The various treatments did not affect the levels of DIMBOA-glucoside (DIMBOA-Glc). Analysis of phenolic compounds by ultra performance LC (UPLC)-MS showed that caffeic acid production was suppressed following infestation by either of the insects. However, chlorogenic acid was induced directly only by *Spodoptera*, but prior infestation of the root system with *Diabrotica* primed the leaf tissues to produce more chlorogenic acid upon *Spodoptera* challenge. Interestingly, direct induction of DIMBOA and priming of chlorogenic acid accumulation in the leaves can

be mimicked by applying abscisic acid (ABA) to the roots of the maize plants. However, the involvement of additional metabolites or mechanisms must be assumed since root treatment of maize plants with ABA alone did not induce resistance against against *Spodoptera littoralis* (Erb et al., 2009). Recent findings uncovered a dual role of BX in inducible herbivore resistance (Glauser et al., 2011). Both *Spodoptera littoralis* and *Spodoptera frugiperda* were shown to be able to detoxify DIMBOA, which was rapidly released from its corresponding glucoside in the primary response against herbivores. In contrast, the highly unstable 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA), which is released in a second step during herbivore attack, functions as deterrent to *Spodoptera littoralis* and *Spodoptera frugiperda* and is quickly degraded in the insect guts.

Besides their direct toxic effect, BX seem to also have a regulatory role in innate immunity. Ahmad et al. (2011) compared the expression of basal resistance in BENZOXAZINELESS1 (*BX1*) wild type and *bx1* mutant maize lines. The *bx1* mutants, besides being less resistant to the fungal pathogen *Setosphaeria turcica*, supported a better development of the cereal aphid *Rhopalosiphum padi.* Already during early infestation stages by *R. padi* an increased accumulation of DIMBOA-Glc, DIMBOA itself, and HDMBOA-glucoside (HDMBOA-Glc) was measured in the leaves. Leaf infiltration with chitosan, an elicitor of defense produced by deacetylation of chitin, a structural element in the skeleton of insects or the cell wall of fungi, also enhanced the accumulation of DIMBOA and HDMBOA-Glc. The expression of genes in the biosynthetic pathway leading to BX however was down-regulated downstream of BX1 by chitosan. Additionally, in *bx1* mutants, callose deposition elicited by chitosan infiltration was reduced compared to wild type. These findings all point toward a role for DIMBOA as a signal in the regulation of maize innate immunity.

An additional role for BX in cereal defense has been suggested in the protection against nematodes. Rye (*Secale cereale*) planted as an annual winter cover crop, is able to reduce insect and nematode infestation in the following crop (Zasada et al., 2005). Since a biocidal action of low molecular weight aliphatic organic acids from such rye plants against *Meloidogyne incognita* had been ruled out (McBride et al., 2000), other possibly involved metabolites were tested. Based on reports that the BX DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and its breakdown product benzoxazolin-2(3*H*)-one (BOA) as well as DIMBOA and its degradation product 6-methoxy-BOA (MBOA) in rye had allelopathic properties (Barnes and Putnam, 1987; Rice et al., 2005), these substances were also tested as to their influence on nematodes (Zasada et al., 2005). DIBOA was shown to be more toxic than DIMBOA. In contrast to corn and wheat, where DIMBOA is the main metabolite, in rye, DIBOA predominates (Friebe, 2001; Rice et al., 2005), making it a possible candidate for nematode control. DIBOA caused a higher mortality than DIMBOA in both plant parasitic nematodes *Xiphinema americanum* and *Meloidogyne incognita,* respectively, whereas eggs were less affected than adults and juveniles (Zasada et al., 2005). Such *in vitro* toxicity studies have to be relativized since it was shown later by the same research group that, based on the fate of DIBOA in agricultural soils, the actually present concentration might be too low to be a major factor in containing nematode populations.

FLAVONOIDS

Flavonoids such as the C-glycosyl flavones maysin and apimaysin found in corn silk for instance have been shown to inhibit the growth of corn earworm (*Helicoverpa zea)* larvae (Lee et al., 1998). Based on QTL analysis, 55–65% of phenotypic variance against the corn earworm could be attributed to maysin or apimaysin, respectively. Interestingly, the two QTLs did not interfere with each other concerning the synthesis of the two substances, pointing to an independent synthesis of the two closely related compounds (Lee et al., 1998). Another flavonoid with activity against *Helicoverpa zea* is isoorientin. In a corn inbred line with high concentrations of isoorientin in the silk it was shown that this was based on the presence of a single recessive gene (Widstrom and Snook, 1998).

In response to nematode invasion, oats (*Avena sativa*) reacts with the induction of flavone-C-glycosides as identified by MS. One of these compounds, O-methyl-apigenin-C-deoxyhexoside-O-hexoside, turned out to be an effective protectant against two major nematodes of cereals, *Pratylenchus* and *Heterodera* (Soriano et al., 2004).

ALKALOIDS

The best-known alkaloids of grasses are hordenine (*N,N*-dimethyltriamine) and gramine (*N,N*-dimethylindolemethylamine), respectively. Hordenine is found in many plant species and in cereals it has been reported in barley, millet (*Panicum miliaceum*) and sorghum (*Sorghum vulgare*; Smith, 1977). Both alkaloids have been shown to act as feeding deterrents against grasshoppers (Hinks and Olfert, 1992). Feeding tests with specialist (*Heliothis subflexa*) and generalist (*Heliothis virescens*) caterpillars also showed deterring effect of hordenine on the feeding behavior and, interestingly, *Heliothis subflexa* was more affected than *Heliothis virescens* (Bernays et al., 2000). Gramine also influences the feeding behavior of aphids. Feeding experiments with *Schizaphis graminum* and *Rhopalosiphum padi* on barley seedlings revealed that the concentration of gramine in the plant and also its tissue location were affecting the feeding behavior (Zúñiga et al., 1988).

These above-mentioned examples were not based on metabolome-covering studies but concentrated specifically on compounds acting as feeding deterrents or with toxic properties. A recent attempt to get a more global picture of herbivore-induced changes in the metabolome of maize was published by Marti et al. (2013). Using UHPLC LC-time-of-flight mass spectrometry (UHPLC-TOF-MS) they took an unbiased approach to determine changes in the metabolite profile at the local and systemic level in maize plants infestated with *Spodoptera littoralis*, thus revealing 32 differentially regulated compounds. It is to be expected that the availability of novel methodologies will speed up our knowledge on the changes occurring at the metabolic level in various plant–insect interactions.

CURRENT METABOLOMIC TECHNOLOGIES

Few analytical techniques are able to profile a broad range of metabolites in a single analysis. An ideal method that would

detect, quantify, and identify all metabolites present in a given plant with high sensitivity, dynamic range, and reproducibility, does not exist (Dunn, 2008; Wolfender et al., 2009). The most comprehensive methods can detect a few thousands of markers, of which only a small portion may be identified (Obata and Fernie, 2012). Amongst the detectors that may be considered for metabolomics, two unarguably stand out from the crowd, namely MS and NMR. In this section, a brief description of both methods is presented with an emphasis on their advantages and limitations and the latest developments in the respective fields.

MS-BASED METHODS

Mass spectrometry involves the generation of ions and the measurement of their mass-to-charge ratio, providing structural information on the detected molecules. MS may be used either alone or coupled with separation techniques including gas chromatography (GC), LC, and capillary electrophoresis (CE). Contrary to NMR (see below), MS is a highly versatile technique with numerous combinations of ionization sources and analyzers possible. However, no combination is as universal as NMR and therefore the chosen approach may have a strong impact on the classes of metabolites detected. The main advantage of MS over NMR is its extreme sensitivity that allows for detection of metabolites present in trace amounts (Dettmer et al., 2007). Another advantage, in particular when hyphenated to separation techniques, is its capacity to separate compounds in complex mixtures with high resolution. Finally, MS has proved very efficient for the analysis of certain specific classes of metabolites such as lipids and is thus accepted as the method of choice in lipidomics. In contrast, absolute quantification of signals is not possible in the absence of reference standards because ionization is compound-dependent. Finally, in comparison to NMR, the relatively poor reproducibility of MS may render its use in long-term studies problematic when samples cannot be stored for a prolonged period of time (Glauser et al., 2013).

DIRECT MS

Direct MS represents an interesting approach for high-throughput fingerprinting of large numbers of biological samples. In general, high resolution mass spectrometers are employed because of their important separative power (Dettmer et al., 2007). Three types of analyzers may be employed: TOF, electrostatic trap, or Orbitrap, and Fourier transform ion cyclotron resonance (FT-ICR). Currently, TOF, Orbitrap, and FT-ICR can attain maximal resolving powers of 30'000–60'000, 240'000, and >1'000'000, respectively. Contrary to TOFs, the two latter technologies allow for the resolution of fine isotopic distributions (e.g., $^{13}C_2$ and ^{34}S isotopes) and are certainly the methods of choice in direct MS metabolomics. However, such resolving powers can only be achieved at low scanning rates, preventing their use in combination with fast chromatographic techniques (Hopfgartner, 2011; Glauser et al., 2012). This is obviously not an issue in direct MS where scan times of 2–5 s may easily be implemented without sacrificing throughput or resolution.

Several ionization methods may be used, including atmospheric pressure ionization (API) methods such as electrospray (ESI), AP chemical ionization (APCI), and AP photo-ionization (APPI) where samples are usually either injected in the so-called flow-injection (FI) mode, or infused at a constant flow rate, a process referred to as direct infusion (DI) mode. Recently, ambient approaches have been developed for the analysis of liquid or solid samples, e.g., desorption-ESI (DESI), desorption-APCI (DAPCI), or extractive-ESI (EESI), and represent promising tools for direct MS metabolomics. Direct analysis in real time (DART) which also operates at AP but relies on different phenomena, also presents interesting features for metabolomics. However, these techniques have only been used in a very limited number of studies (Lee et al., 2012) and more evidence of their applicability to comprehensive plant metabolomics is needed. Another technique complementary to API methods is matrix assisted laser desorption/ionization (MALDI). While MALDI has been traditionally used in proteomics due to its capacity to analyze biomolecules, its use in plant metabolomics has been so far rather limited. The main reasons are the difficulty to produce ions from the relatively hydrophobic species present in plant tissues (Cha et al., 2008), and the fact that the matrices necessary for MALDI generate high background noise in the low mass region of the spectra which may interfere with small metabolites (Shroff et al., 2009). Nevertheless, ion-free matrices (e.g., DIOS, for desorption/ionization on silicon) or rational protocols for matrix selection (Shroff et al., 2009), are potential alternatives for the use of laser induced desorption/ionization in metabolomics. Moreover, as DESI, MALDI can be employed as a "microscope" by collecting mass spectra over a sample surface and reconstructing MS data as an image, a process called MALDI imaging. This method shows great promise for the study of the spatial distribution of metabolites within plant tissues or at the single cell level and is expected to play an increasing role in the future.

HYPHENATION TO SEPARATIVE METHODS

The coupling of MS to separative methods is a powerful means to improve resolution and marker detection by providing multidimensional data (e.g., 3D data consisting of *m/z* ratios, retention times and peak areas). Isomers may be distinguished, and ion suppression effects much reduced. GC and LC are the two most frequently used chromatographic techniques in MS-based metabolomics. Moreover, another separation method, CE, is gaining interest for the analysis of polar metabolites.

The coupling between GC and MS was achieved long before that of LC and MS and was already used in the early 1970s for human metabolite profiling (Horning et al., 1971) and in the 1980s for plant analysis (Sauter et al., 1991). In the domain of crops, it has been used e.g., to screen wheat cultivars resistant to FHB (Hamzehzarghani et al., 2005). Only volatile and thermally stable molecules can be analyzed by GC-MS. In other words, volatile metabolites such as mono- or sesquiterpenes, small aldehydes, and alcohols may be directly analyzed without chemical modification. However, the vast majority of plant metabolites is not volatile and requires chemical derivatization to increase volatility and thermal stability before GC-MS analysis. This is for instance the case for primary metabolites such as mono- or disaccharides, amino acids, organic acid, and fatty acids. Most often, a two-stage derivatization process is employed: carbonyl groups are first converted to oximes derivatives using

e.g., methoxyamine hydrochloride-HCl, followed by formation of trimethylsilyl (TMS) esters with silylating reagents, typically N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Lisec et al., 2006). It has been shown that temperature and derivatization time may affect the outcome of the results, that a range of derivatization products may be formed from a single metabolite, and that the sample stability is a concern (Dunn and Ellis, 2005). Despite these facts, GC-MS after derivatization is nowadays accepted as a gold standard in the field of metabolomics. This is certainly due to the fact that, when coupled through electron ionization, GC-MS yields reproducible and typical spectra, which has enabled the creation of spectral libraries containing hundreds of thousands of mass spectra. By performing mass spectral searches against these libraries, metabolite identification may be successful. However, these libraries are not totally exhaustive and structural identification *via* the interpretation of fragment ions is sometimes necessary. Recent trends in the field of GC comprise the development of 2D GC (GCXGC) metabolomic methods for increased resolution and selectivity (Pierce et al., 2006), and that of fast GC methods using shorter and narrower columns for increased throughput (Jonsson et al., 2004).

In plants, a large portion of metabolites remains inaccessible to GC-MS. For example, flavonoid glycosides or BX glycosides are two important classes of defense secondary metabolites that cannot be volatilized even after derivatization. In such context, the use of LC-MS as an alternative to GC-MS must be considered. With LC-MS, minimal sample preparation is required and the range of metabolites that can be covered is theoretically much wider than that of GC-MS. In principle, LC-MS may detect most organic compounds except extremely volatile ones. For this, several different chromatographic modes shall be employed. Reverse-phase (RP) chromatography using C18 columns has been largely adopted in metabolomic studies. This mode is suitable for most plant secondary metabolites that generally display mildly polar properties (Allwood and Goodacre, 2010). However, very polar and very hydrophobic species require other modes of LC. The former are best analyzed by hydrophilic interaction LC (HILIC; Tolstikov and Fiehn, 2002), while the latter are traditionally separated by normal phase (NP) LC using non-polar solvents such as tert-butyl methylether or hexane. LC and MS are usually interfaced with API sources, predominantly ESI and less often APCI or APPI. These soft ionization techniques yield ions of the molecular species $(M+H)^+$ in positive mode, and $(M-H)^-$ in negative mode, and various adducts, multimers or multiply charged ions. In APCI and APPI, radical cations $(M)^+$ or anions $(M)^-$ may also be formed. Recently, sub-2 μm stationary phases and chromatographs able to withstand pressures up to 1300 bars have been introduced on the market. Such systems are referred to as UHPLC and offer a substantial improvement in chromatographic performances, either for the enhanced resolution of complex extracts or the analysis of numerous samples in a short time (5–15 min per sample (Eugster et al., 2011)). The number of publications which report the use of UHPLC-MS for metabolomic studies has grown exponentially over the last years and the trend will definitely not be reversed in the near future. Still, whatever powerful they are, LC-MS and UHPLC-MS cannot replace all other techniques because they also present some limitations, such as the problem of ion suppression and the lack of reproducibility of fragmentation spectra which complicates the creation of mass spectral libraries based on LC-MS data (Glauser et al., 2013).

Capillary electrophoresis mass spectrometry can be viewed as an alternative to HILIC-MS for polar or charged metabolites. The principle of CE involves the separation of molecules according to their mass-to-charge ratio under the influence of an electric field. To date, CE-MS has been relatively rarely employed in plant metabolomics (Sato et al., 2004). Nevertheless, its different selectivity compared to GC and LC makes it a promising tool for the analysis of charged species and further applications may be anticipated in the future (Ramautar et al., 2009).

NMR-BASED METHODS

Nuclear magnetic resonance is a universal non-destructive and high-throughput technique that requires minimal sample preparation. Generally, plant samples are either freeze-dried and directly extracted in a mixture of D_2O-CD_3OD buffered at e.g., pH 6.0 (Kim et al., 2010), or extracted fresh with $HClO_4$ 1M with subsequent freeze-drying and redissolution in D_2O (Kruger et al., 2008). Standard extracts such as those prepared for LC-MS analysis may also simply be evaporated and redissolved in an appropriate deuterated solvent provided that they are concentrated enough. The identification of markers of interest relies on the comparison of specific NMR chemical shifts for plant metabolites with those of reference compounds under identical solvent conditions. A main advantage of NMR over MS is that the signal intensities can be directly linked to the concentration of metabolites, which makes NMR an absolute quantitative method. A majority of applications has used ^{1}H-NMR due to the omnipresence of hydrogen atoms in organic molecules, the relatively good sensitivity of NMR for their detection compared to ^{13}C or ^{15}N, and the speed of analysis. However, ^{1}H-NMR spectra are often crowded and the detection of certain metabolites may be hindered or biased due to overlapping signals (Kim et al., 2010). An increase in resolution is therefore desirable and may be achieved by the use of stronger magnets (up to 1 GHz for hydrogen atoms), complementary 2D experiments such as *J*-resolved (requiring longer analysis times), or LC-NMR approaches. Another drawback is the lack of sensitivity (several orders or magnitude lower than that of MS), although the use of cryogenic and/or micro probes may increase sensitivity by a factor of 20 (Kim et al., 2010). Still, NMR is superior to MS in terms of reproducibility (Verpoorte et al., 2007; Schripsema, 2010), which makes it an interesting tool for the measurement of predominant constituents of plants such as sugars, amino acids, organic acids, and major secondary metabolites (Wolfender et al., 2013). Recently, an interesting study reported the comparison of GC-MS and NMR performances for metabolite profiling of rice samples (Barding et al., 2012). While GC-MS proved as expected much more sensitive and could detect several minor primary metabolites not observed by NMR, it also presented some limitations including low dynamic range and failure to detect certain metabolites such as dipeptides. Finally, NMR analysis may also be used to complement UHPLC-MS to assess the functional groups and the final identity of purified compounds, such as the BX derivatives in maize (Ahmad et al., 2011).

DATA PROCESSING AND MINING

All "omics" approaches heavily rely on bioinformatic tools for the analysis of the large datasets generated and metabolomics is not an exception. In the case of GC-MS or LC-MS datasets for example, raw data must be recorded and converted to appropriate formats for further data handling, including noise filtering, peak detection, and alignment. Such processing procedure aims to obtain homogenous information for a straightforward comparison of multiple samples by statistical methods. Results are displayed in the form of a marker table containing sample names, variables (characterized by *m/z* and retention time values) and peak intensities or areas. Each sample should ideally be defined by the same number of variables and each variable should correspond to the same metabolite. This peak picking procedure may be achieved using a range of free packages, e.g., MarVis[1], MzMine (Katajamaa and Oresic, 2005), XCMS (Smith et al., 2006), MetAlign (Lommen, 2009), or commercial softwares, e.g., Markerlynx[2].

In a second step, multivariate analysis methods may be used to reduce the dimensionality of data, revealing clusters of samples, and discriminatory variables. Prior to this, a pre-treatment of the data is often carried out to provide suitable data for further analysis. Normalization to the total integrated area or to a given internal standard may or may not be applied to the dataset depending on the biological model studied. Scaling enables the adjustment of the weight of each variable in the model (e.g., unit variance or Pareto scaling). Principal component analysis (PCA) is a common unsupervised multivariate method used for exploratory analyses by building principal components describing the maximal variance of data (Hotelling, 1933). PCA has been employed in the majority of metabolomic studies and represents a good starting point for exploring metabolomic data. Projection to latent structures by means of partial least squares (PLS; Wold et al., 2001) is a well-known supervised regression method and is often employed to maximize the separation between classes. Several other statistical methods exist and interested readers are invited to consult specialized literature for further information (e.g., Boccard et al., 2010; Liland, 2011).

CONCLUSION AND OUTLOOK

The work summarized in this review illustrates the pivotal role of metabolites in cereals during various biotic stresses. Within plant-biotroph interactions such as *Magnaporthe grisea* infection on rice, amino acids as well as sugars are known to be induced (Jones et al., 2011). A more extensive knowledge is also available for maize–pathogen interactions. Analysis of tissue-specific infections of maize with *Ustilago maydis* uncovered a prominent induction of the shikimate and flavonoid pathways in response to fungal attack (Doehlemann et al., 2008). Recently, the organ-specific metabolome changes of maize during infections with the hemibiotrophic fungus *C. graminicola* have been described (Balmer et al., 2013), uncovering higher levels of defense-associated metabolites including flavonoids in roots compared to leaves. In response to FHB, resistant barley lines were found to employ much higher levels of metabolites belonging to the flavonoid, phenylpropanoid, fatty acid, and terpenoid pathways compared to susceptible lines (Choo, 2006). Interestingly, recent evidence was also presented that BX, in addition to their toxic effects, function as a signal in maize immunity (Ahmad et al., 2011).

Recent advances in transcriptomic and metabolomic technologies facilitate a novel trend of integrated "omics," where cereals are screened in regard to pathogen-resistant genotypes as well as biochemical phenotypes (Langridge and Fleury, 2011). A combined transcriptomics/metabolomics analysis of maize and barley infected with different pathogens showed that the transcriptional reprogramming upon pathogen attack does not necessarily correlate with adaptation of the primary metabolism (Voll et al., 2011). Moreover, metabolomic profiling techniques are also applicable for evaluating genetically modified cereals (Ricroch et al., 2011). For instance, a transgenic barley line expressing a chitinase was compared to non-transgenic lines (Kogel et al., 2010). In a recent study of genetically modified maize, Barros et al. (2010) compared the transcriptome, proteome, and metabolome of different lines exposed to variable environmental factors. In this particular example, these factors affected the metabolome much stronger than genetic modification. Nevertheless, metabolomics is a useful tool for screening crops for pathogen resistance, as shown in the case of barley lines screened for resistance against *Gibberella zeae* (Kumaraswamy et al., 2011). There, 161 metabolites could be associated with less susceptible barley lines, including linoleic acid, p-coumaric acid, and naringenin. Besides its utility to screen for resistance traits, metabolomics is also widely applied as diagnostic tool. For instance, metabolomic analysis of naturally contaminated oat, rye and barley grains yielded distinct patterns of metabolites in infected versus non-infected grains (Perkowski et al., 2012). Moreover, in the same study, mycotoxins could also be analyzed in parallel to the plant metabolites, demonstrating the power of metabolomics as diagnostic aid.

Considering the great potential of cereal metabolomics in pathogen and pest resistance, it is not surprising that targeting metabolomic pathways is part of recent transgenic strategies in different cereals, mainly in rice. For instance, a series of momilactone A over-accumulating lines were generated (Sawada et al., 2004; Mori et al., 2007; Kurusu et al., 2010). Some of these lines exhibit enhanced resistance against *Magnaporthe grisea* and *Xanthomonas oryzae*. Similarly, overexpression of sakuranetin in rice resulted in an increased resistance to *Magnaporthe grisea* (Kim et al., 2009). Thus, manipulating biosynthetic pathways of metabolites appears to be an opportunistic strategy in transgenic crop enhancing programs. However, this approach is considered to also have a major drawback, namely the possible manipulation of metabolomic fluxes (Hassan and Mathesius, 2012). For instance, manipulating the phenylpropanoid metabolism in *Medicago truncatula* affected lignin synthesis in roots (Laffont et al., 2010). Moreover, an imbalance of secondary metabolites could possibly result in negative effects for the plant, including disadvantageous transport or exudation defects, as well as negative physiological costs (Hassan and Mathesius, 2012).

Metabolomics research is also accompanied by major limitations, the most important one being the current inability to analyze the entire metabolome. The number of plant metabolites

[1]http://marvis.gobics.de

[2]http://www.waters.com

is estimated to 200'000 or more (Trethewey, 2004; Saito and Matsuda, 2010) and the identified compounds summarized in public databases represent only a very little sample of this great variety. Thus, most of the compounds detected in current metabolomics studies remain unidentified. Despite the existence of public MS-databases such as KNApSAcK[3], KEGG[4], or BRENDA[5], updating and combining the information is one of the major future challenges. An additional drawback is the limited range of metabolites that can be analyzed simultaneously. For instance, excessive levels of sugars can interfere with the detection of flavonoids (Sumner et al., 2003). Moreover, metabolite profiling techniques usually need to be adapted according to the compounds of interest; for example, oligosaccharides are difficult to analyze using LC/MS (Sumner et al., 2003). Finally, it has to be considered that diseased plant material poses a special challenge to the methodology that can be applied and might require specific approaches (Allwood et al., 2012). As a consequence, analytical approaches need to be optimized for a given experimental setup. Another main challenge of metabolomics is the bioinformatics aspect, including data analysis and storage. As a high-throughput technology, current metabolomics generates massive amounts of datasets. The examination of such sets requires appropriate statistical models, as well as appropriate data visualization approaches (Sumner et al., 2007). In addition, the challenge is also to filter biological meaning out of massive datasets, especially when looking at entire metabolomes rather than selected markers. Further advances in bioinformatic tools combining general "omics" will contribute to a better understanding of the role of cereal metabolites during biotic stresses. This knowledge is expected to have a great impact in designing future cereal crop enhancement projects.

[3]www.kanaya.naist.jp/KNApSAcK/

[4]www.genome.ad.jp/kegg/kegg2.html

[5]www.brenda.uni-koeln.de

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support by the National Centre of Competence in Research (NCCR) 'Plant Survival' and SNF Grant 31003A_140593, both research programs of the Swiss National Science Foundation, and the financial support from the Plan de Promoción de la Investigación de la Universitat Jaume I ref:P1.1B2010-06.

REFERENCES

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., et al. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol.* 157, 317–327.

Allwood, J. W., Clarke, A., Goodacre, R., and Mur, L. A. J. (2010). Dual metabolomics: a novel approach to understand plant–pathogen interactions. *Phytochemistry* 71, 590–597.

Allwood, J. W., and Goodacre, R. (2010). An introduction to liquid chromatography-mass spectrometry instrumentation applied in plant metabolomic analyses. *Phytochem. Anal.* 21, 33–47.

Allwood, J. W., Heald, J., Lloyd, A. J., Goodacre, R., and Mur, L. A. J. (2012). Separating the inseparable: the metabolomic analysis of plant–pathogen interactions. *Methods Mol. Biol.* 860, 2012, 157–176.

Balmer, D., de Papajewski, D. V., Planchamp, C., Glauser, G., and Mauch-Mani, B. (2013). Induced resistance in maize is based on organ-specific defence responses. *Plant J.* doi: 10.1111/tpj.12114 [Epub ahead of print].

Barding, G. A. Jr., Fukao, T., Béni, S., Bailey-Serres, J., and Larive, C. K. (2012). Differential metabolic regulation governed by the rice SUB1A gene during submergence stress and identification of alanylglycine by 1H NMR spectroscopy. *J. Proteome Res.* 11, 320–330.

Barnes, J. P., and Putnam, A. R. (1987). Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13, 889–906.

Barros, E. L. S., Lezar, S., Anttonen, M. J., van Dijk, J. P., Röhlig, R. M., Kok, E. J., et al. (2010). Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. *Plant Biotechnol. J.* 8, 436–451.

Bernays, E. A., Oppenheim, S., Chapman, R. F., Kwon, H., and Gould, F. (2000). Taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists: a behavioral test of the hypothesis with two closely related caterpillars. *J. Chem. Ecol.* 26, 547–563.

Boccard, J., Veuthey, J. L., and Rudaz, S. (2010). Knowledge discovery in metabolomics: an overview of MS data handling. *J. Sep. Sci.* 33, 290–304.

Bollina, V., Kumaraswamy, G. K., Kushalappa, A. C., Choo, T. M., Dion, Y., Rioux, S., et al. (2010). Mass spectrometry based metabolomics application to identify quantitative resistance related metabolites in barley against *Fusarium* head blight. *Mol. Plant Pathol.* 11, 769–782.

Cha, S., Zhang, H., Ilarslan, H. I., Wurtele, E. S., Brachova, L., Nikolau, B. J., et al. (2008). Direct profiling and imaging of plant metabolites in intact tissues by using colloidal graphite-assisted laser desorption ionization mass spectrometry. *Plant J.* 55, 348–360.

Choo, T. M. (2006) Breeding barley for resistance to *fusarium* head blight and mycotoxin accumulation. *Plant Breed. Rev.* 26, 125–169.

Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* 26, 51–78.

Doehlemann, G., Wahl, R., Horst, R. J., Voll, L. M., Usadel, B., Poree F., et al. (2008). Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. *Plant J.* 56, 181–195.

Du Fall, L. A., and Solomon, P. S. (2011). Role of cereal secondary metabolites involved in mediating the outcome of plant–pathogen interactions. *Metabolites* 1, 64–78.

Dunn, W. B. (2008). Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. *Phys. Biol.* 5, 011001.

Dunn, W. B., and Ellis, D. I. (2005). Metabolomics: current analytical platforms and methodologies. *Trends Analyt. Chem.* 24, 285–294.

Erb, M., Flors, V., Karlen, D., de Lange, E., Planchamp, C., D'Alessandro, M., et al. (2009). Signal signature of aboveground-induced resistance upon belowground herbivory in maize. *Plant J.* 59, 292–302.

Erb, M., Gordon-Weeks, R., Flors, V., Camañes, G., Turlings, T. C., and Ton, J. (2009). Belowground ABA boosts aboveground production of DIMBOA and primes induction of chlorogenic acid in maize. *Plant Signal. Behav.* 4, 639–641.

Eugster, P. J., Guillarme, D., Rudaz, S., Veuthey, J. L., Carrupt, P. A., and Wolfender, J. L. (2011). Ultra high pressure liquid chromatography for crude plant extract profiling. *J. AOAC Int.* 94, 51–70.

Frey, T. J., Weldekidan, T., Colbert, T., Wolters, P. J. C. C., and Hawk, J. A. (2011). Fitness evaluation of Rcg1, a locus that confers resistance to *Colletotrichum graminicola* (Ces.) G.W. Wils. using near-isogenic maize hybrids. *Crop Sci.* 51, 1551–1563.

Friebe, A. (2001) "Role of benzoxazinones in cereals", in *Allelopathy in Agroecosystems*, eds R. K. Kohli, H. P. Singh, and D. R. Batish (Binghamton: Hawthorn Press), 379–400.

Glauser, G., Boccard, J., Wolfender, J. L., Rudaz, S. (2013) "Metabolomics: application in plant sciences", in *Wiley-VCH book project "Metabolomics in Practice"*, eds M. Laemmerhofer and W. Weckwerth (Weinheim: Wiley VCH Verlag GmbH & Co).

Glauser, G., Marti, G., Villard, N., Doyen, G. A., Wolfender, J. L., Turlings, T. C., et al. (2011). Induction and detoxification of maize 1,4-benzoxazin-3-ones by insect herbivores. *Plant J.* 68, 901–911.

Glauser, G., Veyrat, N., Rochat, B., Wolfender, J. L., and Turlings, T. C. (2012). Ultra-high pressure liquid chromatography-mass spectrometry

for plant metabolomics: a systematic comparison of high-resolution quadrupole-time-of-flight and single stage Orbitrap mass spectrometers. *J. Chromatogr. A pii:* S0021–S9673.

Goff, S. A., Ricke, D., and Lan, T. H., Presting, G., Wang, R., Dunn, M., et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica). Science* 296, 92–100.

González-Lamothe, R., Mitchell, G., Gattuso, M., Diarra, M. S., Malouin, F., and Bouarab, K. (2009). Plant antimicrobial agents and their effects on plant and human pathogens. *Int. J. Mol. Sci.* 10, 3400–3419.

Großkinsky, D. K., van der Graaff, E., and Roitsch, T. (2012). Phytoalexin transgenics in crop protection – fairy tale with a happy end? *Plant Sci.* 195, 54–70.

Gunnaiah, R., Kushalappa, A. C., Duggavathi, R., Fox, S., and Somers, D. J. (2012). Integrated metabolo-proteomic approach to decipher the mechanisms by which wheat QTL (Fhb1) contributes to resistance against *Fusarium graminearum. PLoS ONE* 7:e40695. doi: 10.1371/journal.pone.0040695

Hammerschmidt, R. (1999). Phytoalexins: what have we learned after 60 years. *Annu. Rev. Phytopathol.* 37, 285–306.

Hamzehzarghani, H., Kushalappa, A. C., Dion, Y., Rioux, S., Comeau, A., Yaylayan, V., et al. (2005). Metabolic profiling and factor analysis to discriminate quantitative resistance in wheat cultivars against *fusarium* head blight. *Physiol. Mol. Plant Pathol.* 66, 119–133.

Hassan, S., and Mathesius, U. (2012). The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant–microbe interactions. *J. Exp. Bot.* 63, 3429–3444.

Hinks, C. F., and Olfert, O. (1992). Cultivar resistance to grasshoppers in temperate cereal crops and grasses: a Review. *J. Orthop. Res.* 1, 1–9.

Hopfgartner, G. (2011). Can MS fully exploit the benefits of fast chromatography? *Bioanalysis* 3, 121–123.

Horning, E. C., Devaux, P. G., Moffat, A. C., Pfaffenberger, C. D., Sakauchi, N., and Horning, M. G. (1971). Gas phase analytical separation techniques applicable to problems in clinical chemistry. *Clin. Chim. Acta* 34, 135–144.

Hotelling, H. (1933). Analysis of a complex of statistical variables into principal components. *J. Educ. Psychol.* 24, 417–441.

Jansen, J., Von Wettstein, D., Schäfer, W., Kogel, K. H., Felk, A., and Maier, F. J. (2005). Infection patterns in barley and wheat spikes inoculated with wild type and trichodiene synthase gene disrupted *Fusarium graminearum. Proc. Natl. Acad. Sci. U.S.A.* 102, 16892–16897.

Jones, O. A. H., Maguire, M. L., Griffin, J. L., Jung, Y. H., Shibato, J., Jwa, N. S., et al. (2011). Using metabolic profiling to assess plant–pathogen interactions: an example using rice (*Oryza sativa*) and the blast pathogen *Magnaporthe grisea. Eur. J. Plant Pathol.* 129, 539–554.

Jonsson, P., Gullberg, J., Nordström, A., Kusano, M., Kowalczyk, M., Sjöström, M., et al. (2004). A strategy for identifying differences in large series of metabolomic samples analyzed by GC/MS. *Anal. Chem.* 76, 1738–1745.

Kankanala, P., Czymmek, K., and Valenta, B. (2007). Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* 19, 706–724.

Katajamaa, M., and Oresic, M. (2005). Processing methods for differential analysis of LC/MS profile data. *BMC Bioinforma.* 6:179. doi: 10.1186/1471-2105-6-179

Kim, A., Cho, K., Singh, R., Jung, Y. H., Jeong, S. H., Kim, S. H., et al. (2009). Rice OsACDR1 (*Oryza sativa* accelerated cell death and resistance 1) is a potential positive regulator of fungal disease resistance. *Mol. Cells* 28, 431–490.

Kim, H. K., Choi, Y. H., and Verpoorte, R. (2010). NMR-based metabolomic analysis of plants. *Nat. Protoc.* 5, 536–549.

Koga, H., Dohi, K., Nakayachi, O., and Mori, M. (2004). A novel inoculation method of *Magnaporthe grisea* for cytological observation of the infection process using intact leaf sheaths of rice plants. *Physiol. Mol. Plant Pathol.* 64, 67–72.

Kogel, K. H., Voll, L. M., Schäfer, P., Jansen, C., Wu, Y., Langen, G., et al. (2010). Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variances. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6198–6203.

Kruger, N. J., Troncoso-Ponce, M. A., and Ratcliffe, R. G. (2008). 1H NMR metabolite fingerprinting and metabolomic analysis of perchloric acid extracts from plant tissues. *Nat Protoc.* 3, 1001–1012.

Kumaraswamy, G. K., Kushalappa, A. C., Choo, T. M., Dion, Y., and Rioux, S. (2012), Differential metabolic response of barley genotypes, varying in resistance, to trichothecene-producing and -nonproducing (tri5-) isolates of *Fusarium graminearum. Plant Pathol.* 61, 509–521.

Kumaraswamy, K. G., Kushalappa, A. C., Choo, T. M., Dion, Y., and Rioux, S. (2011). Mass spectrometry based metabolomics to identify potential biomarkers for resistance in barley against *fusarium* head blight (*Fusarium graminearum*). *J. Chem. Ecol.* 37, 846–856.

Kurusu, T., Hamada, J., Nokajima, H., Kitagawa, Y., Kiyoduka, M., Takahashi, A., et al. (2010). Regulation of microbe-associated molecular pattern-induced hypersensitive cell death, phytoalexin production, and defense gene expression by calcineurin B-like protein-interacting protein kinases, OsCIPK14/15, in rice cultured cells. *Plant Physiol.* 153, 678–692.

Laffont, C., Blanchet, S., Lapierre, C., Brocard, L., Ratet, P., Crespi, M., et al. (2010). The compact root architecture1 gene regulates lignification, flavonoid production, and polar auxin transport in *Medicago truncatula. Plant Physiol.* 153, 1597–1607.

Langridge, P., and Fleury, D. (2011). Making the most of 'omics' for crop breeding. *Trends Biotechnol.* 29, 33–40.

Lee, E. A., Byrne, P. F., McMullen, M. D., Snook, M. E., Wiseman, B. R., Widstrom, N. W., et al. (1998). Genetic mechanisms underlying apimaysin and maysin synthesis and corn earworm antibiosis in maize (*Zea mays* L.). *Genetics* 149, 1997–2006.

Lee, S. M., Kim, H. J., and Jang, Y. P. (2012). Chemometric classification of morphologically similar Umbelliferae medicinal herbs by DART-TOF-MS fingerprint. *Phytochem. Anal.* 23, 508–512.

Lemmens, M., Scholz, U., Berthiller, F., D'all Asta, C., Koutnik, A., Krska, R., et al. (2005). The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus. *Mol. Plant Microbe Interact.* 18, 1318–1324.

Liland, K. H. (2011). Multivariate methods in metabolomics – from preprocessing to dimension reduction and statistical analysis. *Trac Trends Anal. Chem.* 30, 827–841.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., and Fernie, A. R. (2006). Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat. Protoc.* 1, 387–396.

Lommen, A. (2009). MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Anal. Chem.* 81, 3079–3086.

Marti, G., Erb, M., Boccard, J., Glauser, G., Doyen, G. R., Villard, N., et al. (2013). Metabolomics reveals herbivore-induced metabolites of resistance and susceptibility in maize leaves and roots. *Plant Cell Environ.* 36, 621–639.

McBride, R. G., Mikkelsen, R. L., and Barker, K. R. (2000). The role of low molecular weight organic acids from decomposing rye in inhibiting root-knot nematode populations in soil. *Appl. Soil Ecol.* 15, 243–251.

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., et al. (2012). Effector-mediated suppression of chitin-triggered immunity by *magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* 24, 322–335.

Morant, A. V., Jørgensen, K., Jørgensen, C., Paquette, S. M., Sánchez-Pérez, R., Møller, B. L., et al. (2008). Beta-glucosidases as detonators of plant chemical defense. *Phytochemistry* 69, 1795–1813.

Mori, M., Tomita, C., Sugimoto, K., Hasegawa, M., Hayashi, N., Dubouzet, J. G., et al. (2007). Isolation and molecular characterization of a spotted leaf 18 mutant by modified activation-tagging in rice. *Plant Mol. Biol.* 63, 847–860.

Niemeyer, H. M. (1988). Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* 27, 3349–3358.

Niemeyer, H. M. (2009). Hydroxamic acids derived from 2-hydroxy-2H-1,4-benzoxazin-(4H)-one: key defense chemicals of cereals. *J. Agric. Food Chem.* 57, 1677–1696.

Obata, T., and Fernie, A. R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. *Cell. Mol. Life Sci.* 69, 3225–3243.

Osbourn, A. E. (2003). Saponins in cereals. *Phytochemistry* 62, 1–4.

Parker, D., Beckmann, M., Zubair, H., Enot, D. P., Caracuel-Rios, Z., Overy, D. P., et al. (2009), Metabolomic analysis reveals a common pattern of metabolic re-programming during invasion of three host plant species by *Magnaporthe grisea. Plant J.* 59, 723–737.

Perkowski, J., Stupera, K., Buśkoa, M., Góralb, T., Kaczmarekc, A., and Jeleńd, H. (2012). Differences in metabolomic profiles of the naturally

contaminated grain of barley, oats and rye. *J. Cereal Sci.* 56, 544–551.

Pierce, K. M., Hope, J. L., Hoggard, J. C., and Synovec, R. E. (2006). A principal component analysis based method to discover chemical differences in comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-TOFMS) separations of metabolites in plant samples. *Talanta* 70, 797–804.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316.

Ramautar, R., Somsen, G. W., and de Jong, G. J. (2009). CE-MS in metabolomics. *Electrophoresis* 30, 276–291.

Rice, C. P., Park, Y. B., Adam, F., Abdul-Baki, A. A., and Teasdale, J. R. (2005). Hydroxamic acid content and toxicity of rye at selected growth stages. *J. Chem. Ecol.* 31, 1887–1905.

Ricroch, A. E., Bergé, J. B., and Kuntz, M. (2011). Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. *Plant Physiol.* 155, 1752–1761.

Saito, K., and Matsuda, F. (2010). Metabolomics for functional genomics, systems biology, and biotechnology. *Annu. Rev. Plant. Biol.* 61, 463–489.

Sato, S., Soga, T., Nishioka, T., and Tomita, M. (2004). Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. *Plant J.* 40, 151–163.

Sauter, H., Lauer, M., and Fritsch, H. (1991) "Metabolic profiling of plants – A new diagnostic technique", in *Synthesis and Chemistry of Agrochemicals II*, eds D. R. Baker, J. G. Fenyes and W. K. Moberg (Washington, DC: American Chemical Society), 288–299.

Sawada, K., Hasegawa, M., Tokuda, L., Kameyama, J., Kodama, O., Kohchi, T., et al. (2004). Enhanced resistance to blast fungus and bacterial blight in transgenic rice constitutively expressing OsSBP, a rice homologue of mammalian selenium-binding proteins. *Biosci. Biotechnol. Biochem.* 68, 873–880.

Schauer, N., and Fernie, A. R. (2006). Plant metabolomics: towards biological function and mechanism. *Trends Plant Sci.* 11, 508–516.

Schripsema, J. (2010). Application of NMR in plant metabolomics: techniques, problems and prospects. *Phytochem. Anal.* 21, 14–21.

Schroeder, H. W., and Christensen, J. J. (1963). Factors affecting resistance of wheat to scab by *Gibberella zeae*. *Phytopathology* 53, 831–838.

Shroff, R., Rulísek, L., Doubsky, J., and Svatos, A. (2009). Acid-base-driven matrix-assisted mass spectrometry for targeted metabolomics. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10092–10096.

Sicker, D., and Schulz, M. (2002). Benzoxazinones in plants: occurrence, synthetic access, and biological activity. *Stud. Nat. Products Chem.* 27, 185–232.

Smith, A. (1977). Phenethylamine and related compounds in plants. *Phytochemistry* 16, 9–18.

Smith, C. A., Want, E. J., O'Maille, G., Abagyan, R., and Siuzdak, G. (2006). XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* 78, 779–787.

Soriano, I. R., Asenstorfer, R. E., Schmidt, O., and Riley, T. I. (2004). Inducible flavone in oats (*Avena sativa*) is a novel defense against plant-parasitic nematodes. *Phytopathology* 94, 1207–1214.

Sumner, L. W., Huhman, D. V., Urbanczyk-Wochniak, E., and Lei, Z. (2007). Methods, applications and concepts of metabolite profiling: secondary metabolism. *EXS* 97, 195–212.

Sumner, L. W., Mendes, P., and Dixon, R. A. (2003). Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry* 62, 817–836.

Tolstikov, V. V., and Fiehn, O. (2002). Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. *Anal. Biochem.* 301, 298–307.

Trethewey, R. N. (2004). Metabolite profiling as an aid to metabolic engineering in plants. *Curr. Opin. Plant Biol.* 7, 196–201.

Verpoorte, R., Choi, Y. H., and Kim, H. K. (2007). NMR-based metabolomics at work in phytochemistry. *Phytochem. Rev.* 6, 3–14.

Voll, L. M., Horst, R. J., Voitsik, A. M., Zajic, D., Samans, B., Pons-Kühnemann, J., et al. (2011). Common motifs in the response of cereal primary metabolism to fungal pathogens are not based on similar transcriptional reprogramming. *Front. Plant Sci.* 2:39. doi: 10.3389/fpls.2011.00039

Widstrom, N. W., and Snook, M. E. (1998). A gene controlling biosynthesis of isoorientin, a compound in corn silks antibiotic to the corn earworm. *Entomol. Exp. Appl.* 89, 119–124.

Wold, S., Sjostrom, M., and Eriksson, L. (2001). PLS-regression, a basic tool of chemometrics. *Chemometr. Intell. Lab. Syst.* 58, 109–130.

Wolfender, J. L., Glauser, G., Boccard, J., and Rudaz, S. (2009). MS-based plant metabolomic approaches for biomarker discovery. *Nat. Prod. Commun.* 4, 1417–1430.

Wolfender, J. L., Rudaz, S., Choi, Y. H., and Kim, H. K. (2013) Plant metabolomics: from holistic data to relevant biomarkers. *Curr. Med. Chem.* 20, 1056–1090.

Zasada, I. A., Meyer, S. L. F., Halbrendt, J. M., and Rice, C. (2005). Activity of hydroxamic acids from *Secale cereale* against the plantparasitic nematodes *Meloidogyne incognita* and *Xiphinema americanum*. *Phytopathology* 95, 1116–1121.

Zúñiga, G. E., Varanda, E. M., and Corcuera, L. J. (1988). Effect of gramine on the feeding behavior of the aphids *Schizaphis graminum* and *Rhopalosiphum padi*. *Entomol. Exp. Appl.* 47, 161–165.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 19 February 2013; paper pending published: 04 March 2013; accepted: 20 March 2013; published online: 23 April 2013.

Citation: Balmer D, Flors V, Glauser G and Mauch-Mani B (2013) Metabolomics of cereals under biotic stress: current knowledge and techniques. Front. Plant Sci. ***4****:82. doi: 10.3389/fpls.2013.00082*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Balmer, Flors, Glauser and Mauch-Mani. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 22 February 2013
doi: 10.3389/fpls.2013.00024

Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae*

Tony Reglinski[1]*, Joel L. Vanneste[1], Kirstin Wurms[1], Elaine Gould[1], Francesco Spinelli[2] and Erik Rikkerink[1]

[1] The New Zealand Institute for Plant and Food Research Limited, Hamilton, New Zealand
[2] Department of Agricultural Sciences, University of Bologna, Bologna, Italy
*Correspondence: tony.reglinski@plantandfood.co.nz

Edited by:
Corné M. Pieterse, Utrecht University, Netherlands

Reviewed by:
Corné M. Pieterse, Utrecht University, Netherlands

Pseudomonas syringae pv. *actinidiae* (Psa) which causes bacterial canker of kiwifruit (*Actinidia deliciosa* and *A. chinensis*) was first isolated in Japan in 1984 (Takikawa et al., 1989), and soon after in Korea (Koh et al., 1994) and Italy (Scortichini, 1994). The economic impact on the global production of kiwifruit of those early occurrences was relatively limited (Vanneste et al., 2011). However, the latest outbreak of Psa which started in Italy in 2008 and rapidly spread throughout most of the kiwifruit growing regions of the world, represents a major threat to the global kiwifruit industry (Vanneste, 2012). The pathovar *actinidiae* is not a genetically homogeneous pathovar; strains can be grouped in four biovars based on their molecular, microbiological and pathogenic characteristics (Vanneste et al., 2013) which is consistent with MLST and whole genome sequence analysis (Ferrante and Scortichini, 2010; Mazzaglia et al., 2011; Chapman et al., 2012). The recent outbreak of bacterial canker on kiwifruit in Europe and New Zealand is caused by the same biovar of Psa (biovar 3) (Chapman et al., 2012; Vanneste et al., 2013). During the 2 years that the pathogen has been present in New Zealand, over 60% of the area planted in kiwifruit has been affected (Kiwifruit Vine Health, 2012). This rapid spread may be attributable to the virulence of biovar 3 and to the scarcity of products available for control of plant pathogenic bacteria in general, and Psa in particular. Many products used for control of plant pathogenic bacteria contain antibiotics (mostly streptomycin) or heavy metals (mostly copper). Both types of products do have limitations because of phytotoxicity or because they are not authorized in some countries (e.g., antibiotics in Europe). This has led to a large screening programme in New Zealand for the identification of potentially effective products to control Psa. The products tested included a number of commercially available potential elicitors of host resistance. One of the most effective elicitors in glasshouse trials on *A. chinensis* and *A. deliciosa* was acibenzolar-S-methyl [ASM], sold under the names of Bion® or Actigard® (Syngenta).

ASM belongs to the benzothiadiazole chemical group and operates as a functional analogue of salicylic acid. It has demonstrated good efficacy against bacterial diseases, including bacterial spot (*Xanthomonas axonopodis* pv. *vesicatoria*) and bacterial speck (*P. syringae* pv. *tomato*) in tomato (Louws et al., 2001), fire blight (*Erwinia amylovora*) in apples (Bastas and Maden, 2007), pear (Spinelli et al., 2006) and quince (Bastas and Maden, 2007), and xanthomonas leaf blight (*X. axonopodis* pv. *allii*) in onions (Gent and Schwartz, 2005). However, while elicitors can be very effective in controlled conditions, the host response can be highly variable in the field, thus raising questions about their potential for disease management. Furthermore, there is evidence that induced resistance, whether via the use of chemical elicitors or by constitutive expression of inducible defenses, can be accompanied by reduced fruit production and/or quality (Walters and Heil, 2007; Cipollini and Heil, 2010). These observations are consistent with the theory that induced resistance evolved as a strategy to minimize the metabolic costs associated with defense (Karban, 2011). Plant genotype and environment factors can also affect the relative benefits and costs of induced resistance (Cipollini and Heil, 2010; Walters et al., 2011) and a greater understanding of these dynamic interactions is necessary to facilitate more effective use of elicitors for disease control.

Complementary studies that target both fundamental and applied aspects of plant innate immunity are critical to realize the potential of induced resistance. Typically, inducible defenses are triggered upon recognition of pathogen-derived molecules. These molecules were historically termed elicitors or avirulence factors, but have more recently been renamed microbe-associated molecular patterns (MAMPs) and effectors, respectively (Jones and Dangl, 2006; Bent and Mackey, 2007). Phytohormone-mediated signaling pathways play a key role in orchestrating the plant response, with cross-talk between salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways providing means whereby the plant can tailor its defense response to different pathogens and pests (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). The SA and JA/ET defense pathways are often mutually antagonistic. However, synergistic interactions have been reported in some pathosystems (Pieterse et al., 2009). Abscisic acid (ABA) has also been shown to interact with defense-signaling pathways and it is proposed that ABA operates as a global regulator and co-ordinates the plant response to simultaneous multiple stresses (Ton et al., 2009). ABA-regulated

stomatal closure is a key element of pre-invasion SA-regulated innate immunity to *P. syringae* in *Arabidopsis* (Melotto et al., 2006) and therefore its role in the kiwifruit/Psa interaction is of interest given that glasshouse studies indicate that kiwifruit resistance to Psa is mediated via the SA signaling pathway. Incidence of the disease was significantly decreased ($p < 0.05$)_on *A. chinensis* seedlings previously treated with ASM as a foliar application (spray) while a significant increase in disease was observed on plants treated with methyl jasmonate (**Figure 1**). Moreover, histological evidence suggests that the pathogen is less able to colonize ASM-treated leaves than untreated leaves (Spinelli et al., 2011).

The number of tools available to analyse and probe the relationships between these host response pathways has grown considerably in recent times. In addition, the affordability of techniques such as Next Generation Sequencing (NGS) has improved considerably and, as a result, these tools can now be applied to many different situations. Increasingly these tools are helping us to understand the suite of genes affected by biotic and abiotic elicitors and the host response associated with major gene resistance (e.g., Kim et al., 2011; Gyetvai et al., 2012). Inevitably, some of the genes involved in these responses are in common, allowing researchers to look for potential synergy or antagonism between these responses. To increase our understanding, we are employing several molecular tools including: (1) NGS to measure total RNA expression in response to time and application of different elicitors on different cultivars; (2) quantitative PCR (qPCR) to study in depth the responses of putative resistance and defense response genes that have already been shown to play a role in kiwifruit interactions with other pests and diseases (Wurms et al., 2011a,b), (3) gene mining of the extensive database of the kiwifruit genome (Crowhurst et al., 2008) to identify novel gene candidates for study, and (4) transformation studies involving up- or down-regulation of specific genes of interest to assess their roles in the kiwifruit-Psa interaction. To date, our qPCR studies on a small set of candidate genes have identified several transcripts that are induced by Psa on its own and by ASM on its own. Moreover, the expression of these genes is enhanced further when ASM-treated plants are inoculated with Psa; this response correlates with decreased disease expression and is consistent with the phenomenon of priming whereby elicitor-treated plants react more rapidly and/or strongly to pathogen attack (Conrath, 2009). Up-regulated genes in this qPCR study included phenylalanine ammonia lyase (PAL), a key regulatory enzyme in the production of antimicrobial

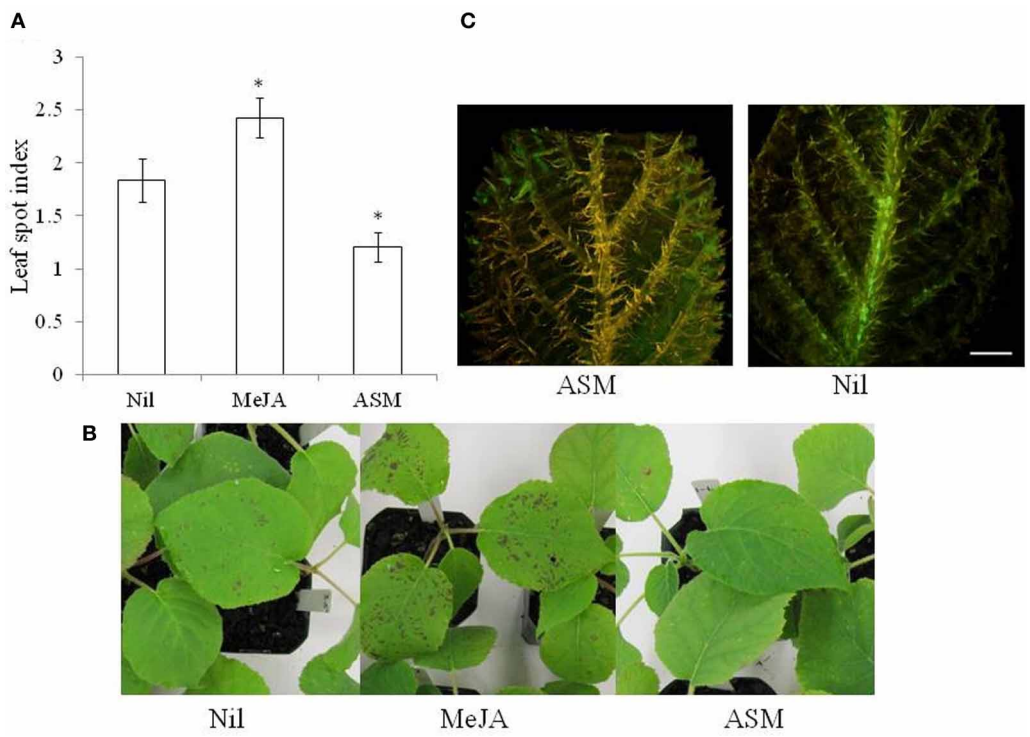


FIGURE 1 | (A,B) Effect of foliar spray with 1.7 mM acibenzolar-S-methyl (ASM) and 1.1 mM methyl jasmonate (MeJA) on Psa infection in *Actinidia chinensis* **(A,B)**. Treatments were applied 1 week before spray inoculation with a suspension containing 10^9 cfu ml^{-1} of *Pseudomonas syringae* pv. *actinidiae* (strain 10627). Plants were assessed 2 weeks later and the leaf spotting was recorded according to the following index, 0 = 0% leaf area, 1 ≤ 10%, 2 = 10–25%, 3 = 25–50%, 4 ≥ 50%. The data are presented as means ± standard error ($n = 9$) and the asterisk indicates a significant difference between the treatment and the untreated control (LSD = 0.53, $P < 0.05$). **(C)** Fluorescent stereomicroscopy of *A. deliciosa* leaves inoculated with GFPuv labeled Psa (strain CFBP7286). Inoculation was performed by cutting the leaf tip with scissor dipped in a bacterial suspension (10^9 cfu ml^{-1}). The photos were taken 2 weeks after inoculation. Measuring bar = 2 mm.

phytoalexins (Naoumkina et al., 2010), a hypersensitivity-induced response protein, a protein that interacts with putative plant R genes (Jung and Hwang, 2007; Jung et al., 2008), and RIN4—a protein thought to play a key role in defense against bacterial pathogens such as *Pseudomonas* spp., and which is involved in both MAMP-triggered and effector-triggered immunity (Afzal et al., 2011).

The analysis of plant immunity in Arabidopsis and tomato model systems has provided basic knowledge of pathogen virulence factors (e.g., effectors), the host proteins/pathways targeted by some of these virulence factors, and how manipulation events are detected by major resistance genes (R genes) (Jones and Dangl, 2006; Dangl, 2007; Nishimura and Dangl, 2010; Schwessinger and Ronald, 2012). This information has been instrumental in shaping the current study by identifying potential targets that can be examined in the context of the Psa-kiwifruit interaction. It is suggested that pathogen effectors might converge on a limited set of host proteins with important regulatory roles in plant immune signaling (Mukhtar et al., 2011; Spoel and Dong, 2012). Psa contains several effectors (Marcelletti et al., 2011) that are known to interfere with RIN4 in the model pathosystem *Pseudomonas-Arabidopsis*. AvrRPM1 is known to induce phosphorylation of RIN4 (Mackey et al., 2002), while HopF2 interferes with the resistance triggered by RIN4s interaction with another effector-AvrRpt2 (Wilton et al., 2010). The exact mechanism of these interactions is not yet understood. Our genome analysis has also identified other candidates that possibly interfere with this protein, such as a distantly related member of the AvrRPM1 effector family (AvrRPM2). It is likely that RIN4 is not the only host target of Psa and the use of NGS and other approaches may identify additional host targets affected by ASM and/or Psa. Further expression studies by qPCR, NGS, and transformation studies will determine whether expression of these genes can be used as a marker in breeding and/or elicitor selection. Other tools, such as yeast 2-hybrid and *in planta* protein-protein interaction screening tools, are also being employed to decipher how pathogen and host proteins interact. Together this new knowledge may also allow us to fine tune elicitor-based strategies (e.g., delivery, timing, and frequency) in order to maximize their impact for the control of plant disease.

The project combines applied and fundamental research to identify methods to protect commercial kiwifruit production from the threat posed by Psa. By integrating these approaches, we can harness the true potential of elicitors both to protect existing kiwifruit cultivars and to develop new cultivars with increased resistance to Psa. For example, as our knowledge about the targets of effectors increases, so will our understanding of which of these targets are involved in other pertinent host pathways, e.g., in response to elicitors or plant hormones. As these effectors are also key components of recognition by R genes, this should allow us to postulate both favorable and unfavorable interactions between elicitor-induced pathways and certain R gene strategies. As the effectors AvrRPM1 and HopF2 both target RIN4, and RIN4 RNA expression appears to be affected by ASM elicitation, there is potential for the perturbation of resistance responses that rely on these effectors by ASM. Depending on the magnitude of the ASM effect on the amount of RIN4 protein, and the nature of the molecular mechanisms involved, the end result could either be neutral, beneficial to, or detrimental to such a resistance response. This simple example illustrates how future fundamental research is needed to reveal the nature of these mechanisms, and to complement resistance breeding and crop protection strategies.

ACKNOWLEDGMENTS

The authors wish to thank Antonio Cellini and Luca Fiorentini (University of Bologna) for fluorescence microscopy and Janet Yu and Deirdre Cornish (Plant and Food Research) for contributions to inoculation studies. We also thank the New Zealand Ministry for Business, Innovation and Employment (C06X0810), and also ZESPRI Innovation Limited (project V11227) for funding different elements of this research.

REFERENCES

Afzal, A. J., da Cunha, L., and Mackey, D. (2011). Separable fragments and membrane tethering of Arabidopsis RIN4 regulate its suppression of PAMP-triggered immunity. *Plant Cell* 23, 3798–3811.

Bastas, K. K., and Maden, S. (2007). Evaluation of host resistance inducers and conventional products for fire blight management in loquat and quince. *Phytoprotection* 88, 93–101.

Bent, A. F., and Mackey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopath.* 45, 399–436.

Chapman, J. R., Taylor, R. K., Weir, B. S., Romberg, M. K., Vanneste, J. L., Luck, J., et al. (2012). Phylogenetic relationships among global populations of *Pseudomonas syringae pv. actinidiae*. *Phytopathology* 102, 1034–1044.

Cipollini, D., and Heil, M. (2010). Costs and benefits of induced resistance to herbivores and pathogens in plants. *CAB Rev. Pers. Agric. Vet. Sci. Nutr. Nat. Res.* 5, 51–25.

Conrath, U. (2009). Priming of induced plant defense responses. *Plant Innate Immun.* 51, 361–395.

Crowhurst, R. N., Gleave, A. P., MacRae, E. A., Ampomah-Dwamena, C., Atkinson, R. G., Beuning, L. L., et al. (2008). Analysis of expressed sequence tags from Actinidia: applications of a cross species EST database for gene discovery in the areas of flavor, health, color and ripening. *BMC Genomics* 9:351. doi: 10.1186/1471-2164-9-351

Dangl, J. L. (2007). Nibbling at the plant cell nucleus. *Science* 315, 1088–1089.

Ferrante, P., and Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathol.* 59, 954–962.

Gent, D. H., and Schwartz, H. F. (2005). Management of Xanthomonas leaf blight of onion with a plant activator, biological control agents, and copper bactericides. *Plant Dis.* 89, 631–639.

Gyetvai, G., Sønderkær, M., Göbel, U., Basekow, R., Ballvora, A., Imhoff, M., et al. (2012). The transcriptome of compatible and incompatible interactions of potato (*Solanum tuberosum*) with *Phytophthora infestans* revealed by deepSAGE analysis. *PLoS ONE* 7:e31526. doi: 10.1371/journal.pone.0031526

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Jung, H. W., and Hwang, B. K. (2007). The leucine-rich repeat (LRR) protein, CaLRR1, interacts with the hypersensitive induced reaction (LIR) protein, Ca HIR1, and suppresses cell death induced by the CaHIR1 protein. *Mol. Plant Pathol.* 8, 503–514.

Jung, H. W., Lim, C. W., Lee, S. C., Choi, H. W., and Hwang, B. K. (2008). Distinct roles of the pepper hypersensitive induced reaction protein gene CaHIR1 in disease and osmotic stress, as determined by comparative transcriptome and proteome analyses. *Planta* 227, 409–425.

Karban, R. (2011). The ecology and evolution of induced resistance against herbivores. *Funct. Ecol.* 25, 339–347.

Kiwifruit Vine Health. (2012). *Psa Statistics Update 28 November 2012*. Available online at: http://

www.kvh.org.nz/vdb/document/453 [Accessed 01 December 2012].

Koh, Y. J., Cha, B. J., Chung, H. J., and Lee, D. H. (1994). Outbreak and spread of bacterial canker in kiwifruit. *Korean J. Plant Pathol.* 10, 68–72.

Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., et al. (2001). Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Dis.* 85, 481–488.

Kim, K. H., Kang, Y. J., Kim, D. H., Yoon, M. Y., Moon, Y.-K., Kim, M. Y., et al. (2011). RNA-Seq analysis of a soybean near-isogenic line carrying bacterial leaf pustule-resistant and -susceptible alleles. *DNA Res.* 18, 483–497.

Mackey, D., Holt, B. F., Wiig, A., and Dangl, J. L. (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell* 108, 743–754.

Marcelletti, S., Ferrante, P., Petriccione, M., Firrao, G., and Scortichini, M. (2011). *Pseudomonas syringae* pv. actinidiae draft genomes comparison reveal strain-specific features involved in adaptation and virulence to actinidia species. *PLoS ONE* 6:e27297. doi: 10.1371/journal.pone.0027297

Mazzaglia, A., Renzi, M., and Balestra, G. M. (2011). Comparison and utilization of different PCR-based approaches for molecular typing of *Pseudomonas syringae* pv. *actinidiae* strains from Italy. *Can. J. Plant Pathol.* 33, 8–18.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* 126, 969–980.

Mukhtar, M. S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601.

Naoumkina, M. A., Zhao, Q., Gallego-Giraldo, L., Dai, X., Zhao, P. X., and Dixon, R. A. (2010). Genome-wide analysis of phenylpropanoid defence pathways. *Mol. Plant Pathol.* 11, 829–846.

Nishimura, M. T., and Dangl, J. D. L. (2010). Arabidopsis and the plant immune system. *Plant J.* 61, 1053–1066.

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521.

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Ann. Rev. Phytopathol.* 49, 317–343.

Scortichini, M. (1994). Occurrence of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Italy. *Plant Pathol.* 43, 1035–1038.

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482.

Spinelli, F., Andreotti, C., Sabatini, E., Costa, G., Spada, G., Ponti, I., et al. (2006). "Chemical control of fire blight in pear: application of prohexadione-calcium, Acibenzolar-S-Methyl, and copper preparations *in vitro* and under field conditions," in *Proceedings of the Xth International Workshop on Fire Blight,* eds C. Bazzi and U. Mazzucchi (Bologna), 233–237.

Spinelli, F., Donati, I., Vanneste, J. L., Costa, M., and Costa, G. (2011). Real time monitoring of the interactions between *Pseudomonas syringae* pv *actinidiae* and *Actinidia* species. *Acta Hortic.* 913, 461–465.

Spoel, S. H., and Dong, X. N. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100.

Takikawa, Y., Serizawa, S., Ichikawa, T., Tsuyumu, S., and Goto, M. (1989). *Pseudomonas syringae* pv. *actinidiae* pv. nov.: the causal bacterium of canker of kiwifruit in Japan. *Ann. Phytopathol. Soc. Jpn.* 55, 437–444.

Ton, J., Flors, V., and Mauch-Mani, B. (2009). The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* 14, 310–317.

Vanneste, J. L. (2012). *Pseudomonas syringae* pv. actinidiae(Psa): a threat to the New Zealand and global kiwifruit industry. *N. Z. J. Crop Hortic. Sci.* 40, 265–267.

Vanneste, J. L., Kay, C., Onorato, R., Yu, J., Cornish, D. A., Spinelli, F., et al. (2011). Recent advances in the characterisation and control of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker on kiwifruit. *Acta Hortic.* 913, 443–456.

Vanneste, J. L., Yu, J., Cornish, D. A., Tanner, D. J., Windner, R., Chapman, J. R., et al. (2013). Identification, virulence and distribution of two biovars of *Pseudomonas syringae* pv. *actinidiae* in New Zealand. *Plant Dis.* [Online].

Walters, D., and Heil, M. (2007). Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol.* 71, 3–17.

Walters, D. R., Havis, N. D., Paterson, L., Taylor, J., and Walsh, D. J. (2011). Cultivar effects on the expression of induced resistance in spring barley. *Plant Dis.* 95, 595–600.

Wilton, M., Subramaniam, R., Elmore, J., Felsensteiner, C., Coaker, G., and Desveaux, D. (2010). The type III effector HopF2Pto targets Arabidopsis RIN4 protein to promote *Pseudomonas syringae* virulence. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2349–2354.

Wurms, K. V., Cui, W., Ah-Chee, A., Rees-George, J., Bublin, M., and Breiteneder, H. (2011a). Down regulation of putative defence-associated transcripts correlates with ripe rot symptoms on kiwifruit (*Actinidia chinensis*). *J. Phytopath.* 159, 435–442.

Wurms, K. V., Reglinski, T., Taylor, J. T., Ah Chee, A., and Cui, W. (2011b). Up-regulation of putative defence-associated transcripts correlates with elicitor-induced ripe rot reduction in 'Hort16A' kiwifruit. *Acta Hortic.* 913, 525–528.

Received: 21 December 2012; accepted: 04 February 2013; published online: 22 February 2013.

Citation: Reglinski T, Vanneste JL, Wurms K, Gould E, Spinelli F and Rikkerink E (2013) Using fundamental knowledge of induced resistance to develop control strategies for bacterial canker of kiwifruit caused by Pseudomonas syringae pv. actinidiae. Front. Plant Sci. ***4****:24. doi: 10.3389/fpls.2013.00024*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Reglinski, Vanneste, Wurms, Gould, Spinelli and Rikkerink. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 18 February 2013
doi: 10.3389/fpls.2013.00019

Modeling induced resistance to plant disease using a dynamical systems approach

Nurul S. Abdul Latif[1,2], Graeme C. Wake[2], Tony Reglinski[3]*, Philip A. G. Elmer[3] and Joseph T. Taylor[3]

[1] Faculty of Agro Based Industry, Universiti Malaysia Kelantan, Jeli, Kelantan, Malaysia
[2] Institute of Information and Mathematical Sciences, Massey University, Auckland, New Zealand
[3] The New Zealand Institute for Plant and Food Research Limited, Hamilton, New Zealand
*Correspondence: tony.reglinski@plantandfood.co.nz

Edited by:
Saskia C. M. van Wees, Utrecht University, Netherlands

Reviewed by:
Michael Jeger, Imperial College London, UK

Standard epidemiological models describe that **Susceptible** plants (S) will become infected and develop **Disease** (D) after inoculation with a compatible pathogen under appropriate environmental conditions. These dynamic relationships can be affected by subtle changes to any one parameter and may result in a proportion of the plant population being able to exhibit **Resistance** (R) to infection. An example of this is the use of elicitors to promote an increase in plant basal resistance and so enable a proportion of formerly susceptible plants to express resistance i.e., a shift in the population from S to R. This phenomenon is termed induced resistance (IR). In this paper, a prototype mathematical model of IR is presented to describe the effects of a chemical elicitor compound, methyl jasmonate (MeJA), on the resistance of *Pinus radiata* seedlings to *Diplodia pinea* the causal agent of pine stem canker and tip dieback. Pine seedlings were sprayed with 0.1% MeJA at 27, 13, 6, or 3 days before inoculation with *D. pinea* using methods previously described by Gould et al. (2008). Disease assessments commenced at 1 week after inoculation and continued at 3–4 day intervals thereafter for 5 weeks. Disease development on the MeJA-treated seedlings was compared to that on a cohort of untreated plants. In this model system, the IR response is transient and it is modeled here using a forward-and-backward kinetics framework to describe the temporal nature of the phenomenon.

Because the expression of IR can only be detected after pathogen challenge, the model is formulated with the treated plants divided into two regimes: (1) pre-inoculation and (2) post-inoculation. The assumptions for the model's formulation can be summarized as follows. The plant population is divided into three compartments according to the above definitions where $S + R + D = 1$. At the time when plants are treated with an elicitor ($t = 0$), a proportion of the plant population will exhibit natural resistance (R_i). The induction period (t_p) describes the time interval between elicitor application and pathogen inoculation. Upon inoculation, a proportion of plants (D_i) will become infected immediately. This prototype IR model is based on the model by Jeger et al. (2009) and Xu et al. (2010). The model's equations for the **treated** plants are as follows:

Pre-inoculation: For $0 \le t < t_p$

$$\frac{dR}{dt} = (e(t) - \gamma R)(1 - R); \quad R(0) = R_i \tag{1}$$

Post-inoculation: For $t_p \le t \le T$

$$\frac{dR}{dt} = (e(t) - \gamma R)(1 - R - D); \quad R(t_p) = R_p \tag{2}$$

$$\frac{dD}{dt} = \beta D(1 - R - D); \quad D(t_p) = D_i \tag{3}$$

where we take $e(t) = \frac{kt}{t^2 + L^2}$ [days^{-1}] as the elicitor effectiveness in the plants where $\frac{k}{2L}$ [days^{-1}] is the maximum elicitor effect and L [days] is the time where this is at its peak, γ [days^{-1}] is the rate that resistant tissue becomes susceptible, and also β [days^{-1}] is the rate of disease development. The form for $e(t)$ is chosen to reflect the temporal nature of IR with an initial increase in resistance which then decays over time. R_p is the degree of the resistance at the time of the pathogen inoculation obtained from Equation (1). Also, T is a finite (sufficiently large) time after the pathogen inoculation. The Equations (1)–(3) are based on the assumption that the rate of change of R and D are directly proportional to the amount of S available at a particular time (that is, $S = 1 - R - D$). For Equation (1), D is not in the equation because the pathogen is absent during this period. Pathogen is inoculated at time t_p, therefore the D term is occurs only in Equations (2) and (3). For the untreated plants, they share the same parameter values, especially β, R_i, D_i, since these untreated plants are characterized as the control group. The untreated plants will not have the $e(t)$ term in their model equations and so are autonomous, that is, independent of time except implicitly. Therefore, the model's equations for the **untreated** plants are as follows:

$$\frac{dR}{dt} = \alpha R(1 - R - D); \quad R(0) = R_i \tag{4}$$

$$\frac{dD}{dt} = \beta D(1 - R - D); \quad D(0) = D_i \tag{5}$$

where α [days^{-1}] is the rate that untreated susceptible tissue becomes resistant.

The seven unknown parameters (α, β, γ, k, L, R_i, D_i) are determined by matching data to the model using computer-based algorithms. The following figure (**Figure 1**) was plotted using the optimal parameter values, and it illustrates the characteristics of the two compartments R and D for the Equations (1)–(5).

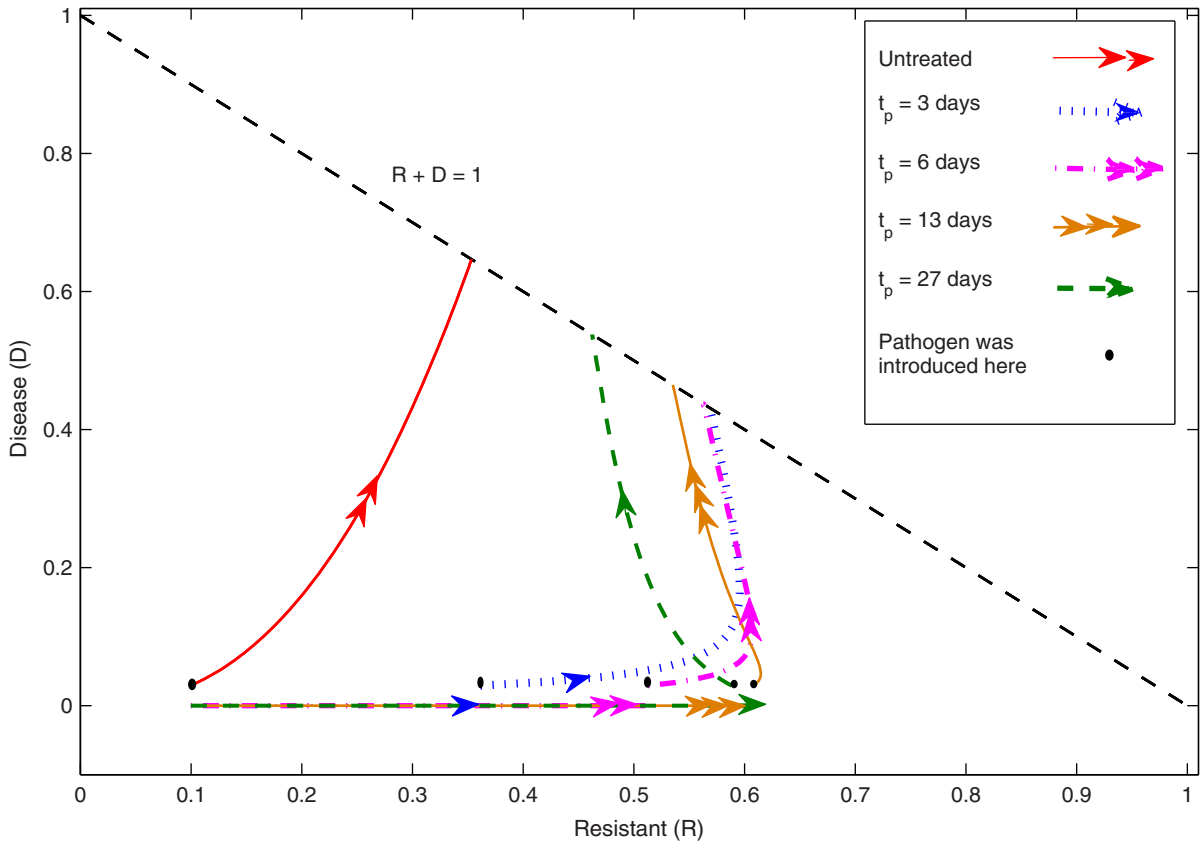


FIGURE 1 | The phase-plane for the induced resistance (IR) model. These trajectories of the R, D compartments are plotted in the feasible region $(R, D > 0, R + D < 1)$. The lines schematically represent the values of the two compartments R and D as time passes for the Equations (1)–(5) based on each induction case t_p and the untreated case. When $D = 0$, the straight lines illustrate the dynamics of the R compartment before the pathogen inoculation. These lines will have a discontinuity when the pathogen is introduced at time $t = t_p$, and it shows the state of the R compartment at that particular time. As can be seen in the figure, there is a jump in the D values of D_0 at $t = t_p$ and the trajectories of the R, D compartments will continue to approach the straight line $R + D = 1$. For the untreated case, the dynamics of the R, D compartments will depend on the initial condition of the systems i.e., R_i and D_i.

The flows in the figure indicate the time-evolution of the system of the differential equations described above based on the different values of t_p and the untreated case. It shows that when t_p is between 3 and 6 days, the subsequent development of disease is less severe than with the other induction times and with the untreated plants. That is, the resistance induced by the elicitor application is at its peak. The figure shows that the trajectories for each induction case will eventually approach that straight line $R + D = 1$. The model has three compartments (S, R, D), but the data sets have only two $([S + R], D)$. The Susceptible cohort is given by $S = 1 - R - D$ and can be separated out by this model. The model has a line of equilibrium states in the R, D plane which are attracting, and predicts that disease development depends on the induction time t_p.

CONCLUSION

The management of plant diseases involves an assessment of the risks and the costs, both economic and environmental, associated with the implementation of different control measures. Various disease risk prediction models have been developed as decision support tools to facilitate more efficient use of management options; these are generally based on the rationale that pest and disease development follow predictable life cycles and that by monitoring key epidemiological parameters it is possible to target more accurately events critical for management (Gent et al., 2011). Disease risk prediction models may prove critical for coordination of elicitor application in crop production systems because of the importance of early intervention when relying on IR for disease control. In this study we discuss the development of a prototype mathematical model to predict the temporal dynamics of chemically-IR. The current model offers the potential to quantitatively estimate the effectiveness of elicitor treatment and to predict the relative proportion of plants exhibiting IR to pathogen inoculation. Moreover, the model is generic and will be applicable for a range of plant-pathogen-elicitor scenarios. For future work, this prototype IR model will be extended to predict the required t_p to achieve optimum disease control. In addition, it will be interesting to observe the dynamics of the system when there are multiple elicitor applications to plants, a scenario

which may be needed in practice. This is important in practical terms because successful application of elicitors will require knowledge of the onset and duration of the IR response. This new model will complement and extend the value of risk prediction models by providing decision support on the timing and frequency of elicitor applications for management of disease.

ACKNOWLEDGMENTS

The authors wish to thank the New Zealand Ministry for Business, Innovation and Employment (LINX0804 "Ecosystem Bioprotection") and Ministry of Higher Education of Malaysia (KPTBS-851110105416) for funding to support this study.

REFERENCES

Gent, D. H., De Wolf, E., and Pethybridge, S. J. (2011). Perceptions of risk, risk aversion, and barriers to adoption of decision support systems and integrated pest management: an introduction. *Phytopathology* 101, 640–643.

Gould, N., Reglinski, T., Spiers, M., and Taylor, J. T. (2008). Physiological trade-offs associated with methyl jasmonate – induced resistance in *Pinus radiata*. *Can. J. For. Res.* 38, 677–684.

Jeger, M. J., Jefferies, P., Elad, Y., and Xu, X. M. (2009). A generic theoretical model for biological control of foliar plant disease. *J. Theor. Biol.* 256, 201–214.

Xu, X. M., Salama, N., Jeffries, P., and Jeger, M. J. (2010). Numerical studies of biocontrol efficacies of foliar plant pathogens in relation to the characteristics of a biocontrol agent. *Am. Phytopathol. Soc.* 100, 814–821.

Received: 19 December 2012; accepted: 25 January 2013; published online: 18 February 2013.
*Citation: Abdul Latif NS, Wake GC, Reglinski T, Elmer PAG and Taylor JT (2013) Modeling induced resistance to plant disease using a dynamical systems approach. Front. Plant Sci. **4**:19. doi: 10.3389/fpls.2013.00019*
This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.
Copyright © 2013 Abdul Latif, Wake, Reglinski, Elmer and Taylor. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 12 April 2013
doi: 10.3389/fpls.2013.00087

Plant systemic induced responses mediate interactions between root parasitic nematodes and aboveground herbivorous insects

Mesfin Wondafrash [1,2]***, Nicole M. Van Dam*** [1] ***and Tom O. G. Tytgat*** [1]******

[1] *Department of Ecogenomics, Institute for Water and Wetland Research, Radboud University Nijmegen, Nijmegen, Netherlands*
[2] *School of Plant Sciences, Haramaya University, Dire Dawa, Ethiopia*

Edited by:
Erik Poelman, Wageningen University, Netherlands

Reviewed by:
Matthias Erb, Max Planck Institute for Chemical Ecology, Germany
Ian Kaplan, Purdue University, USA

****Correspondence:***
Tom O. G. Tytgat, Department of Ecogenomics, Institute for Water and Wetland Research, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, Netherlands.
e-mail: t.tytgat@science.ru.nl

Insects and nematodes are the most diverse and abundant groups of multicellular animals feeding on plants on either side of the soil–air interface. Several herbivore-induced responses are systemic, and hence can influence the preference and performance of organisms in other plant organs. Recent studies show that plants mediate interactions between belowground plant parasitic nematodes (PPNs) and aboveground herbivorous insects. Based on the knowledge of plant responses to pathogens, we review the emerging insights on plant systemic responses against root-feeding nematodes and shoot-feeding insects. We discuss the potential mechanisms of plant-mediated indirect interactions between both groups of organisms and point to gaps in our knowledge. Root-feeding nematodes can positively or negatively affect shoot herbivorous insects, and *vice versa*. The outcomes of the interactions between these spatially separated herbivore communities appear to be influenced by the feeding strategy of the nematodes and the insects, as well as by host plant susceptibility to both herbivores. The potential mechanisms for these interactions include systemic induced plant defense, interference with the translocation and dynamics of locally induced secondary metabolites, and reallocation of plant nutritional reserves. During evolution, PPNs as well as herbivorous insects have acquired effectors that modify plant defense responses and resource allocation patterns to their advantage. However, it is also known that plants under herbivore attack change the allocation of their resources, e.g., for compensatory growth responses, which may affect the performance of other organisms feeding on the plant. Studying the chemical and molecular basis of these interactions will reveal the molecular mechanisms that are involved. Moreover, it will lead to a better understanding of the ecological relevance of aboveground–belowground interactions, as well as support the development of sustainable pest management technologies.

Keywords: aboveground–belowground interactions, signaling interactions, systemic induced plant defense, secondary plant compounds, herbivores

INTRODUCTION

Under natural conditions plants are constantly exposed to various herbivorous organisms feeding on above- and belowground parts. The influence of root-feeders on shoot defense and the patterns of aboveground herbivory and *vice versa* remained unrecognized for a long time (Kaplan et al., 2008a). Most of the earlier knowledge on plant–herbivore interactions emanated from studies conducted on leaf herbivory alone, thereby neglecting plant-mediated interactions between the two herbivore communities (Johnson et al., 2006). However, during the last decade, studies on the interactions between these two spatially separated communities, below- and aboveground herbivores, have substantially increased after scientists began to realize that host plants are serving as mediators of these interactions. The outcomes of such studies witnessed that these herbivore communities rarely function independently, but rather interact continuously with each other via their host plants (Bezemer and van Dam, 2005; Kaplan et al., 2009). Belowground feeding organisms such as insects, nematodes, root pathogens, and ectomycorrhizal fungi are known to influence the concentration of plant defense compounds such as terpenoids, glucosinolates, or phenolics, both in the roots as well as in aboveground plant tissues (Manninen et al., 1998; Bezemer et al., 2004; Kaplan et al., 2009; van Dam, 2009).

Plant parasitic nematodes (PPNs) are so abundant and diverse, that plants almost always interact with them during their lifetime (Sohlenius, 1980). Recent studies have shown that due to their omnipresence, PPN are a key driving force of plant succession in natural environments (De Deyn et al., 2003). They also pose a significant threat to global food production, with annual crop losses due to PPN estimated to be more than a 100 billion US$ (Chitwood, 2003). Similarly, about half of all insect species feed on plants (Schoonhoven et al., 2005). With only a few exceptions, PPN are root-feeders, while the majority of insects feed on aboveground plant parts, which have a higher nutritive quality than

roots (Hunter, 2001; van Dam, 2009). Therefore, both groups of herbivores are very suitable to investigate the mechanisms of plant-mediated above–below ground interactions. Recently, the first studies were performed that analyzed the interactions of PPN and insects (Wardle et al., 2004; van Dam et al., 2005; De Deyn et al., 2007; Wurst and van der Putten, 2007; Kaplan et al., 2008a, 2009; Olson et al., 2008; Lohmann et al., 2009; Hong et al., 2010; Vandegehuchte et al., 2010). It seems that the outcome of the interaction between both groups of plant feeders can either be negative or positive. However, the knowledge on the occurrence of interactions between these spatially separated herbivore communities remains scattered and poorly documented. Therefore, in this review we discuss the current knowledge on the plant defense against PPN and herbivorous insects, present some examples of the plant-mediated interactions between both groups, indicate the gaps in knowledge and finally identify future research directions.

FUNCTIONAL DIVERSITY OF NEMATODES AND INSECTS

It has been suggested that the feeding habit (functional guild) of herbivorous insects and plant-parasitic nematodes involved may be one of the factors that determine the outcome of plant-mediate insect–nematode interactions (Mateille, 1994; van Dam et al., 2003, 2005; Bezemer and van Dam, 2005; Wurst and van der Putten, 2007). Therefore, it is important to discuss the diversity in feeding habits of both groups of herbivores and the specific process involved in each feeding habit.

NEMATODES

Although the basic body plan of all nematodes is highly similar, the genetic diversity is enormous and reflects the long evolutionary trajectory of the phylum (Blaxter, 1998). Phylogenetic analysis revealed that within the Nematoda, plant parasitism evolved at least three times (Blaxter et al., 1998). However, all PPN have common features that arose by convergent evolution to adapt to plant parasitism (Hussey, 1989). They all possess a hollow protrusible stylet that is used to puncture cell walls, inject secretions, and ingest nutrients from the plant cell. The stylet secretions are synthesized in unicellular pharyngeal glands that are much more developed in PPN than in free living nematodes. According to their feeding habit, PPN can be classified into ectoparasites, migratory endoparasites, and sedentary endoparasites (Sijmons et al., 1994; Tytgat et al., 2000).

Ectoparasitic nematodes do not enter the host tissues with their body, but rather puncture plant cells using their stylet and feed on the content of the cells. Depending on the species, feeding can prolong for a few hours till several days. The size of the stylet determines where and how the nematodes in this group feed. Ectoparasitic nematodes with a short stylet (e.g., Trichodoridae, *Tylenchorhynchus* spp.) often feed on root hairs and epidermal cells, while those with a long stylet (e.g., Longidoridae, *Belonolaimus*, *Helicotylenchus* spp.) feed on cortical or even endodermal cells. They insert their stylet into the host cell, inject glandular secretions that dissolve the cell content, and ingest the cytoplasmic contents. Depending on the species, these actions lead to wounding, extensive necrosis, or even gall formation of the root tissue (Sijmons et al., 1994).

Migratory endoparasites are equipped with a robust stylet, which renders them the ability to penetrate and continuously migrate through the root while feeding on the cytoplasm of cortical cells (Sijmons et al., 1994). With the exception of some shoot parasites (Anguinidae and Aphelenchoididae), they all belong to the family of Pratylenchidae (e.g., *Pratylenchus* spp., *Radophulus* spp.). Migration inside the roots is aided by the release of cell wall degrading enzymes via the stylet (Haegeman et al., 2012). Extensive necrosis and sometimes galling or swelling of the root tissue are typical symptoms that develop as a result of infection with such nematodes.

Sedentary endoparasitic nematodes have the most evolved interactions with their host. After root penetration and migration, they induce permanent feeding cells inside the vascular cylinder. The best studied are the cyst nematodes and root-knot nematodes (Hussey, 1989; Davis and Mitchum, 2005). Freshly hatched juveniles penetrate the roots close to the root tip, and migrate intracellularly (cyst nematodes) or intercellularly (root-knot nematodes) toward the vascular cylinder. Also here, migration is performed by vigorous stylet thrusting and secretion of a mix of cell wall degrading enzymes (Davis et al., 2008; Haegeman et al., 2012). After arrival at the vascular cylinder, they puncture the cell wall of a certain cell and start repeated cycles of stylet secretion release into the cytoplasm and ingestion of cytoplasmic content (Wyss, 2002). The initial feeding cell responds with an extensive change in gene expression and morphology (Gheysen and Fenoll, 2002; Caillaud et al., 2008). The cells become hypertrophic and show a huge proliferation of all organelles. Root-knot nematodes induce six to seven giant cells, which become multinucleated by repeated mitosis without cytokinesis. In contrast, cyst nematodes induce a syncytium, which is formed by cell wall dissolution of the initial feeding cell and fusion with the neighboring cells. In clear contrast to the migratory endoparasitic and ectoparasitic nematodes that mostly kill the cells on which they feed, the sedentary endoparasitic nematodes maintain their feeding cells healthy and metabolically active throughout their life cycle. Once they started feeding, they even lose their locomotory muscles, and become completely depended on the hypertrophic cells for further development. Though the giant cells and the syncytia are distinct in their development, functionally they are similar in that they serve as transfer cells of nutrients derived from the phloem (Offler et al., 2003; Hoth et al., 2005, 2008). While cyst nematodes species show specificity for certain plant families, root-knot nematodes such as *Meloidogyne incognita* for instance have an extremely wide host range comprising almost all families of flowering plants (Trudgill and Blok, 2001).

INSECTS

Based on their feeding habits herbivorous insects are classified into leaf chewing, mining and boring, sap-sucking, gall inducing, and seed predating (Schoonhoven et al., 2005; Gullan and Cranston, 2010). The majority of leaf chewing insects belong to the family Lepidoptera, Coleoptera, Orthoptera, and Hymenoptera. Different developmental stages, such as the caterpillars of lepidopterous (moths and butterflies), the larvae and adults (beetles) of coleopterous insects and the nymphs and adults of orthopterous insects feed on the leaves of plants. Other plant parts such as

roots, shoots, stems, flowers, or fruits are also eaten by this group of insects (Schoonhoven et al., 2005; Gullan and Cranston, 2010).

Larvae of plant mining and boring insects live and feed on the internal tissues of plants. For instance, leaf mining insects live and feed between the two epidermal layers of a leaf and ultimately they leave behind a thin layer of dry epidermis (Connor and Taverner, 1997; Sinclair and Hughes, 2010). The damage due to this group of insects often appears as tunnels, blisters, or blotches on the leaf. This leaf mining habit is confined only to insects belonging to the orders Diptera, Lepidoptera, Coleoptera, and Hymenoptera (Gullan and Cranston, 2010). Plant boring insects exhibit a broad range of feeding habits, which can be categorized based on the plant part they damage. These could be stalk borers that attack grasses and more succulent plants; wood borers feeding on twigs, stems, and/or trunks of woody plants; borers that damage roots and belowground plant storage organs such as tubers, corms, and bulbs; fruit borers that destroy or reduce the reproductive output of many plants because the larvae consume the fruit tissues (Schoonhoven et al., 2005; Gullan and Cranston, 2010).

Typical for the sap-sucking insects are the modified mouth parts that are fused to form a stylet (Labandeira, 1997). The stylet is used to penetrate the host tissue, to inject saliva into the host tissue and to retrieve sap from the plant. Depending on the species, the stylet may penetrate superficially into a leaf or deep into the plant tissue, following either intra- or intercellular paths (Kaloshian and Walling, 2005). Because a different reaction toward systemic plant responses can be expected, a functional distinction should be made between cell content and phloem or xylem feeders. Many Heteroptera and thrips feed on the cell content of epidermal or parenchymal cells (Heming, 1993; Schoonhoven et al., 2005). Phloem feeding is performed by most aphids, mealy bugs, soft scales, psyllids, and leafhoppers, while spittle bugs and cicadas feed on xylem sap (Gullan and Cranston, 2010). Because only the stylet penetrates the plant tissue, sap-sucking insects inflict less mechanical damage than leaf chewing or mining and boring insects. However, some sap-sucking insect species may transmit viruses or cause deformation and stunting of shoots (Schoonhoven et al., 2005).

Gall-induction is another feeding habit of herbivorous insects. Generally, galls are defined as pathologically developed cells, tissues, or organs of plants that have arisen by hypertrophy (increase in cell size) and/or hyperplasia (increase in cell number) as a result of stimulation from foreign organisms (Redfern, 1997; Raman, 2012). The formation of the gall is believed to be beneficial to the insects, rather than a defensive response of the plant to insect attack (Stone and Schönrogge, 2003). Most galls serve as sinks of plant assimilates, thereby, providing high quality food to the insect (Bagatto et al., 1996; Koyama et al., 2004). Galls also provide a protective microenvironment to sedentary feeders such as aphids and psyllids compared to normal plant surfaces. Some galls are also known for protecting certain insects from their parasitoids (Gullan and Cranston, 2010). Usually galls are initiated from young leaves, flower buds, stems, and roots. They are rarely initiated on mature plant parts. Continued stimulation of the cells of the plants by the insect determines the development and growth of insect-induced galls. The involvements of oral secretions, anal excreta, and accessory gland secretions have been emphasized in the initiation and growth of galls. Salivary substances such as amino acids, growth regulators, phenolic compounds, and phenol oxidases may have a role in the formation of galls or in overcoming the plant defense (Schoonhoven et al., 2005; Gullan and Cranston, 2010). The involvement of plant hormones such as auxins and cytokinins in the formation of galls is very likely, though it is not clear yet whether such hormones are produced by the insect or the plant under attack (Raman, 2012). Genetic entities such as viruses, plasmids, or transposons, which can be transferred from the insect to the plant, may also play role in the formation of certain complex galls (Gullan and Cranston, 2010).

MOLECULAR MECHANISMS OF PLANT IMMUNITY

Plants have several preformed physical (e.g., wax layer, trichomes) and chemical (toxins) barriers, to ward off pathogens and herbivores. In case these barriers are overcome by the attackers, plants activate a multilayered innate immune system to suppress the infection (Jones and Dangl, 2006). Most of our knowledge about the plant immune system is derived from studies on plant interactions with pathogens (bacteria, viruses, fungi). Now that insights in the herbivore–plant and nematode–plant interactions begin to appear, it becomes clear that although there are several specificities in these interactions, also considerable similarities with pathogen-induced plant responses exist (Kaloshian and Walling, 2005). Therefore, the knowledge about the molecular mechanisms involved in plant responses to pathogens cannot only help to interpret the observations on nematode and herbivore plant interactions, but also provide inspiration for new experiments. Hence, before discussing the nematode–plant and herbivore–plant interactions, we give a brief overview of the basic mechanisms of plant immunity against pathogens.

The first layer of plant's innate immunity consists of a system that is directed against so called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPS), which are conserved molecules characteristic for a big phylogenetic group of pathogenic and non-pathogenic microbes and are often located at the external surface (e.g., fungal chitin or bacterial lipopolysaccharides). PAMP-triggered immunity (PTI) starts with detection of these PAMPs by pattern recognition receptors, which are transmembrane proteins located at the cell surface. They typically consist of an extracellular leucine-rich-repeat, a transmembrane domain, and a cytoplasmic kinase domain (Nurnberger and Kemmerling, 2006; Zipfel, 2008; Monaghan and Zipfel, 2012). Binding of these immune receptors to PAMPs causes a cellular signal cascade of ion influxes and mitogen-activated protein (MAP) kinase activation resulting in production of reactive oxygen species, changes in gene expression and cell wall reinforcements (Schwessinger and Zipfel, 2008; Antolin-Llovera et al., 2012; Schwessinger and Ronald, 2012). In response to the plant defenses, pathogens and herbivores have in turn evolved several mechanisms to evade or suppress the plant's innate immune system (Jones and Dangl, 2006). This is accomplished by secretion of proteins, the so-called "effectors." Besides evasion or suppression of the host defense system, effectors can also be involved in manipulation of the host developmental program, for example when galls are

formed (Gheysen and Mitchum, 2011). In the course of evolution, plants have developed a second layer of immunity that responds to the presence of these effectors and is called Effector-triggered immunity (ETI). The immune receptors for ETI, called resistance (R) proteins, consist of a leucine-rich-repeat domain attached to a nucleotide binding domain with a coiled–coiled or toll-interleukin receptor N-terminal domain (van Ooijen et al., 2007). They are mostly located inside the cytoplasm, but a few also reside on the plasma membrane with their leucine-rich-repeat facing the apoplast. ETI results in a very fast defense response at the site of invasion, which is marked by a rapid calcium and potassium influx, activation of MAP kinase pathways, formation of reactive oxygen species, and ultimately a local programmed cell death, also known as the hypersensitive response (Jones and Dangl, 2006; Spoel and Dong, 2012). Neighboring cells respond by producing toxic compounds and strengthening of their cell walls. An ETI response is much stronger than a PTI response and often blocks the pathogen at the site of invasion.

Originally, it was thought that R proteins interact directly with a certain effector. Because effectors are very species specific, ETI would only be effective against closely related strains of pathogens or herbivores, while PTI is directed against a broader phylogenetic range of pathogens and herbivores possessing conserved PAMPs. Although there are some examples of R proteins directly interacting with effectors, recent insights suggest that the majority of the R proteins monitor modifications caused by the effectors on own proteins (Jones and Takemoto, 2004; van der Hoorn and Kamoun, 2008). This guarding of self-proteins has the advantage that with a limited number of R proteins the activity of numerous effectors can be sensed. Indeed, whole genome sequencing of several plant species demonstrated that the number of different R proteins is much smaller than the number of effectors that they can encounter after attack by different species of pathogens and herbivores (Goff et al., 2002; Meyers et al., 2003). Protein interaction studies have also shown that effectors tend to be mostly directed against the same "hubs" in the immune reaction signaling pathway (Mukhtar et al., 2011), and therefore guarding the modification of a limited number of these hub self-proteins by the R proteins is sufficient to provide resistance against a broad spectrum of invaders.

Induction of PTI and ETI also lead to activation of hormonal signaling pathways, such as the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathway. In general, the SA pathway is induced by biotrophic pathogens, while the JA and ET pathways are induced by wounding or necrotrophic pathogens (Pieterse and van Loon, 1999). However, recent experiments with the pathogen *Pseudomonas syringae* have demonstrated that all three hormonal pathways are important for PTI as well as ETI (Tsuda and Katagiri, 2010). The authors propose a model whereby plants would initially activate all three pathways at low levels, and when the pathogen remains, priority would be given to the most effective pathway only later (Katagiri and Tsuda, 2010). Crucial in this model are indications that the three hormonal pathways consist of several sectorial signaling cascades, whereby some of these sectors are shared between the different hormonal pathways. Depending on the kind of pathogen, the plant would strongly activate those signaling sectors that are the most efficient in suppressing the intruder. It's an intriguing idea and would explain why each pathogen or herbivore species seems to induce a different but partially overlapping transcriptome profiles that strongly depend on their feeding habit (De Vos et al., 2005; Bidart-Bouzat and Kliebenstein, 2011). Although it still needs to be investigated whether this holds true for other pathogens and herbivores as well, this initial low level activation of all three pathways could have significant implications for consecutive infections with other pathogen species.

SYSTEMIC INDUCED RESISTANCE

In addition to the local PTI and ETI responses, a systemic response occurs. This systemic response, for which the signal is not always known, can either directly alter the defensive state of the undamaged organs, or it can "prime" or prepare distant tissues for upcoming attacks. Typical for the primed state is a stronger and faster cellular immune response after a second infection (Conrath et al., 2002, 2006). While the exact molecular mechanism of priming is not completely resolved yet, it probably is based on epigenetic modifications that suppress or enhance the transcription of key regulators of the immune response (Bruce et al., 2007; van den Burg and Takken, 2009). A well-known example of priming is the enhanced broad spectrum resistance against pathogens and herbivores after infection with beneficial soil microorganisms, such as plant growth promoting bacteria and mycorrhizal fungi (Van Wees et al., 2008).

Similar to the local responses, systemic induced defense responses are mainly controlled by the plant hormones SA, JA, and ET (Pieterse et al., 2012). Recently, it was discovered that the JA pathway consists of two antagonistic branches (Verhage et al., 2011). The first branch, activated by herbivorous insects, is controlled by the MYC2 transcription factor and – in *Arabidopsis thaliana* – is characterized by the strong induction of the marker gene *VSP2*. The second branch, called the ERF branch, provides resistance against necrotrophic pathogens in *A. thaliana* and is controlled by the ORA59 transcription factor with *PDF1.2* as the marker gene.

Considerable cross-talk occurs between the different hormonal pathways (Bostock, 2005; Pieterse et al., 2012). In general, SA is known to suppress the JA pathway (Spoel et al., 2003). This can lead to trade-offs in the defense responses when plants are attacked simultaneously by different pathogens. For instance, prior infection with the SA-inducing biotrophic pathogen *Hyaloperonospora arabidopsidis* suppresses the JA-controlled defense against caterpillars of *Pieris rapae* (Koornneef et al., 2008). However, this antagonistic interaction between SA and JA seems to be dependent on the concentration of both hormones, whereby a low concentration of both results in a synergistic effect, while high concentrations lead to antagonism (Mur et al., 2006). The SA–JA antagonism seems also to be dependent on the time that has passed between the induction of both hormonal pathways (Koornneef et al., 2008). Moreover, ET signaling prevents SA-mediated suppression of the JA pathway (Leon-Reyes et al., 2010).

Systemic induced defense signaling ultimately results in the activation of a wide range of different defensive traits. These could be morphological changes (e.g., formation of trichomes), production of defensive proteins (e.g., chitinase, proteinase inhibitors)

or toxins (phytoalexins, alkaloids, glucosinolates), or release of volatiles that either have a repellent effect or attract predators of the attacking herbivores (Bezemer and van Dam, 2005; Kaplan et al., 2008a; Dicke et al., 2009).

PLANT DEFENSE AGAINST NEMATODES

LOCAL DEFENSES AGAINST NEMATODES

Because many PPN species invade the host and remain inside for several weeks to months, they inevitably expose themselves to being detected by PAMP immune receptors. The nematode body is protected on the outside by a cuticle consisting of highly conserved collagens, which may serve as cues (epitopes) for the plant's defense system. However, until now no PAMP receptors have been identified that are directed against conserved epitopes of PPN. Most likely, this is due to several strategies developed by nematodes to evade or suppress PTI (Nobre and Evans, 1998; Davies and Curtis, 2011). First, once inside the plant root, the developing nematodes undergo three consecutive molts. The composition of the new cuticle changes after each molt, thereby creating a new challenge to the plant immune system. Second, the nematode cuticle is covered by a carbohydrate-rich surface coat that is constantly shed and changes in composition, thereby creating a moving and variable target for the plant immune system. Third, the surface coat contains lectin-like proteins, which are capable of binding plant carbohydrates (Spiegel et al., 1995). Although not directly demonstrated, it might well be that, just like animal parasitic nematodes (Blaxter et al., 1992; Maizels et al., 2001), PPN cover themselves with host derived carbohydrates and thereby prevent being recognized by the plant immune system as a non-self-entity.

Despite the strategies to avoid recognition, nematode invasion also activates the plant's immune response. As early as 12 h after root penetration by the root-knot nematode *Meloidogyne incognita*, which is still during the root migration phase (Wyss et al., 1992), peroxidases, cell wall modification enzymes, *LOX* genes, and proteinase inhibitors were induced (Gheysen and Fenoll, 2002). Production of reactive oxygen species, callose deposition, and cell wall thickening were also observed during cyst nematode root migration (Waetzig et al., 1999). Whether these resemble typical wounding responses controlled by JA or PTI induced by PAMP detection remains to be investigated. Because endoparasitic PPN are armed with a robust stylet and a cocktail of cell wall degrading enzymes, cell wall thickening is certainly not sufficient to provide complete resistance against PPN. Nevertheless, root inoculation experiments with sedentary endoparasitic juveniles clearly show that only a fraction of them succeeds in penetrating the roots and reach the vascular cylinder where they can induce a feeding site (Wyss et al., 1992; Tytgat et al., 2002). Moreover, even a weak PTI defense might generate a first systemic signal and prime for a stronger defense at later time points against the same or different pathogens or herbivores.

Numerous effectors have been identified in different PPN by RNA profiling, EST (expressed sequence tag) or whole genome sequencing (Vanholme et al., 2004; Abad et al., 2008; Davis et al., 2008; Opperman et al., 2008; Bellafiore and Briggs, 2010; Haegeman et al., 2012). In a recent experiment, mass spectrometric analysis of nematode secretory proteins identified 486 proteins secreted by pre-parasitic *M. incognita* juveniles, which illustrates the complexity of the effector repertoire used by nematodes (Bellafiore et al., 2008). Numerous effector proteins are cell wall degrading enzymes such as cellulases, pectate lyases, polygalacturonases, xylanases, and expansins. They are found in ectoparasitic, migratory, and sedentary endoparasitic nematodes and are involved in cell wall softening mainly during root migration. Several secreted enzymes, such as glutathione peroxidase and peroxiredoxin, protect against reactive oxygen species that may be formed in response to infestation (Jones et al., 2004; Dubreuil et al., 2011). In the potato cyst nematode *Globodera rostochiensis* a protein called SPRYSEC19 was identified that blocks the activation and consecutive hypersensitive response of several know resistance proteins (Postma et al., 2012). Other effectors are thought to suppress SA or JA production or interfere with the plant's ubiquitin-proteasome pathway (Haegeman et al., 2012). The latter is a mechanism that is often used by pathogens to suppress the plant immune system (Angot et al., 2007). Specific for the sedentary nematodes are also numerous proteins that are thought to be involved in feeding cell induction (Davis et al., 2008; Gheysen and Mitchum, 2011; Haegeman et al., 2012).

Several R genes are identified in different plant species that render resistance against sedentary endoparasitic cyst and root-knot nematodes (Tomczak et al., 2009). None of them seems to be effective during the root migration phase of the nematode, but rather block the development of the feeding site, where after the nematode dies due to starvation (Bakker et al., 2006). Interestingly, the tomato Mi-1 R protein that renders resistance to root-knot nematodes (*Meloidogyne* spp.) also provides resistance to potato aphids (*Macrosiphum euphorbiae*) and whiteflies (*Bemisia tabaci*; Rossi et al., 1998; Vos et al., 1998; Nombela et al., 2003). It was demonstrated that this ETI resistance requires SA, but not JA (Branch et al., 2004; Li et al., 2006; Bhattarai et al., 2007, 2008). Strikingly, host plants without the *Mi-1* gene were more susceptible in a choice experiment to potato aphids when the JA pathway was blocked (Bhattarai et al., 2007). However, no effect of JA on the fecundity or survival of the potato aphids was found. This illustrates that in the absence of *Mi-1*-conferred ETI, plants still can have another form of defense, in this case a JA-dependent defense that renders them less attractive to the aphids.

SYSTEMIC INDUCED RESPONSES AFTER NEMATODE INFESTATION

While several gene expression studies have been performed on PPN-infected plants, most of them were designed to analyse the local response, mainly feeding site development of sedentary endoparasitic nematodes. Only recently a few studies have been published providing information on the systemic induced defense after PPN infection. A microarray analysis of *A. thaliana* after infection with the cyst nematode *Heterodera schachtii* revealed a strong induction of *VSP2,* a marker gene for the MYC2 branch of the JA defense pathway, in the whole root system at day 3 after nematode inoculation (Puthoff et al., 2003). At that time point, juveniles have penetrated the roots and reached the vascular cylinder where they just have started inducing the feeding site. Transcriptome analysis during a time course experiment (6 h till 8 days after infection) of soybean with the soybean cyst nematode, *Heterodera glycines*, also found a clear induction

of the JA pathway in the whole root system at all time points (Alkharouf et al., 2006). A similarly clear systemic induction of the JA pathway in soybean roots was observed at day 2, 5, and 10 after *H. glycines* infection (Ithal et al., 2007a), however, locally in the developing syncytia the JA-controlled defense was suppressed (Ithal et al., 2007b).

A comparison of systemic defense signaling after rice infection with a root-knot nematode and a migratory endoparasitic nematode was performed (Kyndt et al., 2012). Infection with the migratory endoparasitic nematode *Hirschmanniella oryzae* activates a systemic JA and ET signaling at day 3, while the SA pathway is suppressed. However, by day 7 the JA and ET signaling is repressed again. At day 3, infection with the root-knot nematode *Meloidogyne graminicola* activates in the systemic root tissue SA and JA, but suppresses ET. By day 7, also the JA pathway is largely suppressed. In contrast, in the shoot tissue all three hormonal defense pathways are suppressed already at day 3 by this nematode. Foliar application of the hormones SA, JA, or ET and mutants analysis showed that *M. graminicola* is mainly sensitive to a JA- and ET-induced defense, but only slightly to SA-induced defense, while *H. oryzae* was sensitive to all the defenses controlled by all three hormones (Nahar et al., 2011; Nahar et al., 2012). In tomato plants, spraying with methyl jasmonate also results in a lower infection rate of *M. incognita* (Fujimoto et al., 2011).

Similar observations of an early shoot defense suppression after *M. incognita* infection were performed in *A. thaliana* where several marker genes for the SA and JA pathway were measured after 5 till 14 days (Hamamouch et al., 2011). In the roots, *M. incognita* infection of *A. thaliana* strongly induces the SA-controlled defense at day 9 and 14, but not at day 5. In addition, a weak response of JA-controlled defense markers was observed at day 9. *A. thaliana* infection with the cyst nematode *H. schachtii* strongly induces the SA, but not the JA marker genes in the roots starting from day 5. In the shoots, however, also some JA marker genes are induced. Moreover, SA-deficient *A. thaliana* mutants exhibit an increased susceptibility to *H. schachtii,* whereas ectopic application of SA renders wild type plants less susceptible (Wubben et al., 2008). In conclusion, sedentary endoparasitic nematodes seem to initially induce the JA, ET, and SA pathways, but very quickly, especially after *M. incognita* infection, parts of these pathways are repressed again.

PLANT DEFENSES AGAINST INSECTS

Insect herbivore-induced immune responses have been reviewed extensively recently (Hilker and Meiners, 2010; Wu and Baldwin, 2010; Arimura et al., 2011; Bonaventure et al., 2011; Hogenhout and Bos, 2011; Kim et al., 2011; Erb et al., 2012; Kerchev et al., 2012; Mithofer and Boland, 2012; Smith and Clement, 2012). Therefore, we only summarize the main points that are essential for understanding how interactions between root-feeding nematodes and shoot-feeding herbivores may occur.

Similar to pathogens, insect herbivores are also detected in two ways. First, wounding by chewing insect causes a release of cellular components that are otherwise compartmentalized. Some of these components act as elicitors of defense reactions and are called damage-associated molecular patterns (DAMPS; Boller and Felix, 2009; Koo and Howe, 2009). Second, herbivore elicitors present in oral or ovipositor secretions, called herbivore-associated molecular patterns (HAMPS), are detected by the plant (Mithöfer and Boland, 2008).

Both DAMPS and HAMPS may trigger the production of a plethora of plant defense responses, ranging from increases in morphological defenses, such as trichomes (Mathur et al., 2012) or chemical defense, such as phenolics, alkaloids, terpenoids, or glucosinolates (van Dam et al., 2009; Turlings et al., 2012). These induced responses may directly contribute to plant resistance by deterring herbivore feeding or indirectly by attracting the herbivores' enemies, e.g., predators and parasitoids, to the plant (Dicke et al., 2009). Which of the many defense compounds are produced upon damage – and thus the effect on the herbivore or its parasitoids – is determined early in the induction process by cross-talk between the JA, SA, and ET pathways. It is thought that cross-talk between signaling pathways is essential for the fine-tuning of plant responses to specific attackers. Each herbivore species may elicit a specific signal signature, which triggers the transcription of distinctive sets of genes (De Vos et al., 2005; Diezel et al., 2009; Verhage et al., 2011). The feeding strategy of the herbivores may greatly co-determine the transcription profile after induction: both specialist and generalist aphids induce transcription profiles in *A. thaliana* that are more similar to each other than to the transcription profiles induced by different species of caterpillars (Bidart-Bouzat and Kliebenstein, 2011). Similar as for pathogen-induced defense responses, herbivore-induced defense responses are often systemic thus affecting the preference and performance of other herbivores feeding elsewhere on the plant (Bezemer and van Dam, 2005; Kaplan et al., 2008a,b; Rasmann et al., 2011).

CHANGES IN PRIMARY METABOLISM

In addition to the increased production of defense compounds, herbivore- and pathogen-induced responses may also alter the plant's primary metabolism, and, consequently, the preference and performance of root and shoot herbivores feeding on the plant. Several recent studies investigated the induced changes of resource allocation after herbivore attack or application of defense-related hormones. Radio-actively labeled CO_2 was used to track assimilated carbon in *Populus* spp. after JA treatment of the leaves (Babst et al., 2005). An increased export of photosynthate toward the stem and roots was observed. An increased allocation of photosynthate to the roots was also observed in *Nicotiana attenuata* after simulated herbivory (Schwachtje et al., 2006). In tomato, JA treatment of the leaves resulted in an increased export of photosynthate and amino acids out of the treated leaves and resulted in an increased amino acid content in the roots (Gómez et al., 2010).

A comparison of leaf and root JA application showed that leaves had a lower total sugar and amino acid concentration after leaf JA application, but only a lower total amino acid concentration after root JA application (van Dam and Oomen, 2008). Measurement of 56 primary metabolites in different tissues of tomato after herbivory with two caterpillar species demonstrated rapid changes that are tissue and herbivore species specific (Steinbrenner et al., 2011). Also PPN seem to influence the primary metabolism of

its host. Metabolic analysis after infection with the cyst nematode *H. schachtii* resulted in decreased amino acid levels in the shoots, but a higher concentration of glyceric acid, gluconic acid, trehalose, 1-kestose, and raffinose (Hofmann et al., 2010).

It is suggested that this change in primary metabolites after herbivore or pathogen infection can have several reasons (Schwachtje and Baldwin, 2008): (i) primary metabolites are used to synthesize defensive secondary metabolites (Smith and Stitt, 2007; Bolton, 2009), (ii) reallocation of resources away from the site of attack my safeguard them for future plant regrowth (Utsumi and Ohgushi, 2007; Steinbrenner et al., 2011), (iii) primary metabolites, such as trehalose, may serve as a signal in the defense pathway (Ahn and Lee, 2003; Bolton, 2009), (iv) primary metabolites may have a defensive function themselves (Lou and Baldwin, 2004). Moreover, recent findings indicate that insect herbivory reduces photosynthesis by transcriptional reprograming as well as physiological mechanisms (Kerchev et al., 2012). We therefore suggest that when investigating plant-mediated interactions between different pathogens or herbivores, not only defense mechanisms should be considered, but also changes in primary metabolism. For instance, a change in the amino acid or sugar content of the phloem sap could have significant effects on aphid performance.

ABOVEGROUND–BELOWGROUND INTERACTIONS BETWEEN NEMATODES AND INSECT HERBIVORES

As discussed above, there is clearly a scope for interactions between belowground PPN and aboveground herbivores feeding on the same plant. Most of the plant responses induced by either of these herbivore classes are systemic and moreover the induced responses are governed by the same signaling pathways. The feeding strategy of the nematodes, the degree of host plant susceptibility to herbivores and the identity of shoot herbivorous insects have been suggested as important factors determining the outcome of interactions between root-feeding nematodes and shoot herbivorous insects as these may differentially influence the responses of the host plants to attackers (Mateille, 1994; van Dam et al., 2003, 2005; Bezemer and van Dam, 2005; Wurst and van der Putten, 2007). The penetration, migration, induction of feeding sites, formation of lesions, cell death, and other infection processes caused by nematodes and the different feeding habits of herbivorous insects (leaf chewing, sap-sucking, leaf mining and boring, and gall-induction) may elicit different hormonal regulatory responses in their host plants. These differential host responses in turn may lead to different systemic induced responses and thereby have differential effects on herbivores feeding on the other plant compartment. Based on what is currently known about plant responses to different species of nematodes, it has been postulated that migratory endoparasitic and ectoparasitic nematodes influence the host plant's immune system to a lesser extent than sedentary endoparasites (Mateille, 1994; Zinov'eva et al., 2004; Bezemer and van Dam, 2005). This would mean that the effects of sedentary nematodes on aboveground herbivores would be greater than that of migratory nematodes. However, there is little concrete experimental evidence that this is the case. Here, we rather suggest that each nematode species can significantly affect their host plant's defense equally strong, but that the direction of the effects on the aboveground herbivores differ depending on the feeding strategy and species of the nematode.

In the last decade, empirical evidence on the occurrence of plant-mediated interactions between root-feeding nematodes and aboveground herbivorous insects are accumulating (**Table 1**). van Dam et al. (2005) reported that the quality of black mustard, *Brassica nigra*, for the shoot herbivore *Pieris rapae* decreased as a result of root herbivory by the migratory endoparasitic nematode *Pratylenchus penetrans*. The reduced performance of the caterpillar was attributed to the enhanced production of phenolics and glucosinolates following root- and shoot-feeding (van Dam et al., 2005).

In a microcosm experiment, the PPNs, which mainly consisted of ectoparasites and migratory endoparasites, caused a reduced fecundity of the aphids *Rhopalosiphum padi* feeding on *Agrostis capillaris* and *Anthoxanthum odoratum* (Bezemer et al., 2005). A lower amino acid content was observed in the phloem sap of the nematode-infected plants, and was probably one of the causes of this decreased aphid performance. A study on *Plantago lanceolata* also documented negative aphid–nematode interactions with lower aphid fecundity on plants attacked by the nematode *P. penetrans* (Wurst and van der Putten, 2007). Herbivory by *P. penetrans* decreased the number of offspring produced by the aphid *Myzus persicae* by 43.8%. The authors suggested that the nematodes might have affected the aphids via changes in the nutritional quality of the aboveground plant parts and possibly via the induction of the same plant defense pathways as those induced by aphids. The latter idea finds support in the fact that the *Mi-1* gene confers resistance to root-knot nematodes as well as aboveground sucking insects such as white flies and aphids (Vos et al., 1998; Kaloshian, 2004). The possibility of priming is also raised as the earlier infection with nematodes might have enhanced the defense against the subsequent attackers (aphids). Also cell content feeding spider mites (Arthropoda) showed a reduced fecundity when feeding on *P. penetrans*-infected *Phaseolus vulgaris* plants (Bonte et al., 2010).

The influence of an infection with sedentary endoparasitic nematodes on sap-sucking insects is rather variable. An infection with *H. schachtii* of *Beta vulgaris* or *Brassica oleracea* resulted in a reduced growth and fecundity of *Brevicoryne brassicae* and *M. persicae* (Hol et al., 2010). *M. persicae* also showed a reduced growth rate and fecundity when feeding on *M. incognita*-infected *Nicotiana tabacum* (Kaplan et al., 2011). In contrast, no effect on the performance of *B. brassicae* was found by an infection of *B. oleracea* with a mix of different root parasitic nematode species (Kabouw et al., 2011). In *A. thaliana*, a simultaneous inoculation with the sedentary endoparasitic nematode *H. schachtii* and the aphid *B. brassicae* was performed (Kutyniok and Muller, 2012). No effects of the nematode infection were observed on the aphid performance at day 3. In contrast, a lower number of nematodes were found on the aphid-infested plants compared to control plants. Hong et al. (2010) studied the effects of soybean plant infection with soybean cyst nematode (*H. glycines)* on the preference and performance of soybean aphid (*Aphis glycines*; Hong et al., 2011). Soybean aphids prefer uninfected plants compared to plants infected with soybean cyst nematode. However, the effect of soybean cyst nematode on the performance of aphids was not

Table 1 | Overview of the plant-mediated interaction studies between root-feeding nematodes and leaf herbivorous insects, whereby a distinction between the different feeding habits is made.

Plant	Nematode		Insect		Outcome	Suggested mechanism	Reference
	Migratory	Sedentary	Leaf chewer	Phloem feeder			
Brassica nigra	*Pratylenchus penetrans*	–	*Pieris rapae*	–	Reduced caterpillar performance	Enhanced production of phenolics and glucosinolates	van Dam et al. (2005)
Nicotiana tabacum	*Tylenchorhynchus* sp., *Xiphinema* sp. (ectoparasites) *Pratylenchus* sp.	–	*Spodoptera exigua*	–	Higher number of nematodes on insect defoliated plants	–	Kaplan et al. (2009)
Plantago lanceolata	*Pratylenchus penetrans*	–	–	*Myzus persicae*	Reduced aphid fecundity	Systemic induced defense, change in nutritional quality	Wurst and van der Putten (2007)
Nicotiana tabacum	*Tylenchorhynchus* sp., *Xiphinema* sp. (ectoparasites) *Pratylenchus* sp.	–	–	*Myzus persicae*	Negative correlation between nematodes and aphids	–	Kaplan et al. (2009)
Anthoxanthum odoratum	Paratylenchidae and Dolichodoridae (ectoparasites) Pratylenchidae	–	–	*Rhopalosiphum padi*	Reduced aphid fecundity	Reduced amino acid concentrations in phloem sap	Bezemer et al. (2005)
Agrostis capillaris	Paratylenchidae and Dolichodoridae (ectoparasites) Pratylenchidae	–	–	*Rhopalosiphum padi*	Reduced aphid fecundity	Reduced amino acid concentrations in phloem sap	Bezemer et al. (2005)
Phaseolus vulgaris	*Pratylenchus penetrans*	–	–	*Tetranychus urticae* (cell content feeder)	Reduced spider mite fecundity	–	Bonte et al. (2010)
Ammophila arenaria	*Pratylenchus brzeskii*	*Meloidogyne* sp. *Heterodera* sp.	–	*Schizaphis rufula*	Microcosm: lower aphid preference for nematode-infected plants Field: no effect	In fields: additional parameters influence herbivores	Vandegehuchte et al. (2010)

(Continued)

Table 1 | Continued

Plant	Nematode		Insect		Outcome	Suggested mechanism	Reference
	Migratory	**Sedentary**	**Leaf chewer**	**Phloem feeder**			
Glycine max	–	*Heterodera glycines*	–	*Aphis glycines*	Lower aphid preference for nematode-infected plants	Change in volatile emissions	Hong et al. (2010, 2011)
Brassica oleracea	–	*Heterodera schachtii*	–	*Brevicoryne brassicae*	Reduced aphid growth and fecundity	–	Hol et al. (2010)
Beta vulgaris	–	*Heterodera schachtii*	–	*Myzus persicae*	Reduced aphid growth and fecundity	–	Hol et al. (2010)
Brassica oleracea	mix of nematode species		–	*Brevicoryne brassicae*	No effect on aphid population growth	–	Kabouw et al. (2011)
Arabidopsis thaliana	–	*Heterodera schachtii*	–	*Brevicoryne brassicae*	No effect on aphid performance Reduced nematode infection	Aphid induced changes in glucosinolate content	Kutyniok and Muller (2012)
Nicotiana tabacum	–	*Meloidogyne incognita*	–	*Myzus persicae*	Lower aphid growth rate and fecundity No effect on nematode performance	Systemic induced defense	Kaplan et al. (2011)
Glycine max	–	*Heterodera glycines*	*Helicoverpa zea*	–	Increased number of insect larvae on nematode-infected plants	–	Alston et al. (1991)
Glycine max		*Meloidogyne incognita*	*Pseudoplusia includens*	–	No effect on caterpillar development	–	Carter-Wientjes et al. (2004)
Nicotiana tabacum	–	*Meloidogyne incognita*	*Trichoplusia ni* (generalist)	–	Increased caterpillar performance	Interference with nicotine synthesis and transport to the shoots	Kaplan et al. (2008a)
Nicotiana tabacum	–	*Meloidogyne incognita*	*Spodoptera exigua* (generalist)	–	Increased caterpillar performance	Interference with nicotine synthesis and transport to the shoots	Kaplan et al. (2008a)

(Continued)

Table 1 | Continued

Plant	Nematode		Insect		Outcome	Suggested mechanism	Reference
	Migratory	**Sedentary**	**Leaf chewer**	**Phloem feeder**			
Nicotiana tabacum	–	*Meloidogyne incognita*	*Manduca sexta* (specialist)	–	No effect on caterpillar	Specialist insect not sensitive to nicotine	Kaplan et al. (2008a)
Gossypium hirsutum	–	*Meloidogyne incognita*	*Helicoverpa zea*	–	No effect on caterpillar	No induction of toxins, no increased attraction of parasitoids	Olson et al. (2008)
Glycine max	–	*Heterodera glycines*	*Pseudoplusia includens*	–	Increased nematode performance	–	Russin et al. (1989)
Glycine max	–	*Meloidogyne incognita*	*Pseudoplusia includens*	–	Increased nematode performance	–	Russin et al. (1993)
Glycine max	–	*Heterodera glycines*	*Helicoverpa zea*	–	Increased nematode performance	Longer growth season of host plant due to defoliation	Alston et al. (1993)
Zea mays	Mix of nematode species		*Romalea guttata*	–	No effect on nematodes	–	Fu et al. (2001)
Zea mays	–	*Meloidogyne incognita*	*Ostrinia nubilalis* (stalk borer)	–	Reduced nematode root penetration	Systemic induced defense	Tiwari et al. (2009)

significant in one set of experiments and significant in another experiment. Ultimately, the authors concluded that soybean cyst nematode primarily influences the behavior of soybean aphid more than its performance. Similarly, in a controlled microcosm experiment conducted on Marram grass, *Ammophila arenaria*, the aphid *Schizaphis rufula* showed a lower preference for plants infected with mix of *Pratylenchus brzeskii*, *Meloidogyne* and *Heterodera* sp. (Vandegehuchte et al., 2010). However, in the field no significant correlations between the abundances of the two groups of herbivores were detected. The authors argue that in the field, other variables related to plant vitality and water content structure herbivore populations.

Different results were also obtained when studying the plant-mediated effects of sedentary endoparasitic nematodes on leaf chewing insects. In a field study, a higher number of *Helicoverpa zea* larvae was found when *Glycine max* plants were infected with *H. glycines* (Alston et al., 1991). Kaplan et al. (2008a) reported that belowground herbivory by the root-knot nematode *M. incognita* on tobacco plants increased the larval weight of the aboveground generalist caterpillar *Trichoplusia ni* by 29%, whereas herbivory by the same nematode did not significantly affect the performance of the specialist caterpillar *Manduca sexta*. As plants in the genus *Nicotiana* produce alkaloids such as nicotine for constitutive and induced defense against aboveground herbivores (Steppuhn et al., 2004), this facilitation effect of nematode herbivory on aboveground herbivorous insects may result from interference of nematode feeding with the transport and biosynthesis of nicotine which takes place in the roots. This is supported by the higher (>2 times) concentration of leaf nicotine in control plants when compared to *M. incognita*-infested plants (Kaplan et al., 2008a). Furthermore, nematode root herbivory on nicotine producing plants increases the weight gain of the caterpillar *Spodoptera exigua*, whereas the performance of the caterpillar on nicotine-deficient plants is not affected as a result of root herbivory by nematodes (Kaplan et al., 2008a). The same authors have also noticed that nematode-free plants respond to caterpillar feeding by inducing higher levels of nicotine whereas nematode-infected tobacco plants are impaired in their ability to induce nicotine levels upon larval feeding.

Contrary to the above cases, Olson et al. (2008) reported that root herbivory by the root-knot nematode *M. incognita* has little influence on the direct and indirect induced defense of cotton, *Gossypium hirsutum*, against insect herbivory. In this study, the levels of gossypol and gossypol-like compounds, and the emissions of induced local and systemic volatiles were measured in cotton plants that are exposed to either the foliar feeder *H. zea*, the root-feeding nematode *M. incognita* or their combination. The attraction of the parasitic wasp *Microplitis croceipes* to plants exposed to the different treatments was also investigated. Local and systemic induction of volatiles that attract the parasitoid *M. croceipes* occurred two days after leaf herbivory, and an increased level of herbivore-induced volatiles was recorded from plants infested by nematodes. However, these differences in induced volatile emissions did not affect the attraction of *M. croceipes*: plants with nematodes and an aboveground herbivore were equally attractive as plants with caterpillar damage only. None of the treatments led to changes in gossypol and gossypol-like compounds in leaf or root tissues. Also no effects were found on *Pseudoplusia includens* caterpillar development by a *M. incognita* infection on *Glycine max* (Carter-Wientjes et al., 2004).

While the above examples illustrate the influence of PPN on aboveground feeding insects, leaf feeding insects in turn can also influence PPN performance. Using field surveys and experimental field studies, it was demonstrated that nematode performance was influenced by the aboveground insect feeding guild on tobacco plants (Kaplan et al., 2009). This study shows that positive interactions between nematodes and leaf chewing insects (e.g., caterpillars) predominate, whereas negative interactions occur with sap-feeding insects (e.g., aphids). Overall, insect defoliated plants had a 41% higher numbers of tobacco feeding nematodes in the rhizosphere compared to insect-free plants (Kaplan et al., 2009). The total numbers of nematodes were lower in the rhizosphere of aphid-infested plants, but the effects differed between nematodes species. The ectoparasitic nematode *Tylenchorhynchus* was less abundant whereas the density of *Pratylenchus* remained unaffected by aphid herbivory. Similarly, an increased number of *H. glycines* and *M. incognita* were found on *G. max* when they were defoliated by *P. includes* or *H. zea* caterpillars (Russin et al., 1989; Alston et al., 1993; Russin et al., 1993). In contrast, stalk boring by *Ostrinia nubilalis* resulted in a reduced number of *M. incognita* penetrating the roots of *Zea mays* (Tiwari et al., 2009), while defoliation by *Romalea guttata* had no effect on the number of nematodes (Fu et al., 2001).

The above mentioned plant-mediated interactions between root-feeding nematodes and leaf herbivorous insects are summarized in **Table 1**. Although many more data are needed to draw any real conclusions, the outcome of the interaction seems indeed to be at least partially determined by the feeding habits. For instance, migrating endoparasitic nematodes (e.g., *Pratylenchus* sp.) cause a reduced aphid fecundity, while sedentary endoparasitic nematodes (e.g., *Heterodera, Meloidogyne*) rather decrease the attractiveness of the plants for these sap-sucking insects, but can have a variable influence on the aphid performance. This example illustrates also that when studying plant-mediated interactions between nematodes and insects all different possible plant responses against infestation should be considered. It would for instance be interesting to know how a nematode infection influences the oviposition preference of Lepidoptera. Moreover, because indirect defenses, such as attraction of parasitoids and predators, are important mechanisms of plants to deal with herbivore infections, plant-mediated interactions between nematodes and herbivorous insects should preferably be studied in a multitrophic environment.

CONCLUSIONS AND FUTURE OUTLOOK

Given the high abundance of both groups of herbivores in the field, plants will inevitably encounter both root-feeding nematodes and aboveground feeding insects in their lifetime. Both types of herbivores will elicit induced defenses responses, and possibly also shifts in primary metabolites, that are systemic throughout the plant. It is therefore likely that PPN and aboveground insects interact with each other via systemic induced responses in the plant. The evidence for these interactions is slowly accumulating. Given the

observed induction of SA, JA, and ET defense signaling pathways and a partial repression of them later on by the sedentary PPN, it is hard to predict the outcome of a combined infection with PPN and herbivorous insects. Therefore, a more detailed analysis of the plant-mediated interactions between PPN and insects is necessary to better understand how plants integrate the induced responses that are triggered by these different groups of herbivores. This knowledge will also lead to new insights in the regulation of plant-induced responses under multiple attacks, as is common in natural environments. Moreover, analyzing the molecular responses of plants challenged with a combined infection of root parasitic nematodes and shoot herbivores will provide us with new insights on the mechanisms of root-shoot communication. A systems-biological approach, whereby a detailed transcriptomic analysis of the systemic induced defense responses against nematodes and insects in roots and shoots is complemented with metabolic measurements, is necessary to obtain the most comprehensive view on the causes and consequences of double infestations. Surely, the model species *A. thaliana* could be used to investigate certain molecular mechanisms, but the small size and the short life cycle of this model plant makes it rather unsuitable to perform long-term investigations of the direct and indirect defense strategies of plants against these herbivores. Recent advances in genomic analysis of other relevant host plants such as *Brassica* spp., tomato, and potato should make this kind of studies feasible on a more realistic time scale.

Another issue to be resolved is the validity of lab-based experiments for processes in the fields. Therefore we suggest performing the laboratory experiments in such a way that they mimic ecological relevant conditions, including inoculum densities and developmental stage of the insects and plants at the time of infection. The results obtained under (semi)-controlled laboratory conditions should be complemented with field studies. This will not only indicate whether the mechanisms observed in the greenhouse are working under natural conditions, but it will also reveal the effect of additional factors influencing the performance of PPN, the foliar herbivore, and their hosts.

REFERENCES

Abad, P., Gouzy, J., Aury, J. M., Castagnone-Sereno, P., Danchi, E. G. J., Deleury, E., et al. (2008). Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nat. Biotechnol.* 26, 909–915.

Ahn, J. H., and Lee, J. S. (2003). Sugar acts as a regulatory signal on the wound-inducible expression of SbHRGP3::GUS in transgenic plants. *Plant Cell Rep.* 22, 286–293.

Alkharouf, N. W., Klink, V. P., Chouikha, I. B., Beard, H. S., Macdonald, M. H., Meyer, S., et al. (2006). Time-course microarray analyses reveal global changes in gene expression of susceptible *Glycine max* (soybean) roots during infection by *Heterodera glycines* (soybean cyst nematode). *Planta* 224, 838–852.

Alston, D. G., Bradley, J. R., Schmitt, D. P., and Coble, H. D. (1991). Response of *Helicoverpa zea* (Lepidoptera, Noctuidae) populations to canopy development in soybean as influenced by *Heterodera glycines* (Nematode, Heteroderidae) and annual weed population densities. *J. Econ. Entomol.* 84, 267–276.

Alston, D. G., Schmitt, D. P., Bradley, J. R., and Coble, H. D. (1993). Multiple pest interactions in soybean – Effects on *Heterodera glycines* egg populations and crop yield. *J. Nematol.* 25, 42–49.

Angot, A., Vergunst, A., Genin, S., and Peeters, N. (2007). Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. *PLoS Pathog.* 3:e3. doi: 10.1371/journal.ppat.0030003

Antolin-Llovera, M., Ried, M. K., Binder, A., and Parniske, M. (2012). Receptor kinase signaling pathways in plant–microbe interactions, *Annu. Rev. Phytopathol.* 50, 451–473.

Arimura, G. I., Ozawa, R., and Maffei, M. E. (2011). Recent advances in plant early signaling in response to herbivory. *Int. J. Mol. Sci.* 12, 3723–3739.

Babst, B. A., Ferrieri, R. A., Gray, D. W., Lerdau, M., Schlyer, D. J., Schueller, M., et al. (2005). Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. *New Phytol.* 167, 63–72.

Bagatto, G., Paquette, L. C., and Shorthouse, J. D. (1996). Influence of galls of *Phanacis taraxaci* on carbon partitioning within common dandelion, *Taraxacum officinale*. *Entomol. Exp. Appl.* 79, 111–117.

Bakker, E., Dees, R., Bakker, J., and Goverse, A. (2006). "Mechanisms involved in plant resistance to nematodes," in *Multigenic and Induced Systemic Resistance in Plants*, eds T. Sadik and B. Elizabeth (Berlin: Springer), 314–334.

Bellafiore, S., and Briggs, S. P. (2010). Nematode effectors and plant responses to infection. *Curr. Opin. Plant Biol.* 13, 442–448.

Bellafiore, S., Shen, Z. X., Rosso, M. N., Abad, P., Shih, P., and Briggs, S. P. (2008). Direct identification of the *Meloidogyne incognita* secretome reveals proteins with host cell reprogramming potential. *PLoS Pathog.* 4:e1000192. doi: 10.1371/journal.ppat.1000192

Bezemer, T. M., De Deyn, G. B., Bossinga, T. M., Van Dam, N. M., Harvey, J. A., and Van Der Putten, W. H. (2005). Soil community composition drives aboveground plant–herbivore–parasitoid interactions. *Ecol. Lett.* 8, 652–661.

Bezemer, T. M., and van Dam, N. M. (2005). Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.* 20, 617–624.

Bezemer, T. M., Wagenaar, R., Van Dam, N. M., Van Der Putten, W. H., and Wackers, F. L. (2004). Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30, 53–67.

Bhattarai, K. K., Xie, Q. G., Mantelin, S., Bishnoi, U., Girke, T., Navarre, D. A., et al. (2008). Tomato susceptibility to root-knot nematodes requires an intact jasmonic acid signaling pathway. *Mol. Plant Microbe Interact.* 21, 1205–1214.

Bhattarai, K. K., Xie, Q. G., Pourshalimi, D., Younglove, T., and Kaloshian, I. (2007). Coi1-dependent signaling pathway is not required for Mi-1-mediated potato aphid resistance. *Mol. Plant Microbe Interact.* 20, 276–282.

Bidart-Bouzat, M. G., and Kliebenstein, D. (2011). An ecological genomic approach challenging the paradigm of differential plant responses to specialist versus generalist insect herbivores. *Oecologia* 167, 677–689.

Blaxter, M. (1998). *Caenorhabditis elegans* is a nematode. *Science* 282, 2041–2046.

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., et al. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71–75.

Blaxter, M. L., Page, A. P., Rudin, W., and Maizels, R. M. (1992). Nematode surface coats – actively evading immunity. *Parasitol. Today* 8, 243–247.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406.

Bolton, M. D. (2009). Primary metabolism and plant defense—fuel for the fire. *Mol. Plant Microbe Interact.* 22, 487–497.

Bonaventure, G., Vandoorn, A., and Baldwin, I. T. (2011). Herbivore-associated elicitors: FAC signaling and metabolism. *Trends Plant Sci.* 16, 294–299.

Bonte, D., De Roissart, A., Vandegehuchte, M. L., Ballhorn, D. J., Van Leeuwen, T., and De La Pena, E. (2010). Local adaptation of aboveground herbivores towards plant phenotypes induced by soil biota. *PLoS ONE* 5:e11174. doi: 10.1371/journal.pone.0011174

Bostock, R. M. (2005). Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* 43, 545–580.

Branch, C., Hwang, C. F., Navarre, D. A., and Williamson, V. M. (2004). Salicylic acid is part of the Mi-1-mediated defense response to root-knot nematode in tomato. *Mol. Plant Microbe Interact.* 17, 351–356.

Bruce, T. J. A., Matthes, M. C., Napier, J. A., and Pickett, J. A. (2007). Stressful "memories" of plants: evidence and

possible mechanisms. *Plant Sci.* 173, 603–608.

Caillaud, M. C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecornte, P., Engler, J. D., et al. (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *J. Plant Physiol.* 165, 104–113.

Carter-Wientjes, C. H., Russin, J. S., Boethel, D. J., Griffin, J. L., and Mcgawley, E. C. (2004). Feeding and maturation by soybean looper (Lepidoptera : Noctuidae) larvae on soybean affected by weed, fungus, and nematode pests. *J. Econ. Entomol.* 97, 14–20.

Chitwood, D. J. (2003). Research on plant-parasitic nematode biology conducted by the United States Department of Agriculture – Agricultural Research Service. *Pest. Manag. Sci.* 59, 748–753.

Connor, E. F., and Taverner, M. P. (1997). The evolution and adaptive significance of the leaf-mining habit. *Oikos* 79, 6–25.

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant Microbe Interact.* 19, 1062–1071.

Conrath, U., Pieterse, C. M. J., and Mauch-Mani, B. (2002). Priming in plant–pathogen interactions. *Trends Plant Sci.* 7, 210–216.

Davies, K. G., and Curtis, R. H. C. (2011). Cuticle surface coat of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* 49, 135–156.

Davis, E. L., Hussey, R. S., Mitchum, M. G., and Baum, T. J. (2008). Parasitism proteins in nematode-plant interactions. *Curr. Opin. Plant Biol.* 11, 360–366.

Davis, E. L., and Mitchum, M. G. (2005). Nematodes. Sophisticated parasites of legumes. *Plant Physiol.* 137, 1182–1188.

De Deyn, G. B., Raaijmakers, C. E., Zoomer, H. R., Berg, M. P., De Ruiter, P. C., Verhoef, H. A., et al. (2003). Soil invertebrate fauna enhances grassland succession and diversity. *Nature* 422, 711–713.

De Deyn, G. B., Van Ruijven, J., Raaijmakers, C. E., De Ruiter, P. C., and Van Der Putten, W. H. (2007). Above- and belowground insect herbivores differentially affect soil nematode communities in species-rich plant communities. *Oikos* 116, 923–930.

De Vos, M., Van Oosten, V. R., Van Poecke, R. M. P., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., et al. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Interact.* 18, 923–937.

Dicke, M., Van Loon, J. J. A., and Soler, R. (2009). Chemical complexity of volatiles from plants induced by multiple attack. *Nat. Chem. Biol.* 5, 317–324.

Diezel, C., Von Dahl, C. C., Gaquerel, E., and Baldwin, I. T. (2009). Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiol.* 150, 1576–1586.

Dubreuil, G., Deleury, E., Magliano, M., Jaouannet, M., Abad, P., and Rosso, M.-N. (2011). Peroxiredoxins from the plant parasitic root-knot nematode, *Meloidogyne incognita*, are required for successful development within the host. *Int. J. Parasitol.* 41, 385–396.

Erb, M., Meldau, S., and Howe, G. A. (2012). Role of phytohormones in insect-specific plant reactions. *Trends Plant Sci.* 17, 250–259.

Fu, S., Kisselle, K. W., Coleman, D. C., Hendrix, P. F., and Crossley D. A. Jr. (2001). Short-term impacts of aboveground herbivory (grasshopper) on the abundance and 14C activity of soil nematodes in conventional tillage and no-till agroecosytems. *Soil Biol. Biochem.* 33, 1253–1258.

Fujimoto, T., Tomitaka, Y., Abe, H., Tsuda, S., Futai, K., and Mizukubo, T. (2011). Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (*Meloidogyne incognita*) in tomato plants (*Solanum lycopersicum*) after foliar treatment with methyl jasmonate. *J. Plant Physiol.* 168, 1084–1097.

Gheysen, G., and Fenoll, C. (2002). Gene expression in nematode feeding sites. *Annu. Rev. Phytopathol.* 40, 191–219.

Gheysen, G., and Mitchum, M. G. (2011). How nematodes manipulate plant development pathways for infection. *Curr. Opin. Plant Biol.* 14, 415–421.

Goff, S. A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. *ssp. japonica*). *Science* 296, 92–100.

Gómez, S., Ferrieri, R. A., Schueller, M., and Orians, C. M. (2010). Methyl jasmonate elicits rapid changes in carbon and nitrogen dynamics in tomato. *New Phytol.* 188, 835–844.

Gullan, P. J., and Cranston, P. S. (2010). *The Insects: An Outline of Entomology*. Hoboken: Wiley-Blackwell.

Haegeman, A., Mantelin, S., Jones, J. T., and Gheysen, G. (2012). Functional roles of effectors of plant-parasitic nematodes. *Gene* 492, 19–31.

Hamamouch, N., Li, C. Y., Seo, P. J., Park, C. M., and Davis, E. L. (2011). Expression of *Arabidopsis* pathogenesis-related genes during nematode infection. *Mol. Plant Pathol.* 12, 355–364.

Heming, B. S. (1993). "Structure, function, ontogeny, and evolution of feeding in thrips (Thysanoptera)," in *Functionaql Morphology of Insect Feeding*, eds C. W. Schaefer and R. A. B. Leschen (Lanham: Entomological Society of America), 3–41.

Hilker, M., and Meiners, T. (2010). How do plants "notice" attack by herbivorous arthropods? *Biol. Rev.* 85, 267–280.

Hofmann, J., El Ashry, A., Anwar, S., Erban, A., Kopka, J., and Grundler, F. (2010). Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *Plant J.* 62, 1058–1071.

Hogenhout, S. A., and Bos, J. I. B. (2011). Effector proteins that modulate plant-insect interactions. *Curr. Opin. Plant Biol.* 14, 422–428.

Hol, W. H. G., De Boer, W., Termorshuizen, A. J., Meyer, K. M., Schneider, J. H. M., Van Dam, N. M., et al. (2010). Reduction of rare soil microbes modifies plant–herbivore interactions. *Ecol. Lett.* 13, 292–301.

Hong, S. C., Donaldson, J., and Gratton, C. (2010). Soybean cyst nematode effects on soybean aphid preference and performance in the laboratory. *Environ. Entomol.* 39, 1561–1569.

Hong, S. C., Macguidwin, A., and Gratton, C. (2011). Soybean aphid and soybean cyst nematode interactions in the field and effects on soybean yield. *J. Econ. Entomol.* 104, 1568–1574.

Hoth, S., Schneidereit, A., Lauterbach, C., Scholz-Starke, J., and Sauer, N. (2005). Nematode infection triggers the de novo formation of unloading phloem that allows macromolecular trafficking of green fluorescent protein into syncytia. *Plant Physiol.* 138, 383–392.

Hoth, S., Stadler, R., Sauer, N., and Hammes, U. Z. (2008). Differential vascularization of nematode-induced feeding sites. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12617–12622.

Hunter, M. D. (2001). Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. *Agric. For. Entomol.* 3, 3–9.

Hussey, R. S. (1989). Disease-inducing secretions of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* 27, 123–141.

Ithal, N., Recknor, J., Nettleton, D., Hearne, L., Maier, T., Baum, T. J., et al. (2007a). Parallel genome-wide expression profiling of host and pathogen during soybean cyst nematode infection of soybean. *Mol. Plant Microbe Interact.* 20, 293–305.

Ithal, N., Recknor, J., Nettleton, D., Maier, T., Baum, T. J., and Mitchum, M. G. (2007b). Developmental transcript profiling of cyst nematode feeding cells in soybean roots. *Mol. Plant Microbe Interact.* 20, 510–525.

Johnson, S. N., Birch, A. N. E., Gregory, P. J., and Murray, P. J. (2006). The 'mother knows best' principle: should soil insects be included in the preference-performance debate? *Ecol. Entomol.* 31, 395–401.

Jones, D. A., and Takemoto, D. (2004). Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. *Curr. Opin. Immunol.* 16, 48–62.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Jones, J. T., Reavy, B., Smant, G., and Prior, A. E. (2004). Glutathione peroxidases of the potato cyst nematode *Globodera rostochiensis*. *Gene* 324, 47–54.

Kabouw, P., Kos, M., Kleine, S., Vockenhuber, E. A., Van Loon, J. J. A., Van Der Putten, W. H., et al. (2011). Effects of soil organisms on aboveground multitrophic interactions are consistent between plant genotypes mediating the interaction. *Entomol. Exp. Appl.* 139, 197–206.

Kaloshian, I. (2004). Gene-for-gene disease resistance: bridging insect pest and pathogen defense. *J. Chem. Ecol.* 30, 2419–2438.

Kaloshian, I., and Walling, L. L. (2005). Hemipterans as plant pathogens. *Annu. Rev. Phytopathol.* 43, 491–521.

Kaplan, I., Halitschke, R., Kessler, A., Rehill, B. J., Sardanelli, S., and Denno, R. F. (2008a). Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecol. Lett.* 11, 841–851.

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., and Denno, R. F. (2008b). Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* 89, 392–406.

Kaplan, I., Sardanelli, S., and Denno, R. F. (2009). Field evidence for indirect interactions between foliar-feeding insect and root-feeding nematode communities on *Nicotiana tabacum*. *Ecol. Entomol.* 34, 262–270.

Kaplan, I., Sardanelli, S., Rehill, B. J., and Denno, R. F. (2011). Toward a mechanistic understanding of competition in vascular-feeding herbivores: an empirical test of the sink competition hypothesis. *Oecologia* 166, 627–636.

Katagiri, F., and Tsuda, K. (2010). Understanding the plant immune system. *Mol. Plant Microbe Interact.* 23, 1531–1536.

Kerchev, P. I., Fenton, B., Foyer, C. H., and Hancock, R. D. (2012). Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. *Plant Cell Environ.* 35, 441–453.

Kim, J., Quaghebeur, H., and Felton, G. W. (2011). Reiterative and interruptive signaling in induced plant resistance to chewing insects. *Phytochemistry* 72, 1624–1634.

Koo, A. J. K., and Howe, G. A. (2009). The wound hormone jasmonate. *Phytochemistry* 70, 1571–1580.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F. C., Van Loon, L. C., et al. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiol.* 147, 1358–1368.

Koyama, Y., Yao, I., and Akimoto, S.-I. (2004). Aphid galls accumulate high concentrations of amino acids: a support for the nutrition hypothesis for gall formation. *Entomol. Exp. Appl.* 113, 35–44.

Kutyniok, M., and Muller, C. (2012). Crosstalk between above- and belowground herbivores is mediated by minute metabolic responses of the host *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 6199–6210.

Kyndt, T., Nahar, K., Haegeman, A., De Vleesschauwer, D., Hofte, M., and Gheysen, G. (2012). Comparing systemic defence-related gene expression changes upon migratory and sedentary nematode attack in rice. *Plant Biol.* 14, 73–82.

Labandeira, C. C. (1997). Insect mouthparts: ascertaining the paleobiology of insect feeding strategies. *Annu. Rev. Ecol. Syst.* 28, 153–193.

Leon-Reyes, A., Du, Y. J., Koornneef, A., Proietti, S., Korbes, A. P., Memelink, J., et al. (2010). Ethylene signaling renders the jasmonate response of *Arabidopsis* insensitive to future suppression by salicylic acid. *Mol. Plant Microbe Interact.* 23, 187–197.

Li, Q., Xie, Q. G., Smith-Becker, J., Navarre, D. A., and Kaloshian, I. (2006). Mi-1-mediated aphid resistance involves salicylic acid and mitogen-activated protein kinase signaling cascades. *Mol. Plant Microbe Interact.* 19, 655–664.

Lohmann, M., Scheu, S., and Muller, C. (2009). Decomposers and root feeders interactively affect plant defence in *Sinapis alba*. *Oecologia* 160, 289–298.

Lou, Y. G., and Baldwin, I. T. (2004). Nitrogen supply influences herbivore-induced direct and indirect defenses and transcriptional responses to *Nicotiana attenuata*. *Plant Physiol.* 135, 496–506.

Maizels, R. M., Gomez-Escobar, N., Gregory, W. F., Murray, J., and Zang, X. X. (2001). Immune evasion genes from filarial nematodes. *Int. J. Parasitol.* 31, 889–898.

Manninen, A. M., Vuorinen, M., and Holopainen, J. K. (1998). Variation in growth, chemical defense, and herbivore resistance in scots pine provenances. *J. Chem. Ecol.* 24, 1315–1331.

Mateille, T. (1994). Biology of the plant nematode relationship – physiological changes and the defense mehanism of plants. *Nematologica* 40, 276–311.

Mathur, V., Tytgat, T. G., Graaf, R., Kalia, V., Sankara Reddy, A., Vet, L. M., et al. (2012). Dealing with double trouble: consequences of single and double herbivory in *Brassica juncea*. *Chemoecology* 1–12.

Meyers, B. C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R. W. (2003). Genome-wide analysis of NBS-LRR–encoding genes in *Arabidopsis*. *Plant Cell Online* 15, 809–834.

Mithöfer, A., and Boland, W. (2008). Recognition of herbivory-associated molecular patterns. *Plant Physiol.* 146, 825–831.

Mithofer, A., and Boland, W. (2012). "Plant defense against herbivores: chemical aspects," in *Annual Review of Plant Biology*, Vol 63. ed. S. S. Merchant, 431–450.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr. Opin. Plant Biol.* 15, 349–357.

Mukhtar, M. S., Carvunis, A.-R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., et al. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601.

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* 140, 249–262.

Nahar, K., Kyndt, T., De Vleesschauwer, D., Hofte, M., and Gheysen, G. (2011). The jasmonate pathway is a key player in systemically induced defense against root knot nematodes in rice. *Plant Physiol.* 157, 305–316.

Nahar, K., Kyndt, T., Nzogela, Y. B., and Gheysen, G. (2012). Abscisic acid interacts antagonistically with classical defense pathways in rice-migratory nematode interaction. *New Phytol.* 196, 901–913.

Nobre, M. J. G., and Evans, K. (1998). Plant and nematode surfaces: their structure and importance in host–parasite interactions. *Nematologica* 44, 103–124.

Nombela, G., Williamson, V. M., and Muniz, M. (2003). The root-knot nematode resistance gene Mi-1.2 of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol. Plant Microbe Interact.* 16, 645–649.

Nurnberger, T., and Kemmerling, B. (2006). Receptor protein kinases – pattern recognition receptors in plant immunity. *Trends Plant Sci.* 11, 519–522.

Offler, C. E., Mccurdy, D. W., Patrick, J. W., and Talbot, M. J. (2003). Tansfer cells: cells specialized for a special purpose. *Annu. Rev. Plant Biol.* 54, 431–454.

Olson, D. M., Davis, R. F., Wackers, F. L., Rains, G. C., and Potter, T. (2008). Plant-herbivore-carnivore interactions in cotton, *Gossypium hirsutum*: linking belowground and aboveground. *J. Chem. Ecol.* 34, 1341–1348.

Opperman, C. H., Bird, D. M., Williamson, V. M., Rokhsar, D. S., Burke, M., Cohn, J., et al. (2008). Sequence and genetic map of *Meloidogyne hapla*: a compact nematode genome for plant parasitism. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14802–14807.

Pieterse, C. M. J., Van Der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal Modulation of Plant Immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521.

Pieterse, C. M. J., and van Loon, L. C. (1999). Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* 4, 52–58.

Postma, W. J., Slootweg, E. J., Rehman, S., Finkers-Tomczak, A., Tytgat, T. O. G., Van Gelderen, K., et al. (2012). The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in plants. *Plant Physiol.* 160, 944–954.

Puthoff, D. P., Nettleton, D., Rodermel, S. R., and Baum, T. J. (2003). *Arabidopsis* gene expression changes during cyst nematode parasitism revealed by statistical analyses of microarray expression profiles. *Plant J.* 33, 911–921.

Raman, A. (2012). Gall induction by hemipteroid insects. *J. Plant Interact.* 7, 29–44.

Rasmann, S., Bauerle, T. L., Poveda, K., and Vannette, R. (2011). Predicting root defence against herbivores during succession. *Funct. Ecol.* 25, 368–379.

Redfern, M. (1997). Plant galls: an intimate association between animals and plants. *Antenna* 21, 55–63.

Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., and Williamson, V. M. (1998). The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9750–9754.

Russin, J. S., Layton, M. B., Boethel, D. J., Mcgawley, E. C., Snow, J. P., and Berggren, G. T. (1989). Development of *Heterodera glycines* on soybean damaged by soybean looper and stem canker. *J. Nematol.* 21, 108–114.

Russin, J. S., Mcgawley, E. C., and Boethel, D. J. (1993). Population development of *Meloidogyne incognita* on soybean defoliated by Pseudoplusia includens. *J. Nematol.* 25, 50–54.

Schoonhoven, L. M., Van Loon, J. J. A., and Dicke, M. (2005). *Insect-Plant Biology*. Wallingford: Cab publishing.

Schwachtje, J., and Baldwin, I. T. (2008). Why does herbivore attack reconfigure primary metabolism? *Plant Physiol.* 146, 845–851.

Schwachtje, J., Minchin, P. E. H., Jahnke, S., Van Dongen, J. T., Schittko, U., and Baldwin, I. T. (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12935–12940.

Schwessinger, B., and Ronald, P. C. (2012). Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* 63, 451–482.

Schwessinger, B., and Zipfel, C. (2008). News from the frontline: recent insights into PAMP-triggered immunity in plants. *Curr. Opin. Plant Biol.* 11, 389–395.

Sijmons, P. C., Atkinson, H. J., and Wyss, U. (1994). Parasitic strategies of root nematodes and associated host-cell responses. *Annu. Rev. Phytopathol.* 32, 235–259.

Sinclair, R. J., and Hughes, L. (2010). Leaf miners: the hidden herbivores. *Austral Ecol.* 35, 300–313.

Smith, A. M., and Stitt, M. (2007). Coordination of carbon supply and plant growth. *Plant Cell Environ.* 30, 1126–1149.

Smith, C. M., and Clement, S. L. (2012). Molecular bases of plant resistance to arthropods. *Annu. Rev. Entomol.* 57, 309–328.

Sohlenius, B. (1980). Abundance, biomass and contribution to energy-flow by soil nematodes in terrestrial ecosystems. *Oikos* 34, 186–194.

Spiegel, Y., Inbar, J., Kahane, I., and Sharon, E. (1995). Carbohydrate-recognition domains on the surface of phytophagous nematodes. *Exp. Parasitol.* 80, 220–227.

Spoel, S. H., and Dong, X. N. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100.

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15, 760–770.

Steinbrenner, A., Gómez, S., Osorio, S., Fernie, A., and Orians, C. (2011). Herbivore-induced changes in tomato (*Solanum lycopersicum*) primary metabolism: a whole plant perspective. *J. Chem. Ecol.* 37, 1294–1303.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R., and Baldwin, I. T. (2004). Nicotine's defensive function in nature. *PLoS Biol.* 2:e217. doi: 10.1371/journal.pbio.0020217

Stone, G. N., and Schönrogge, K. (2003). The adaptive significance of insect gall morphology. *Trends Ecol. Evol.* 18, 512–522.

Tiwari, S., Youngman, R. R., Lewis, E. E., and Eisenback, J. D. (2009). European corn borer (Lepidoptera: Crambidae) stalk tunneling on root-knot nematode (Tylenchida: Heteroderidae) fitness on corn. *J. Econ. Entomol.* 102, 602–609.

Tomczak, A., Koropacka, K., Smant, G., Goverse, A., and Bakker, E. (2009). "Resistant plant responses", in *Plant Cell Monographs*, Vol. 15, eds R. H. Berg and C. G. Taylor (New York: Springer), 83–113.

Trudgill, D. L., and Blok, V. C. (2001). Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopathol.* 39, 53–77.

Tsuda, K., and Katagiri, F. (2010). Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* 13, 459–465.

Turlings, T. C. J., Hiltpold, I., and Rasmann, S. (2012). The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. *Plant Soil* 358, 47–56.

Tytgat, T., De Meutter, J., Gheysen, G., and Coomans, A. (2000). Sedentary endoparasitic nematodes as a model for other plant parasitic nematodes. *Nematology* 2, 113–121.

Tytgat, T., De Meutter, J., Vanholme, B., Claeys, M., Verreijdt, L., Gheysen, G., et al. (2002). Development and pharyngeal gland activities of *Heterodera schachtii* infecting *Arabidopsis thaliana* roots. *Nematology* 4, 899–908.

Utsumi, S., and Ohgushi, T. (2007). Plant regrowth response to a stem-boring insect: a swift moth-willow system. *Popul. Ecol.* 49, 241–248.

van Dam, N. M. (2009). Belowground herbivory and plant defenses. *Annu. Rev. Ecol. Evol. Syst.* 40, 373–392.

van Dam, N. M., Harvey, J. A., Wackers, F. L., Bezemer, T. M., Van Der Putten, W. H., and Vet, L. E. M. (2003). Interactions between aboveground and belowground induced responses against phytophages. *Basic Appl. Ecol.* 4, 63–77.

van Dam, N. M., and Oomen, M. W. A. T. (2008). Root and shoot jasmonic acid applications differentially affect leaf chemistry and herbivore growth. *Plant Signal. Behav.* 3, 91–98.

van Dam, N. M., Raaijmakers, C. E., and Van Der Putten, W. H. (2005). Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.* 115, 161–170.

van Dam, N. M., Tytgat, T. O. G., and Kirkegaard, J. A. (2009). Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochem. Rev.* 8, 171–186.

van den Burg, H. A., and Takken, F. L. W. (2009). Does chromatin remodeling mark systemic acquired resistance? *Trends Plant Sci.* 14, 286–294.

van der Hoorn, R. A. L., and Kamoun, S. (2008). From guard to decoy: a new model for perception of plant pathogen effectors. *Plant Cell* 20, 2009–2017.

Vandegehuchte, M. L., De La Pena, E., and Bonte, D. (2010). Interactions between root and shoot herbivores of *Ammophila arenaria* in the laboratory do not translate into correlated abundances in the field. *Oikos* 119, 1011–1019.

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., and Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. *Gene* 332, 13–27.

van Ooijen, G., Van Den Burg, H. A., Cornelissen, B. J. C., and Takken, F. L. W. (2007). Structure and function of resistance proteins in solanaceous plants. *Annu. Rev. Phytopathol.* 45, 43–72.

Van Wees, S. C. M., Van Der Ent, S., and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448.

Verhage, A., Vlaardingerbroek, I., Raaijmakers, C., Van Dam, N., Dicke, M., Van Wees, S. C. M., et al. (2011). Rewiring of the jasmonate signaling pathway in *Arabidopsis* during insect herbivory. *Front. Plant Sci.* 2:47. doi: 10.3389/fpls.2011.00047

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., et al. (1998). The tomato Mi-1 gene confers resistance to both root-knot nematodes and potato aphids. *Nat. Biotechnol.* 16, 1365–1369.

Waetzig, G. H., Sobczak, M., and Grundler, F. M. W. (1999). Localization of hydrogen peroxide during the defence response of *Arabidopsis thaliana* against the plant-parasitic nematode *Heterodera glycines*. *Nematology* 1, 681–686.

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., Van Der Putten, W. H., and Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science* 304, 1629–1633.

Wubben, M. J. E., Jin, J., and Baum, T. J. (2008). Cyst nematode parasitism of *Arabidopsis thaliana* is inhibited by salicylic acid (SA) and elicits uncoupled SA-independent pathogenesis-related gene expression in roots. *Mol. Plant Microbe Interact.* 21, 424–432.

Wu, J. Q., and Baldwin, I. T. (2010). New insights into plant responses to the attack from insect herbivores. *Annu. Rev. Genet.* 44, 1–24.

Wurst, S., and van der Putten, W. H. (2007). Root herbivore identity matters in plant-mediated interactions between root and shoot herbivores. *Basic Appl. Ecol.* 8, 491–499.

Wyss, U. (2002). "Feeding behaviour of plant-parasitic nematodes," in *The Biology of Nematodes*, ed. D.L. Lee (London: Taylor and Francis), 233–259.

Wyss, U., Grundler, F. M. W., and Munch, A. (1992). The Parasitic behavior of 2nd-stage juveniles of *Meloidogyne-incognita* in Roots of *Arabidopsis-thaliana*. *Nematologica* 38, 98–111.

Zinov'eva, S. V., Vasyukova, N. I., and Ozeretskovskaya, O. L. (2004). Biochemical aspects of plant interactions with phytoparasitic nematodes: a review. *Appl. Biochem. Microbiol.* 40, 111–119.

Zipfel, C. (2008). Pattern-recognition receptors in plant innate immunity. *Curr. Opin. Immunol.* 20, 10–16.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 December 2012; accepted: 22 March 2013; published online: 12 April 2013.

*Citation: Wondafrash M, Van Dam NM and Tytgat TOG (2013) Plant systemic induced responses mediate interactions between root parasitic nematodes and aboveground herbivorous insects. Front. Plant Sci. **4**:87. doi: 10.3389/fpls.2013.00087*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Wondafrash, Van Dam and Tytgat. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 04 February 2013
doi: 10.3389/fpls.2013.00013

Differential contribution of transcription factors to *Arabidopsis thaliana* defense against *Spodoptera littoralis*

Fabian Schweizer, Natacha Bodenhausen†, Steve Lassueur, Frédéric G. Masclaux and Philippe Reymond*

Department of Plant Molecular Biology, University of Lausanne, Lausanne, Switzerland

Edited by:
Corné M. Pieterse, Utrecht University, Netherlands

Reviewed by:
Peer Schenk, The University of Queensland, Australia
Roberto Solano, Centro Nacional de Biotechnologia-Consejo Superior de Investigaciones Cientificas, Spain

****Correspondence:***
Philippe Reymond, Department of Plant Molecular Biology, University of Lausanne, Biophore Building, 1015 Lausanne, Switzerland.
e-mail: philippe.reymond@unil.ch

*†**Present address:***
Natacha Bodenhausen, Institute of Microbiology, Swiss Federal Institute of Technology Zurich, 8093 Zurich, Switzerland.

In response to insect herbivory, *Arabidopsis* plants activate the synthesis of the phytohormone jasmonate-isoleucine, which binds to a complex consisting of the receptor COI1 and JAZ repressors. Upon proteasome-mediated JAZ degradation, basic helix-loop-helix transcription factors (TFs) MYC2, MYC3, and MYC4 become activated and this results in the expression of defense genes. Although the jasmonate (JA) pathway is known to be essential for the massive transcriptional reprogramming that follows herbivory, there is however little information on other TFs that are required for defense against herbivores and whether they contribute significantly to JA-dependent defense gene expression. By transcriptome profiling, we identified 41 TFs that were induced in response to herbivory by the generalist *Spodoptera littoralis*. Among them, nine genes, including *WRKY18*, *WRKY40*, *ANAC019*, *ANAC055*, *ZAT10*, *ZAT12*, *AZF2*, *ERF13*, and *RRTF1*, were found to play a significant role in resistance to *S. littoralis* herbivory. Compared to the triple mutant *myc234* that is as sensitive as *coi1-1* to herbivory, knockout lines of these nine TFs were only partially more sensitive to *S. littoralis* but, however, some displayed distinct gene expression changes at the whole-genome level. Data thus reveal that MYC2, MYC3, and MYC4 are master regulators of *Arabidopsis* resistance to a generalist herbivore and identify new genes involved in insect defense.

Keywords: *Arabidopsis thaliana*, *Spodoptera littoralis*, transcription factors, defense, MYC2, MYC3, MYC4

INTRODUCTION

During million years of coexistence, plants and insects have evolved different types of interactions. Some relationships like pollination are mutually beneficial, whereas the more common predator-host relationship is highly detrimental to plants (Walling, 2000). As a consequence, plants have developed several defense mechanisms to cope with insect attacks including physical barriers, the production of anti-digestive proteins, or toxic secondary metabolites (Howe and Jander, 2007). Most of these defenses are constitutive but are also highly inducible to minimize the cost of triggering defense in times of peace. In *Arabidopsis* and more generally in the Brassicaceae, the amino-acid derived glucosinolates (GS) have been extensively studied for their insect repellent/deterrent properties (Wittstock and Gershenzon, 2002; Halkier and Gershenzon, 2006). These compounds are generally stored as inactive molecules in the vacuole. Upon tissue or cell disruption, GS are catalyzed by myrosinases into active, highly toxic compounds including isothiocyanates, nitriles, and thiocyanates (Grubb and Abel, 2006; Halkier and Gershenzon, 2006).

Several studies have revealed that the plant hormone jasmonate (JA) is the main signal responsible for the activation of inducible defenses against arthropods and necrotrophic fungi (reviewed in Howe and Jander, 2007). In plants, herbivory triggers a burst of JA which leads to a massive transcriptional reprogramming and expression of defense genes (Reymond et al., 2000, 2004; Halitschke et al., 2001; de Vos et al., 2005; Devoto et al., 2005). The F-box protein COI1 was identified as a major component of the JA-pathway, as *coi1-1* mutants were not responding to JA treatment (Xie et al., 1998) and were impaired in the expression of most JA- and insect-inducible genes, including glucosinolate biosynthesis-genes (Reymond et al., 2004; Devoto et al., 2005; Mewis et al., 2006). Consequently, laboratory and field studies have shown that mutants compromised in JA biosynthesis or perception are highly affected in resistance against a wide range of insect herbivores (Howe et al., 1996; McConn et al., 1997; Baldwin, 1998; Stintzi et al., 2001; Li et al., 2004; Reymond et al., 2004; Paschold et al., 2007).

For years, the precise mode of JA perception had remained elusive until several studies provided evidence that COI1 itself, together with members of the JAZ family of repressors, forms a complex with jasmonate-isoleucine (JA-Ile), an amino-acid conjugated form of JA (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Further work demonstrated that (+)-7-*iso*-Jasmonoyl-L-isoleucine is the natural and bioactive ligand of COI1-JAZ complexes (Fonseca et al., 2009). In the absence of JA-Ile, reflecting the state of non-attacked plants, JAZ proteins interact with the bHLH MYC2 transcription factor (TF) and NINJA, which in turn interacts with TPL to actively repress transcription of MYC2 target genes (TG; Pauwels et al., 2010). Upon herbivory, the accumulation and binding of JA-Ile to COI1 leads to ubiquitination and subsequent degradation of JAZs via the 26S proteasome, allowing MYC2 to activate the expression of JA-responsive genes (Sheard et al., 2010; Pauwels and Goossens, 2011).

While mechanisms of JA perception are being unveiled, relatively little is known on which transcription factors (TFs) are controlling such a massive transcriptional reprogramming and on which downstream genes are important for defense against herbivores. Although MYC2 has been shown to interact with JAZs and therefore potentially activate JA-responsive genes, several studies reported that contrary to *coi1-1* that is male sterile, *myc2* alleles are fully fertile; moreover, they are only partially sensitive to exogenous JA and are only slightly more susceptible to insect herbivory than wild-type plants (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Fernández-Calvo et al., 2011; Verhage et al., 2011). Recently, MYC2 was found to act additively with its closely related homologs MYC3 and MYC4 to control JA responses, including defense against herbivory (Fernández-Calvo et al., 2011). Indeed, a triple mutant *myc2myc3myc4* (*myc234*) was as susceptible as *coi1-1* to the generalist herbivore *Spodoptera littoralis* and had a similar reduced expression of JA marker genes (Fernández-Calvo et al., 2011). Besides MYC factors, the insect-inducible *Arabidopsis* MYB102 was found to be necessary for defense against the specialist *Pieris rapae* (de Vos et al., 2006). A*myb102* mutant showed lower expression of defense- and cell wall-related genes. However, its connection with the JA-pathway was not examined (de Vos et al., 2006). Enhanced expression of *MYB75* (*PAP1*), a gene that controls phenylpropanoid metabolism, by activation-tagging in *Arabidopsis* slowed growth of *Spodoptera frugiperda*, but the molecular mechanism of this response was not investigated (Johnson and Dowd, 2004). Similarly, heterologous expression of MYB12 in tobacco conferred increased resistance to *Spodoptera litura* and *Helicoverpa armigera*, presumably by the enhanced accumulation of flavonoids (Misra et al., 2010). Two WRKY TFs from *Nicotiana attenuata*, WRK3 and WRK6, were found to positively control the accumulation of JA-Ile and susceptibility to *Manduca sexta*, suggesting that these factors play a role upstream of the JA-pathway (Skibbe et al., 2008). Finally, GS biosynthesis is regulated by six R2R3-MYB TFs. MYB28, MYB29, and MYB76 control aliphatic-GS genes (Hirai et al., 2007; Gigolashvili et al., 2008; Sønderby et al., 2010), whereas MYB34, MYB51, and MYB122 control indole-GS genes (Gigolashvili et al., 2007). Overexpression of MYB51 in *Arabidopsis* impaired growth of *Spodoptera exigua* (Gigolashvili et al., 2007) whereas a *myb28myb29* double mutant lacking aliphatic-GS was more susceptible to feeding by *Mamestra brassicae* (Beekwilder et al., 2008).

To identify novel TFs that respond to herbivory and to gain insight on their relative contribution to defense, we carried-out a transcriptomic search of insect-inducible TFs. We found nine TFs that had a significant effect on insect performance and analyzed insect-induced transcriptome changes in respective knockout lines. Our study reveals new players in *Arabidopsis* defense against a generalist herbivore and highlights the predominant role of MYC2, MYC3, and MYC4.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana Col-0 was the genetic background of all mutant lines used in this study. The following T-DNA insertion lines were obtained from the Nottingham *Arabidopsis* Stock Center: *erf13* (GK_121A12), *nac019* (Salk_096295), *nac055* (SALK_014331), *wrky18* (SALK_093916), *zat10* (SALK_054092), *zat12* (SAIL_347_G03), *azf2-1* (SALK_132562), *rap2.6* (SAIL_1225_G09), *rrtf1* (SALK_150614), *myb44* (SALK_039074). Homozygous lines were selected by PCR and absence of transcription of the TG in mutant lines was confirmed by RT-PCR. Specific forward and reverse primers were designed with SIGnAL T-DNA verification tool for all lines[1]. We generated *nac019nac055* by crossing single mutants. Seeds of the triple mutant *myc2myc3myc4* were a gift from Roberto Solano (Centro Nacional de Biotecnología-CSIC, Madrid, Spain). The *coi1-1* (non-glabrous) mutant was obtained from Jane Glazebrook (University of Minnesota, St. Paul, MN, USA) and *wrky40* and *wrky18wrky40* mutants were obtained from Imre Somssich (Department of Plant Microbe Interactions, Max Planck-Institute for Breeding Research, Cologne, Germany).

Col-0 and mutant lines were stratified in water for 4 days at 4°C. The *myc2myc3myc4* mutant was stratified in water containing 0.1 mM gibberellic acid to stimulate germination. Seeds were then transferred to pots containing potting compost. The *coi1-1* mutant was germinated on Murashige and Skoog medium (Sigma, Buchs, Switzerland) containing 3% sucrose and 30 μM JA and incubated under continuous light (150 $\mu mol\ m^{-2}\ s^{-1}$) for 7 days in a growth chamber. Homozygous *coi1-1* mutants showing normal greening of leaves and no inhibition of root growth (Feys et al., 1994) were transferred to pots. Plants were grown in a growth chamber as previously described (Reymond et al., 2000).

INSECT BIOASSAYS

Spodoptera littoralis (Egyptian cotton worm) eggs were obtained from Syngenta (Stein, Switzerland) and were stored at 10°C until further use. Eggs were placed in a beaker covered with plastic film in an incubator (26°C) for 2–3 days to allow hatching. Larvae were then reared on *Arabidopsis* plants. For initial insect challenge, two to three fourth- or fifth-instar *S. littoralis* larvae were allowed to feed on 6-week-old plants for 4–5 h in a transparent plastic box in a growth chamber (20°C, 65% relative humidity, 100 $\mu mol\ m^{-2}\ s^{-1}$, 10/14 h photoperiod) until approximately 20% of leaf area was removed. For each experiment, damaged leaf tissue from 12 challenged plants was harvested and immediately stored in liquid nitrogen. Leaves from 12 control, unchallenged plants were collected at the same time. For longer feeding experiments, newly hatched larvae (three for two plants) were allowed to feed continuously during 8 days until leaves were harvested. Microarray analyses with Col-0 and *coi1-1* plants were performed on at least three independent biological replicates.

For testing the susceptibility of TF mutants, 3-week-old plants were used. Forty newly hatched *S. littoralis* larvae were placed in a transparent plastic box containing 70 plants. After 8 days of feeding, larvae were collected and weighed on a precision balance (Mettler-Toledo, Greifensee, Switzerland) whereas plant tissues from control and treated plants were immediately stored in liquid nitrogen and used for microarray analyses. All experiments were repeated at least three times independently, except for *erf13* and *rrtf1* mutants (two replicates).

[1]http://signal.salk.edu/tdnaprimers.2.html

MICROARRAY EXPERIMENTS AND DATA ANALYSIS

For microarray analysis, total RNA of plant tissues was extracted, reverse-transcribed, and processed according to a previously published procedure (Bodenhausen and Reymond, 2007). Labeled probes were hybridized onto CATMAv4 microarrays containing 32,998 *Arabidopsis* gene-specific tags and gene-family tags (Sclep et al., 2007). Hybridization and scanning have been described previously (Reymond et al., 2004). Data normalization and statistical analyses including false-discovery rate (FDR) correction were carried-out using an interface developed at the University of Lausanne [Gene Expression Data Analysis Interface (GEDAI; Liechti et al., 2010)]. Hierarchical clustering of microarray data as well as gene node heights calculations were done with Multi experiment viewer software[2] using the default options. Microarray data have been submitted to ArrayExpress database under accession E-MTAB-1418[3].

QUANTITATIVE RT-PCR

Leaf samples from 5 to 10 plants were harvested and pooled after 48 h of herbivory by first-instar *S. littoralis* larvae. Tissue samples were ground in liquid nitrogen and total RNA was extracted using RNeasy Plant Mini Kit and treated with DNaseI (Qiagen, Hombrechtikon, Switzerland). Afterward, cDNA was synthesized from 1 μg of RNA using M-MLV reverse transcriptase (Invitrogen, Zug, Switzerland) in a final volume of 25 μl and subsequently diluted fourfold with water. Gene-specific primers were designed to produce amplicons between 80 and 120 bp. Primer efficiencies (E) were evaluated by five-step dilution regression. Quantitative real-time PCR (qRT-PCR) was performed using Brilliant II Fast SYBR-Green qRT-PCR Master Mix. Reactions were done in a final volume of 25 μl containing 12.5 μl of 2× SYBR, 3.75 μl of ROX (1/5000 dilution), 4.25 μl of RNAse-free water, 2.5 μl of primer mix (each primer at 1 μM), and 2 μl of cDNA. A Mx3000P real-time PCR instrument (Agilent, Morges, Switzerland) was used with the following program: 95°C for 5 min, then 40 cycles of 10 s at 95°C, 20 s at 55°C, and 30 s at 60°C. Values were normalized to the house-keeping gene *ACTIN8*. The expression level of a TG was normalized to the reference gene (RG) and calculated as Normalized Relative Quantity (NRQ) as follows: $\text{NRQ} = E^{\text{CtRG}}/E^{\text{CtTG}}$. Each experiment was repeated three times independently.

GLUCOSINOLATE ANALYSIS

For GS extraction, seven 3-week-old plants were challenged for 48 h with two neonate *S. littoralis* larvae per leaf. Unchallenged plants were used as controls. Samples from four biologically independent replicates were analyzed. Extraction method, UHPLC-QTOFMS measurements and analysis have been recently described (Glauser et al., 2012).

RESULTS

IDENTIFICATION OF INSECT-INDUCED TRANSCRIPTION FACTORS

To identify novel TFs that are involved in the response to herbivory, we reasoned that some of these factors might be themselves subjected to transcriptional regulation. We therefore performed a whole-genome microarray analysis of *Arabidopsis* plants challenged with the generalist *S. littoralis* and searched for TF genes that were robustly induced by herbivory. We collected RNA from several independent replicates after 5 h of feeding with fourth–fifth instar larvae and after 8 days of feeding with neonate larvae and analyzed the transcriptome using *Arabidopsis* CATMA microarrays (Sclep et al., 2007). In addition, to evaluate the role of the JA-pathway in regulating these TFs, we used *coi1-1* plants in the same experimental set-up. Induced genes were defined as genes with a mean expression ratio ≥ 2 in Col-0 (adjusted P-value < 0.05). Based on TAIR annotation[4], we identified 41 TFs that were significantly up-regulated by *S. littoralis* herbivory (Table S1 in Supplementary Material). Clustering microarray data of Col-0 and *coi1-1* plants showed that most TFs were not or much less induced in *coi1-1* plants, suggesting that they depend on a functional JA-pathway (**Figure 1**; Table S1 in Supplementary Material). Induced TFs belonged to different classes, including for example several ERF/AP2, bHLH, MYB, WRKY, Zinc-fingers, and NAC factors.

INSECT PERFORMANCE ON TF KNOCKOUT LINES

Larval growth can be used as an outcome of plant defense ability against herbivores. To assess whether the newly identified insect-induced TFs where involved in defense, we obtained T-DNA knockout lines and challenged them with insects. For this assay, 3-week-old plants were subjected to feeding by neonate *S. littoralis* for 8 days. Among the 41 insect-induced TFs, some were already known to be involved in defense against herbivory (MYC2, MYB34, MYB75) and were not tested further. For the other candidates, we obtained 11 homozygous mutant lines, of which nine showed a significantly higher growth of *S. littoralis* larvae (**Figure 2**; **Table 1**). Larval weight was between 27% (*erf13*) and 66% (*zat12*) higher on mutant than on wild-type plants, but this was less pronounced than on *coi1-1* or *myc234* plants (>300%; **Table 1**). Interestingly, all sensitive TF mutants belonged to unrelated gene families like bHLH (*myc234*), WRKY (*wrky18*, *wrky40*), NAC (*nac019*, *nac055*), zinc-finger (*zat10*, *zat12*, *azf2-1*), and ERF/AP2 (*erf13* and *rrtf1*). For some closely related TFs like WRKY18, WRKY40, and NAC019, NAC055, the respective double mutants were also tested. Noteworthy, although both single mutants were significantly more sensitive to herbivory, none of the double mutants showed an additive effect on larval growth (**Figure 2**). A plausible explanation could be that these factors form heterodimers and control the same sets of defense genes.

EXPRESSION OF JA MARKER GENES

Several studies have shown that the JA-pathway positively controls the expression of at least two distinct sets of genes. Herbivory leads to a burst of JA which activates the expression of genes like *JAZ10* and *VSP2* (Reymond et al., 2004; Yan et al., 2007). In response to necrotrophic fungi, plants produce JA and ethylene (ET), which together turn on a set of genes including *PDF1.2* and *ORA59* (Manners et al., 1998; Penninckx et al., 1998; Pré et al., 2008). To test the involvement of insect-responsive TFs in the activation

[2]http://www.tm4.org/mev/

[3]http://www.ebi.ac.uk/arrayexpress/

[4]www.arabidopsis.org

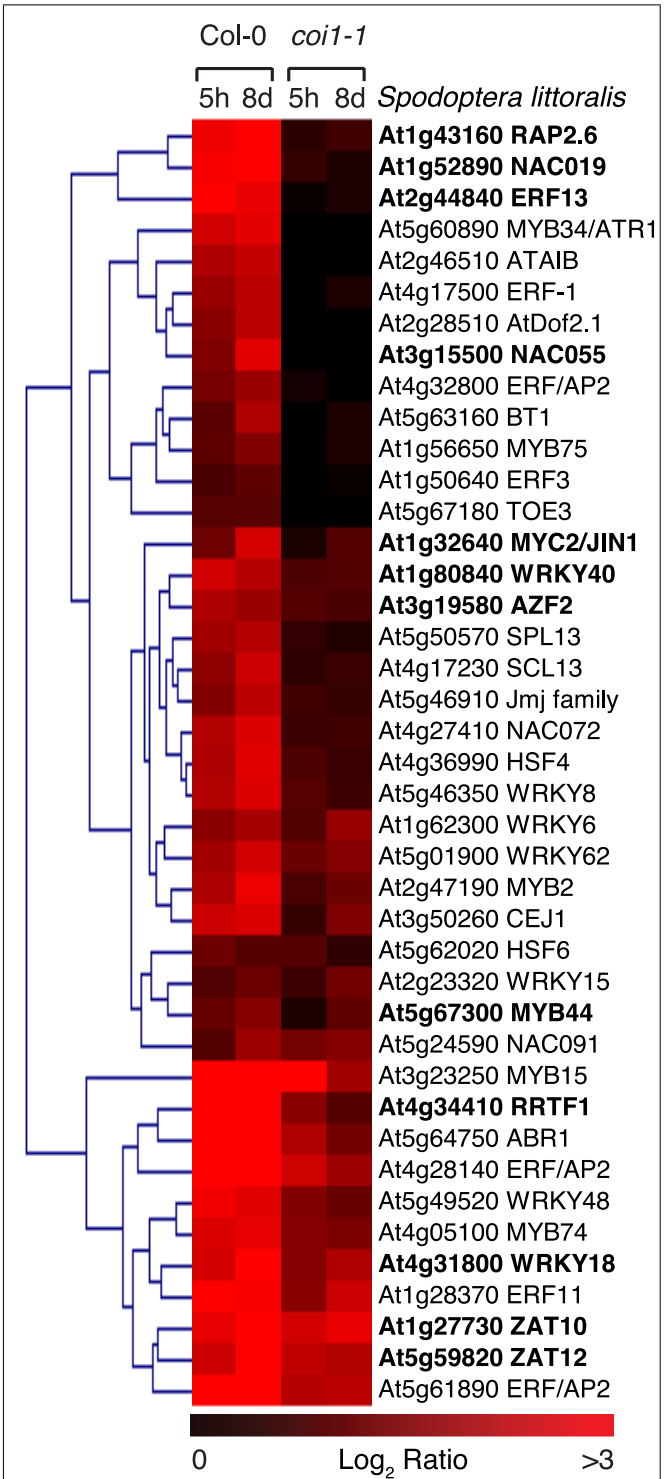


FIGURE 1 | Expression of insect-inducible transcription factors in wild-type and *coi1-1*. Heat map representing transcription factors induced in response to *Spodoptera littoralis* in Col-0 and their expression in the *coi1-1* mutant. Plants were challenged for 5 h with fourth–fifth instar larvae or for 8 days with first-instar larvae. Genes significantly induced ($\log_2$ ratio > 1, *P*-value < 0.05) were represented in a clustered heat map with MultiExperiment Viewer 4.8.1 using Euclidian distance. Genes in bold were analyzed in this study.

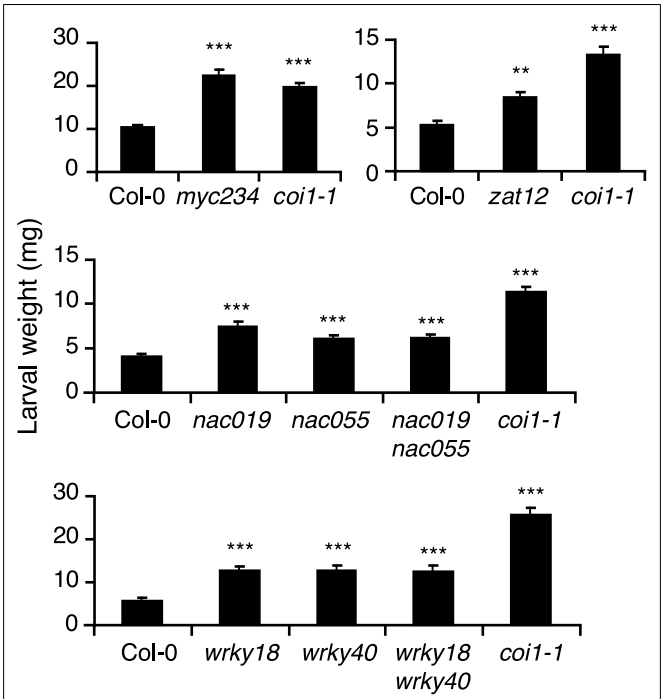


FIGURE 2 | Insect performance on transcription factor mutants. Freshly hatched *S. littoralis* larvae were placed on each genotype and larval weight (mean ± SE) was measured after 8 days of feeding. Asterisks indicate statistically significant differences between mutant plants and Col-0 (Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Similar results were observed in at least three independent replicate experiments.

of these two branches of the JA-pathway, we monitored *VSP2* and *PDF1.2* expression in mutant lines by qRT-PCR. *VSP2* induction by *S. littoralis* was significantly reduced in *nac019*, *nac019nac055*, *wrky18*, *wrky40*, *wrky18wrky40* mutants, although to a lesser extent than in *coi1-1*, but was not affected in *nac055*, *zat10*, *zat12*, and *erf13* mutants (**Figure 3**). Interestingly, up-regulation of *PDF1.2* was higher in all *nac* mutants, as well as in *wrky18*, *wrky18wrky40*, *zat10*, and *zat12*, than in Col-0. On the contrary, *PDF1.2* expression was abolished in *coi1-1* (**Figure 3**). Noteworthy, such opposite expression of *PDF1.2* was previously observed between *myc234* and *coi1-1* in response to JA treatment (Fernández-Calvo et al., 2011). Our results suggest that increased insect susceptibility of some TF mutant lines can be explained by a reduced activation of the JA-pathway that leads to the accumulation of anti-insect proteins, including VSP2.

WHOLE-GENOME ANALYSIS OF TF MUTANTS

To gain more insight on the role of insect-induced TFs on downstream gene expression, we carried-out microarray analyses with mutant lines that showed a higher sensitivity to *S. littoralis*. As controls for highly sensitive mutants, we included *coi1-1* and *myc234*. *S. littoralis* larvae were allowed to feed for 8 days on Col-0 and mutant plants, then RNA was extracted and hybridized to CATMA microarrays. As expected, the majority of genes induced by herbivory in Col-0 were JA-dependent and thereby were not induced in *coi1-1* (**Figure 4A**). In accordance with their similar insect

Table 1 | Insect performance on transcription factor mutants.

Mutant	AGI	Relative weight
coi1-1	At2g39940	3.00 ± 0.23***
myc234	At1g32640/At5g46760/At4g17880	3.10 ± 0.42***
nac019	At1g52890	1.50 ± 0.14***
nac055	At3g15500	1.38 ± 0.17***
nac019nac055	At1g52890/At3g15500	1.40 ± 0.24***
zat10	At1g27730	1.45 ± 0.28***
zat12	At5g59820	1.66 ± 0.05***
azf2-1	At3g19580	1.54 ± 0.07***
wrky18	At4g31800	1.57 ± 0.20***
wrky40	At1g80840	1.54 ± 0.38**
wrky18wrky40	At4g31800/At1g80840	1.46 ± 0.13***
rrtf1-1	At4g34410	1.30 ± 0.08***
erf13	At2g44840	1.27 ± 0.13***
rap2.6	At1g43160	1.32 ± 0.22 n.s.
myb44	At5g67300	1.00 ± 0.11 n.s.

*Relative weight corresponds to the mean weight of neonate S. littoralis larvae feeding on 3-week-old mutants for 8 days divided by the mean weight of larvae feeding on Col-0. Values (±SE) are the mean of several replicates (mutants n ≥ 3; myb44 n = 2; Col-0 n = 17). Asterisks indicate P-value (n.s. not significant; * < 0.05; ** < 0.01; *** < 0.001; Nested ANOVA).*

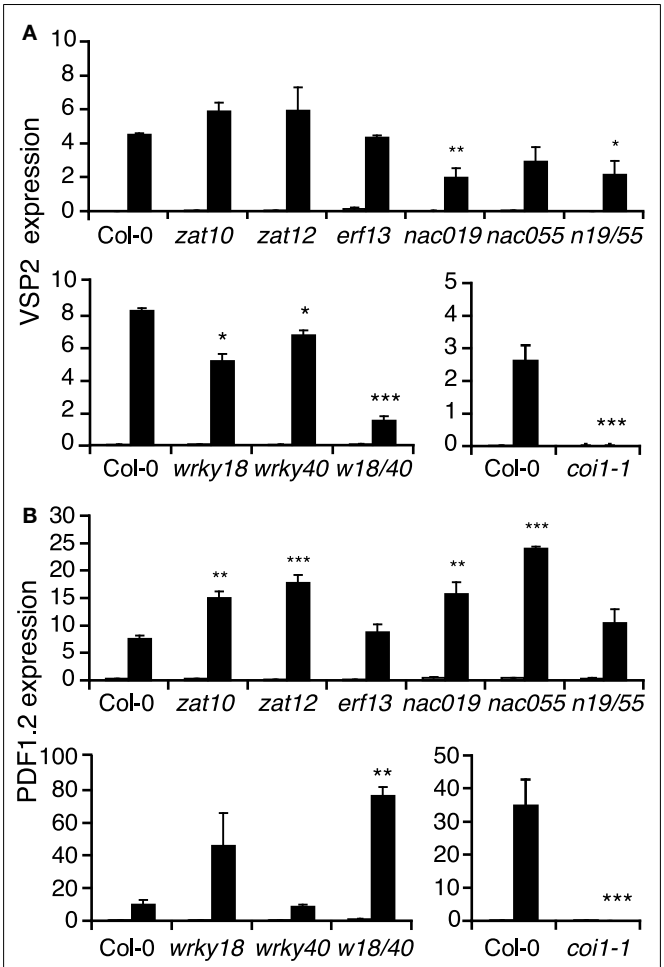


FIGURE 3 | Expression of jasmonate marker genes in transcription factor mutants. Relative expression of *VSP2* **(A)** and *PDF1.2* **(B)** was measured by qRT-PCR in untreated plants (white bars) and in plants challenged for 48 h with *S. littoralis* larvae (black bars). Values are the mean ± SE of three biological replicates. Asterisks indicate statistically significant differences in treated mutant plants compared to treated Col-0 plants (Student's *t*-test, $*P < 0.05$, $**P < 0.01$).

susceptibility, *myc234* and *coi1-1* showed a very similar expression profile, corroborating the additive role of MYC2, MYC3, and MYC4 as general transcriptional regulators acting directly downstream of COI1 to control the expression of JA-responsive genes (Fernández-Calvo et al., 2011).

Although other TF mutants showed an overall expression pattern that was more similar to Col-0, they anyhow displayed altered profiles (**Figure 4A**). A correspondence analysis where the weight distance between different experiments is indicative of their relative similarity indicated that *coi1-1* and *myc234* expression profiles form a distinct subgroup that is distant from a second subgroup containing Col-0 and all TF mutant profiles (**Figure 4B**). In this second subgroup, *wrky18* and *wrky 40* mutants formed a clearly separated branch, as well as *erf13* and *rrtf1*, two members of the B3 sub-family of ERF/AP2 TFs that clustered together, whereas *nac019*, *nac055*, *zat10*, *zat12*, and *azf2-1* were more similar to Col-0 (**Figure 4B**).

Although *coi1-1* and *myc234* expression profiles were globally similar, we could however detect significant differences in the expression of several genes. We observed that some COI1-dependent genes were normally expressed in *myc234*, as for instance *PDF1.2* and a myrosinase-associated protein (At1g54010), or showed a reduced induction, as for instance *VSP2*, *CORI3*, and *MYB75* (**Table 2**; Table S2 in Supplementary Material). Thus, the distance separating transcriptomes of *coi1-1* and *myc234* on the cluster (**Figure 4B**) probably reflects the expression changes of such genes.

A careful examination of the expression profiles of *nac019*, *nac055*, *nac055nac019*, *zat10*, *zat12*, *azf2-1*, *rrtf1*, and *erf13* mutants did not allow to identify candidate defense genes that could easily explain the susceptibility to *S. littoralis*. As illustrated by the clustering of TF mutant expression profiles with Col-0 (**Figure 4B**), the large majority of insect-inducible genes were still up-regulated in the mutants (Table S2 in Supplementary Material). However, consistent with the fact that *wrky* mutants formed a distinguishable group in the cluster, they showed a partially reduced expression of genes from several pathways including general defense (protease inhibitors), JA-biosynthesis (LOX2), GS biosynthesis (MYB34, CYP79B3), and breakdown (TGG2), and phenylpropanoid biosynthesis (DFR, CHS; Table S2 in Supplementary Material). To test whether the transcriptional change in GS biosynthesis-genes could effectively alter GS biosynthesis, we quantified GS in *wrky18wrky40* by UHPLC-QTOFMS (Glauser et al., 2012). Analysis of the most abundant GS showed that, in response to *S. littoralis*, *wrky18wrky40* accumulated significantly more methylthio-GS (4MTB, 7MTH, 8MTO), less methylsulfyl-GS (4MSOB, 8MSOO), and less indole-GS (I3M, 1MO-I3M) than

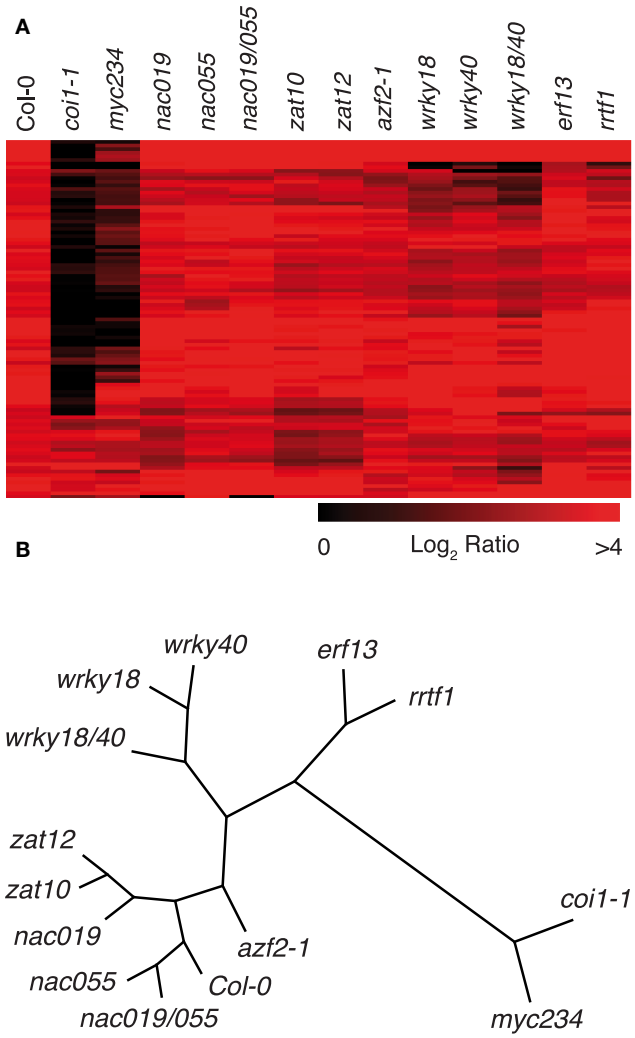


FIGURE 4 | Whole-genome expression profile of transcription factor mutants. (A) Heat map clustering the 100 most highly induced genes in Col-0 plants after 8 days of insect feeding and their respective expression in mutant plants. Heat map was created with MultiExperiment Viewer 4.8.1. **(B)** Correspondence analysis of expression profiles including all insect-induced genes ($\log_2$ ratio > 1, *P*-value < 0.05; $n = 874$). Clustering and node length calculations were performed with MultiExperiment Viewer 4.8.1 and represented as unrooted tree in Treeview 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Col-0 (**Figure 5A**). Thus, although the total GS amount between *wrky18wrky40* and Col-0 was similar, these qualitative differences could contribute to the increased insect susceptibility of the mutant.

To test the involvement of WRKY18 and WRKY40 in the phenylpropanoid pathway, we analyzed three genes involved in the last steps of anthocyanins and flavonols biosynthesis. Expression analysis by qRT-PCR showed clearly that DFR, LDOX, and 3GT were strongly induced by herbivory in Col-0, whereas no significant induction could be observed in *wrky18wrky40* (**Figure 5**). The insect sensitive phenotype of *wrky18wrky40* could therefore be explained in part by a reduced accumulation of metabolites from the phenylpropanoid pathway.

EXPRESSION OF INSECT-INDUCIBLE TFs IN *coi*1-1 AND *myc234*

We found that some TF mutants show an altered expression of JA marker genes but that this was not as severe as in *coi1-1* and *myc234* plants (**Figure 3**, Table S2 in Supplementary Material). We thus wondered whether this regulation was done through the COI1/MYC234 signaling module or whether these TFs were independent modulators of defense gene expression. To address this hypothesis, we analyzed the expression of nine TFs whose mutants were more sensitive to insects in Col-0, *coi1-1*, and *myc234*. All tested TFs were highly induced in response to *S. littoralis*, validating the microarray data (**Figure 6**). Moreover, TF expression pattern in *coi1-1* and *myc234* could be separated into two different types of responses. First, *NAC019*, *NAC055*, *ERF13*, and *RRTF1* were all significantly less induced in *coi1-1* and *myc234* than in Col-0 (**Figure 6**). Interestingly, *NAC019*, *NAC055*, *ERF13*, and *RRTF1* were barely induced in *coi1-1* mutants but did still show a slight induction in *myc234*. Taken together, it seems that these genes depend on a functional JA-pathway and are thus not induced in *coi1-1*, whereas a redundant MYC or other TFs might contribute to their partial expression in *myc234*. The second group included genes whose expression was still induced in *coi1-1* and *myc234*, but somewhat reduced when compared to Col-0 (**Figure 6**). Induction of *ZAT10*, *ZAT12*, *AZF2*, *WRKY18*, and WRKY40 was reduced in *coi1-1* and *myc234* compared to Col-0, although the difference with Col-0 was only statistically significant for *ZAT12* in *coi1-1* and *AZF2* in *myc234*.

Previous reports have shown that MYC2, MYC3, and MYC4 bind preferentially to G-box and G-box like sequences in the promoter of TGs (Dombrecht et al., 2007; Fernández-Calvo et al., 2011; Godoy et al., 2011). We further investigated whether there was a correlation between TF expression patterns and the presence of MYC2 binding *cis*-elements in their respective promoters. Nearly all promoters contained G-box and G-box like sequences, indicating that they might be direct targets of MYCs (**Table 3**). The exception was *WRKY18* and *ERF13* that did not contain any G-box element. Since *ERF13* expression was strongly dependent on COI1 and MYC2, MYC3, MYC4 (**Figure 6**), this gene must thus be indirectly controlled by MYCs. Taken together, our findings suggest that enhanced insect performance on *coi1-1* and *myc234* is explained in part by a reduced expression of downstream TFs that regulate the expression of defense genes.

DISCUSSION

During insect herbivory, plants induce about 1000 genes, of which roughly 65% are regulated by the JA-pathway (Halitschke et al., 2001; Reymond et al., 2004; de Vos et al., 2005; Devoto et al., 2005). Following JA-Ile perception by COI1, repression of MYC2, MYC3, and MYC4 by JAZs is released allowing the transcription of defense genes. Consequently, *coi1-1* and *myc234* mutants display a strong susceptibility to herbivory (Fernández-Calvo et al., 2011). In order to identify novel TFs involved in plant response to herbivory, we performed a whole-genome expression analysis and found 41 TFs that were robustly induced after short- or long-term feeding by the generalist *S. littoralis*. From these, we obtained 11

Table 2 | List of insect-induced genes.

Description	AGI	Expression ratio (log_2)		
		Col-0	*coi1-1*	*myc234*
TI1, trypsin inhibitor	At2g43510	4.79***	3.32**	4.07**
RD20, calcium-binding protein	At2g33380	4.57***	3.33**	3.74**
Protease inhibitor (LTP)	At4g12500	4.27**	4.49**	5.03**
Aldo/keto reductase	At2g37770	4.17***	3.78***	3.51**
CAD8, cinnamyl-alcohol dehydrogenase	At4g37990	3.86**	3.26**	3.10**
Strictosidine synthase	At1g74010	3.64**	3.74*	3.36**
Protease inhibitor (LTP)	At4g12490	3.63**	3.67**	4.03**
Protease inhibitor	At2g38870	3.55**	3.48**	3.72**
PRX52, peroxidase	At5g05340	3.53**	4.17**	4.14**
Trypsin and protease inhibitor	At1g73260	3.51**	2.20*	2.74*
FAD-binding berberine family protein	At4g20860	3.59***	2.26*	2.63**
FAD-binding berberine family protein	At2g34810	3.82***	1.77*	2.04*
PDF1.2, plant defensin	At5g44420	4.33*	0.23	4.09**
Myrosinase-associated protein	At1g54010	4.20**	0.77	3.67**
Oxidoreductase, 2OG-Fe(II) oxygenase	At5g05600	4.57***	0.03	3.50**
GCN5-related N-acetyltransferase (GNAT)	At2g39030	5.57***	−0.34	2.78*
Palmitoyl protein thioesterase	At4g17470	5.25***	−0.05	2.54**
VSP2, acid phosphatase	At5g24770	5.03***	0.81	2.52**
CORI3, cystine lyase	At4g23600	4.16***	0.55	2.15*
BAM5, beta-amylase	At4g15210	3.84***	−0.54	2.44*
PAP1 (MYB75), transcription factor	At1g56650	3.75**	0.58	2.72*
Terpene synthase/cyclase,	At1g61120	4.84***	0.07	1.23*
Oxidoreductase, 2OG-Fe(II) oxygenase	At2g38240	4.54**	−0.24	1.36*
Jacalin lectin	At1g52000	4.42**	−0.39	1.54*
TSA1, calcium-binding protein	At1g52410	4.13**	0.41	1.78**
SSRP1, DNA-binding protein	At3g28730	4.10***	0.77	1.32*
GOLS1, galactinol synthase	At2g47180	4.08***	0.85	1.36*
Expressed protein	At4g02360	4.02***	0.60	1.69
ARGAH2, arginase	At4g08870	3.96**	−0.15	1.54*
DHAR1, dehydroascorbate reductase	At1g19570	3.89***	0.11	1.42*
Cysteine proteinase	At4g11320	3.79***	−0.06	1.54**
ILL6, IAA amino-acid conjugate hydrolase	At1g44350	3.59**	−0.56	1.51*
AOC1, allene oxide cyclase	At3g25760	3.58**	−0.18	1.26*
LOX3, lipoxygenase	At1g17420	3.97**	0.87	0.96*
Protein kinase	At4g10390	3.68**	0.21	0.93*
Oxidoreductase, 2OG-Fe(II) oxygenase	At3g55970	3.90**	0.14	0.99*
jacalin lectin,	At2g39330	4.92***	−0.57	0.99*
FAMT, farnesoic acid methyl transferase	At3g44860	3.79***	−1.17	0.82
VSP1, acid phosphatase	At5g24780	6.48***	0.59	0.47
JAZ10	At5g13220	4.83***	−0.33	0.39
MBP2, myrosinase-binding protein	At1g52030	4.74***	0.33	−0.24
MBP1, myrosinase-binding protein	At1g52040	4.55***	0.39	−0.10
TRAF-like family protein	At5g26260	4.33***	0.40	0.38
UTR3, UDP-galactose transporter	At1g14250	3.86**	−0.42	0.43
Trypsin and protease inhibitor	At1g73325	3.85***	−0.19	0.37
O-methyltransferase	At1g76790	3.74***	−1.09	−0.68
TRAF-like family protein	At3g28220	3.72***	−1.12	0.07
AT14A, transmembrane protein	At3g28300	3.70*	−1.06	−1.09
AT14A, transmembrane protein	At3g28290	3.58*	−1.12	−0.60
PGL5, 6-phosphogluconolactonase	At5g24420	3.56***	−0.83	−0.79*

List of the 50 most highly induced genes in response to S. littoralis in Col-0. Three-week-old plants were challenged for 8 days with first-instar larvae. Values are calculated from several independent biological replicates (Col-0, myc234 n = 4; coi1-1: n = 3). Ratios are color-coded according to intensity: yellow (from 1 to 2), magenta (from 2 to 3), red (>3). Asterisks indicate adjusted P-value (< 0.05; ** < 0.01; *** < 0.001).*

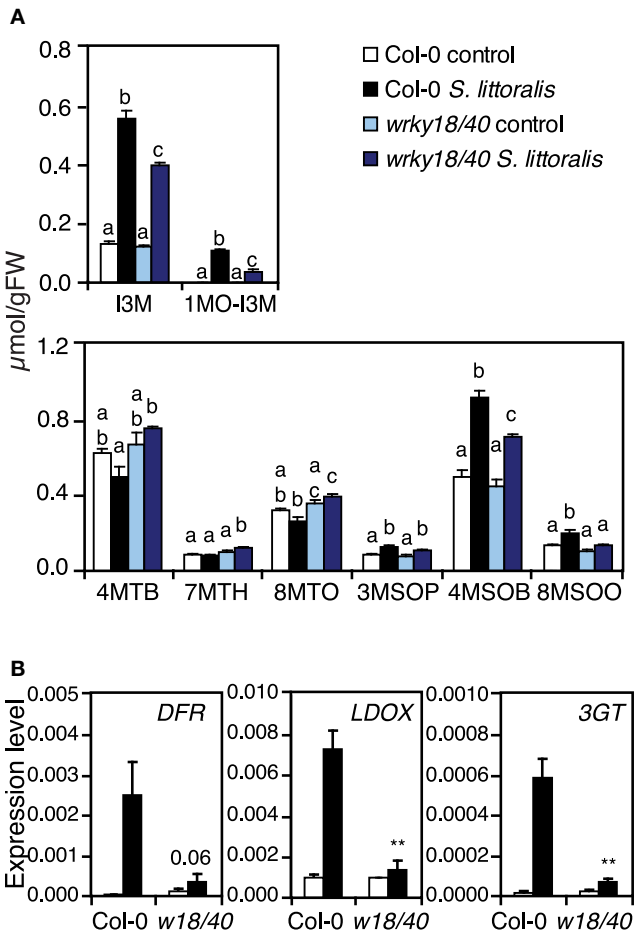


FIGURE 5 | Quantification of glucosinolates and expression of phenylpropanoid pathway genes in *wrky18wrky40* mutant. (A) Levels of eight glucosinolates were quantified in Col-0 and *wrky18wrky40* double mutant. Plants were challenged for 2 days with *S. littoralis* larvae. Unchallenged plants were used as controls. Values are the mean (±SE) of four biological replicates. Bars with different letters differ at $P < 0.05$ (Tukey's HSD test). 4MTB, 4-Methylthiobutyl-GS; 7MTH, 7-Methylthioheptyl-GS; 8MTO, 8-Methylthiooctyl-GS; 3MSOP, 3-Methylsulfinylpropyl-GS; 4MSOB, 4-Methylsulfinylbutyl-GS; 8MSOO, 8-Methylsulfinyloctyl-GS; I3M, Indol-3-ylmethyl-GS; 1MO-I3M, 1-Methoxyindol-3-ylmethyl-GS. **(B)** The *wrky18wrky40* mutant shows altered expression of phenylpropanoid pathway genes *DFR*, *LDOX*, and *3GT* in response to herbivory. Relative expression was measured by qRT-PCR in untreated plants (white bars) and in plants challenged for 48 h with *S. littoralis* larvae (black bars). Values are the mean (±SE) of three replicate experiments. Asterisks indicate statistically significant differences in treated *wrky18wrky40* plants compared to treated Col-0 plants (Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

mutants of which nine were found to increase insect performance. However, mutation in none of these TFs was able to phenocopy the severe susceptibility observed with *coi1-1* and *myc234*, suggesting that these factors only partially contribute to insect defense. One explanation could be that these TFs are downstream targets of MYCs and that they regulate subsets of defense genes. However, analysis of their expression in *coi1-1* and *myc234* revealed that this was not always the case. Whereas *NAC019*, *NAC055*, *ERF13*, and *RRTF1* induction by herbivory was clearly dependent on COI1 and MYC2/MYC3/MYC4, expression of other TFs was not, or only partly, affected in the mutants. We thus propose a model where groups of TFs activates defense gene expression in JA-dependent and JA-independent manner (**Figure 7**). For the JA-dependent pathway, MYC2/MYC3/MYC4 play a quantitatively important role by directly activating defense genes or by activating downstream TFs. In parallel, a JA-independent pathway triggers WRKYs and Zinc-finger TFs expression to provide additional defense. These findings might however represent only a fraction of all TFs involved in defense against herbivory. First, we could only obtain 11 confirmed mutants and the implication of the other insect-induced TFs should be tested. Second, it is also possible that important TFs are not induced by herbivory. For example, expression of MYC3 and MYC4 is not up-regulated by JA treatment (Fernández-Calvo et al., 2011).

Although *myc234* and *coi1-1* were equally sensitive to herbivory, confirming previous observations (Fernández-Calvo et al., 2011), we found that their expression profile was not identical and several potential defense genes were induced in Col-0 and *myc234* but not in *coi1-1*. This suggests that other yet unknown TFs are targets of the COI1-JAIle-JAZs signaling module. Since the JA-pathway is also crucial for defense against necrotrophic fungi (Thomma et al., 2000; Thaler et al., 2004), it is plausible to postulate that specific TFs are involved in this response. Recently, it was shown that JAZs bind to ethylene-stabilized TFs EIN3 and EIL1 to repress the activation of downstream genes *ERF1* and *PDF1.2* (Zhu et al., 2011). Interestingly, *PDF1.2* induction by *S. littoralis* was larger in *myc234*, *wrky18wrky40*, *zat10*, *zat12*, *nac019*, and *nac055* than in Col-0, whereas it was abolished in *coi1-1*, indicating that this branch of the JA-pathway, that requires also ethylene, might be under the negative regulation of the MYC2/MYC3/MYC4 branch. It would be interesting to test the response of *myc234* and TF mutants to necrotrophic fungi.

Global expression profiles of most TF mutants in response to herbivory displayed a moderate change compared to Col-0, whereas *coi1-1* or *myc234* had a marked reduction of defense gene expression. However, these mutants displayed a significant increased sensitivity to *S. littoralis*, indicating that each TF is controlling the expression of important defense genes. One interpretation could be that mutation in these TFs strongly affected the basal expression of defense genes and, whereas the expression ratios were similar between wild-type and mutant plants, the absolute expression level in insect-treated plants might be considerably lower. However, an analysis of expression levels of the most highly insect-induced genes did not show a drastic difference between Col-0 and TF mutants (not shown). Alternatively, the enhanced susceptibility of TF mutant plants might be due to a small but general reduction of defense gene expression. Finally, the downregulation of a few specific genes that have a strong impact on defense could also explain these results. Future research will be required to elucidate which hypothesis is true.

Results from whole-genome expression profiles placed *wrky* mutants in a distinct subgroup. Induction of the anti-insect protein VSP2 (Liu et al., 2005) was partially reduced in *wrky18*, *wrky40*, and more strongly in *wrk18wrky40* mutants. The expression of genes related to several pathways was also affected, in

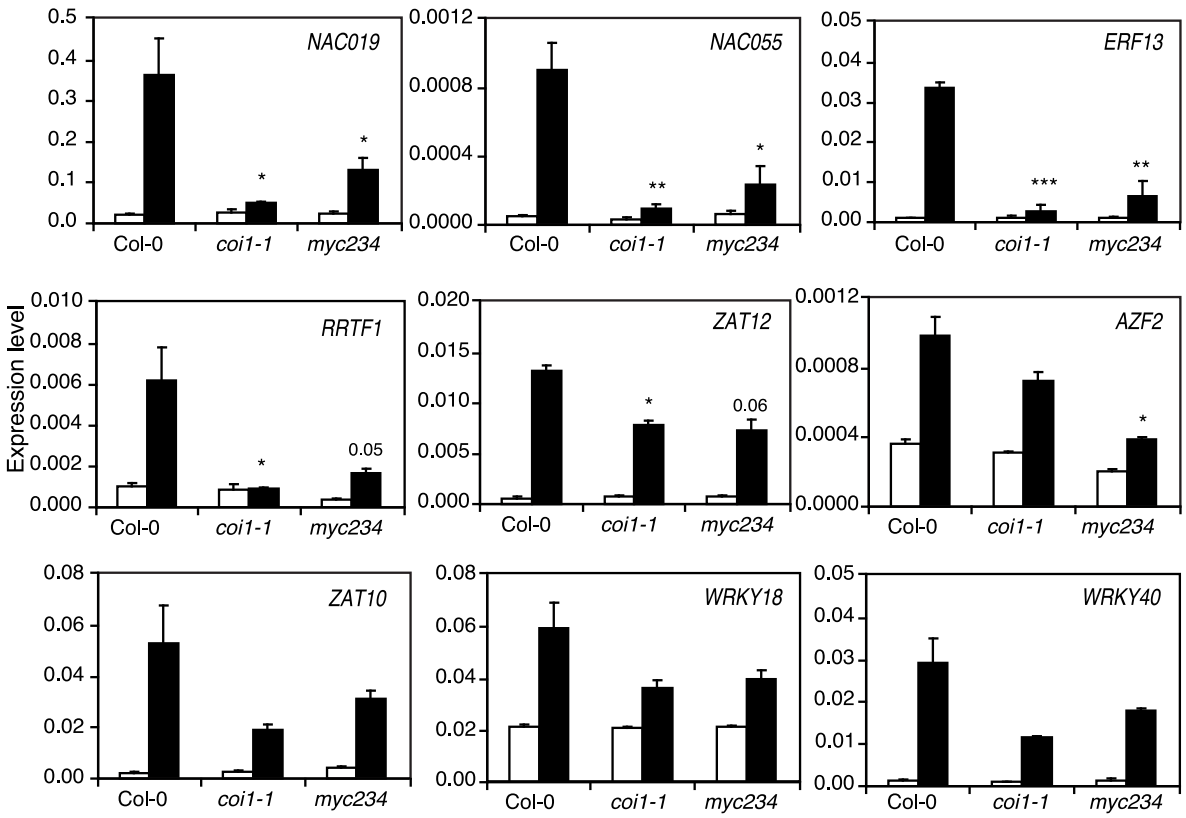


FIGURE 6 | Expression of insect-inducible transcription factors in *coi1-1* and *myc234*. Expression of TFs was measured by qRT-PCR in untreated plants (white bars) and in plants challenged for 48 h with *S. littoralis* larvae (black bars). Values are the mean (±SE) of three biological replicates. Asterisks indicate statistically significant differences in treated plants compared to Col-0 (Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Table 3 | MYC-binding sites in the promoter of insect-induced TFs.

AGI	Gene	CACGTG	AACGTG	CATGTG	CACGAG	CACGCG	CACGGG	CATATG	CACCTG	CACGTC	sum
At1g52890	*ANAC019*	4			1						5
At3g15500	*ANAC055*	2		1	1			1			5
At2g44840	*ERF13*										0
At4g34410	*RRTF1*	2	1	2							5
At4g31800	*WRKY18*										0
At1g80840	*WRKY40*	2						2			4
At3g19580	*AZF2*	1					1				2
At1g27730	*ZAT10*	1	2								3
At5g59820	*ZAT12*				1	1			1	1	4

Number of G-box and G-box like cis-elements in 1 kb5′-upstream region of TFs (TAIR7_upstream_1000) was identified by using Promomer (http://bar.utoronto.ca/welcome.htm). MYC-binding affinities on G-box and G-box like motifs have been previously studied (Godoy et al., 2011). The first sequence from the left represents the canonical G-box with the highest affinity, whereas the eight other sequences represent G-box like motifs with decreasing affinity (Godoy et al., 2011).

particular GS biosynthesis and phenylpropanoid biosynthesis were affected. Since GS are known insect deterrents, the altered GS-profile observed in *wrky* mutants might have contributed to their enhanced susceptibility. The phenylpropanoid pathway provides precursors of various secondary metabolites related to abiotic and biotic stress, including sinapate esters, lignin, suberin, flavonols, and anthocyanins (Vogt, 2010). Polyphenols include also insect repellents like catechin, rotenone, and phaseolin (Schoonhoven et al., 2005). However these compounds are not produced in *Arabidopsis* and it remains still debatable whether anthocyanins or any metabolite derived from the phenylpropanoid pathway have deterrent effects against herbivores. Our observation that genes from the last steps of anthocyanins and flavonols biosynthesis are no longer induced by *S. littoralis* in *wrky18wrky40* suggests that these compounds might be important for defense. It would thus be interesting to perform a targeted metabolic profiling of this mutant.

It was reported previously that WRKY18 and WRKY40 have opposite effects on resistance to necrotrophic and biotrophic pathogens. A *wrky18wrky40* double mutant was more susceptible to *Botrytis cinerea* while it was more resistant to *Pseudomonas syringae* (Xu et al., 2006). Since defense to *B. cinerea* requires a functional JA-pathway (Rowe et al., 2010), these WRKYs might play an important role in JA-mediated responses. In addition, a recent study found that WRKY18 and WRKY40 were involved in early ABA signaling (Shang et al., 2010). Interestingly, ABA deficient mutants have been shown to be more sensitive to herbivore

insects (Bodenhausen and Reymond, 2007). A more detailed analysis of the respective roles of GS, phenylpropanoids and/or defense proteins in the WRKY-dependent response to herbivory will be interesting in the future. In addition, WRKY60, a close homolog of WRKY18, and WRKY40, was shown to form homo- and hetero-complexes with these factors and played a partially redundant role in *Arabidopsis* response to *B. cinerea* and *P. syringae* (Xu et al., 2006). A study of *wrky18/40/60* triple mutant might unveil an increased susceptibility to *S. littoralis* herbivory and a more pronounced alteration of the transcriptome.

Although there were no marked overall differences in expression patterns of *nac019*, *nac055*, and *nac019nac055* mutants compared to Col-0, these profiles were nevertheless not identical. Previously, these two TFs have been shown to be regulated by MYC2, to form homo-and hetero-dimers and to directly control the expression of *VSP1*, a close homolog of *VSP2* (Bu et al., 2008). Besides forming a distinct clade in the NAC protein family, NAC019, NAC055, and their homolog NAC072/RD26 have been shown to bind *in vitro* to the CATGTG motif (Tran et al., 2004), a G-box like motif to which MYC2, MYC3, and MYC4 also bind with high affinity (Fernández-Calvo et al., 2011). This would suggest that MYCs and NACs compete for the same binding site or form a complex. Consistent with the presence of at least two G-boxes in the promoter of *NAC019* and *NAC055*, we found that their induction by herbivory was highly reduced in *myc234*, which suggests that MYC2, MYC3, and MYC4 directly regulate the expression of these genes. Indeed, a recent study showed that MYC2 binds directly to the promoter of *NAC019*, *NAC055*, and *NAC072* and that these TFs positively regulate coronatine-mediated suppression of the salicylic acid (SA) pathway (Zheng et al., 2012). The negative cross-talk between JA and SA is a relatively well-understood process (Pieterse et al., 2012) which could explain the insect sensitive phenotypes of *nac* mutants. In the presence of insects, JA might represses the SA signaling pathway via these NAC TFs, whereas elevated SA, as observed in triple *nac* mutants, repress the JA signaling pathway (Zheng et al., 2012). The fact that whole-genome transcription analysis did not show any major differences between *nac019*, *nac055*, *nac019nac055*, and Col-0 in response to herbivory might have been due to a compensatory effect of NAC072 and may have thus prevented a comprehensive characterization of the role of NACs in insect defense. Further investigations with *nac019/055/072* triple mutants will be needed to determine whether NACs directly regulate insect defense genes or whether this is done indirectly by repressing the SA pathway.

ZAT12 and ZAT10 are known to play a role in plant defense to oxidative stress. It was reported that *zat12* plants are more sensitive to H_2O_2 application and are unable to activate ROS-scavenging transcripts (Rizhsky et al., 2004). In addition, overexpression of ZAT10 elevated the expression of ROS-responsive genes (Mittler et al., 2006). Since ROS production has been implicated in defense against herbivores (Kerchev et al., 2012), the enhanced susceptibility of *zat10* and *zat12* mutants could be explained by a decreased ability to generate ROS. Further experiments will be required to test this hypothesis.

Finally, *erf13* and *rrtf1*, two TF mutants that belong to the same sub-family of ERF/AP2 factors, were clustered separately from Col-0 and other TF mutants. This difference can be attributed to the fact that both mutants had a more pronounced induction of many insect-induced genes. However, since these mutants were more susceptible to *S. littoralis* herbivory, it does not seem that this enhanced expression played a significant role in their response to herbivory.

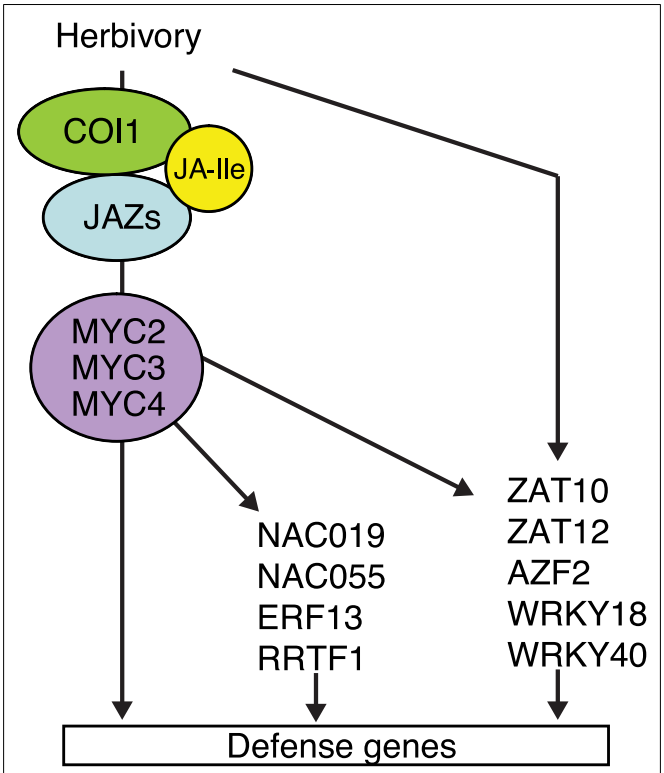


FIGURE 7 | A model for the transcriptional network in defense against chewing insects. In response to herbivory, plants produce JA-Ile. This hormone is detected by its receptor COI1 that in turn degrades JAZ repressors (not shown) to allow the transcriptional activity of MYC2, MYC3, and MYC4 TFs. As a consequence, MYCs activate the expression defense genes and downstream TFs. In addition, herbivory induces the expression of several TFs, which partially depend on COI1 and MYCs. How these TFs are regulated and which are their target genes remains unknown. This model only contains TFs described in this study.

In conclusion, this study demonstrates the involvement of several novel TFs in plant defense against insects. We find that MYC2/MYC3/MYC4 are the main contributors of resistance to a generalist herbivore and that they constitute a central hub that controls the expression of downstream TFs. In addition, JA-independent factors also contribute significantly to defense. In the future, more work will be necessary to identify the complete regulatory network and associated genes that are involved in defense against insects.

ACKNOWLEDGMENTS

We thank Roland Reist (Syngenta, Stein, Switzerland) for providing *S. littoralis* eggs, Blaise Tissot for help in growing plants, and Caroline Gouhier-Darimont for technical support. This work was supported by the Swiss National Science Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant-Microbe_Interaction/10.3389/fpls.2013.00013/abstract

Table S1 | List of insect-induced TFs.

Table S2 | Whole-genome expression data for Col-0 and TF mutants.

Table S3 | List of primers used in this study.

REFERENCES

Baldwin, I. T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8113–8118.

Beekwilder, J., van Leeuwen, W., van Dam, N. M., Bertossi, M., Grandi, V., Mizzi, L., et al. (2008). The impact of the absence of aliphatic glucosinolates on insect herbivory in *Arabidopsis*. *PLoS ONE* 3:e2068. doi:10.1371/journal.pone.0002068

Bodenhausen, N., and Reymond, P. (2007). Signaling pathways controlling induced resistance to insect herbivores in *Arabidopsis*. *Mol. Plant Microbe Interact.* 20, 1406–1420.

Boter, M., Ruíz-Rivero, O., Abdeen, A., and Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes Dev.* 18, 1577–1591.

Bu, Q., Jiang, H., Li, C.-B., Zhai, Q., Zhang, J., and Wu, X. (2008). Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Res.* 18, 756–767.

Chini, A., Fonseca, S., Fernández, G., Adie, B. A. T., Chico, J. M., Lorenzo, O., et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* 448, 666–671.

de Vos, M., Denekamp, M., Dicke, M., Vuylsteke, M., Van Loon, L. C., Smeekens, S. C., et al. (2006). The *Arabidopsis thaliana* transcription factor AtMYB102 functions in defense against the insect herbivore *Pieris rapae*. *Plant Signal. Behav.* 1, 305–311.

de Vos, M., Van Oosten, V. R., Van Poecke, R. M. P., Van Pelt, J. A., Pozo, M. J., Mueller, M. J., et al. (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant Microbe Interact.* 18, 923–937.

Devoto, A., Ellis, C., Magusin, A., Chang, H.-S., Chilcott, C., Zhu, T., et al. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Mol. Biol.* 58, 497–513.

Dombrecht, B., Xue, G. P., Sprague, S. J., Kirkegaard, J. A., Ross, J. J., Reid, J. B., et al. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* 19, 2225–2245.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J. M., Gimenez-Ibanez, S., Geerinck, J., et al. (2011). The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* 23, 701–715.

Feys, B. J., Benedetti, C. E., Penfold, C. N., and Turner, J. G. (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6, 751–759.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., et al. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nat. Chem. Biol.* 5, 344–350.

Gigolashvili, T., Berger, B., Mock, H.-P., Müller, C., Weisshaar, B., and Flügge, U.-I. (2007). The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. *Plant J.* 50, 886–901.

Gigolashvili, T., Engqvist, M., Yatusevich, R., Müller, C., and Flügge, U.-I. (2008). HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. *New Phytol.* 177, 627–642.

Glauser, G., Schweizer, F., Turlings, T. C. J., and Reymond, P. (2012). Rapid profiling of intact glucosinolates in *Arabidopsis* leaves by UHPLC-QTOFMS using a charged surface hybrid column. *Phytochem. Anal.* 23, 520–528.

Godoy, M., Franco-Zorrilla, J. M., Pérez-Pérez, J., Oliveros, J. C., Lorenzo, O., and Solano, R. (2011). Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. *Plant J.* 66, 700–711.

Grubb, C., and Abel, S. (2006). Glucosinolate metabolism and its control. *Trends Plant Sci.* 11, 89–100.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W., and Baldwin, I. T. (2001). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol.* 125, 711–717.

Halkier, B. A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* 57, 303–333.

Hirai, M. Y., Sugiyama, K., Sawada, Y., Tohge, T., Obayashi, T., Suzuki, A., et al. (2007). Omics-based identification of *Arabidopsis* Myb transcription factors regulating aliphatic glucosinolate biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6478–6483.

Howe, G. A., and Jander, G. (2007). Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* 59, 41–66.

Howe, G. A., Lightner, J., Browse, J., and Ryan, C. A. (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell* 8, 2067–2077.

Johnson, E. T., and Dowd, P. F. (2004). Differentially enhanced insect resistance, at a cost, in *Arabidopsis thaliana* constitutively expressing a transcription factor of defensive metabolites. *J. Agric. Food Chem.* 52, 5135–5138.

Kerchev, P. I., Fenton, B., Foyer, C. H., and Hancock, R. D. (2012). Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. *Plant Cell Environ.* 35, 441–453.

Li, L., Zhao, Y., McCaig, B. C., Wingerd, B. A., Wang, J., Whalon, M. E., et al. (2004). The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *Plant Cell* 16, 126–143.

Liechti, R., Csárdi, G., Bergmann, S., Schütz, F., Sengstag, T., Boj, S. F., et al. (2010). EuroDia: a beta-cell gene expression resource. *Database (Oxford)* 2010, baq024.

Liu, Y., Ahn, J.-E., Datta, S., Salzman, R. A., Moon, J., Huyghues-Despointes, B., et al. (2005). *Arabidopsis* vegetative storage protein is an anti-insect acid phosphatase. *Plant Physiol.* 139, 1545–1556.

Lorenzo, O., Chico, J. M., Sánchez-Serrano, J. J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* 16, 1938–1950.

Manners, J. M. J., Penninckx, I. A. I., Vermaere, K. K., Kazan, K. K., Brown, R. L. R., Morgan, A. A., et al. (1998). The promoter of the plant defensin gene PDF1.2 from *Arabidopsis* is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. *Plant Mol. Biol.* 38, 1071–1080.

McConn, M., Creelman, R. A., Bell, E., Mullet, J. E., and Browse, J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5473–5477.

Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C., and Gershenzon, J. (2006). Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67, 2450–2462.

Misra, P., Pandey, A., Tiwari, M., Chandrashekar, K., Sidhu, O. P., Asif, M. H., et al. (2010). Modulation of transcriptome and metabolome of tobacco by *Arabidopsis* transcription factor, AtMYB12, leads to insect resistance. *Plant Physiol.* 152, 2258–2268.

Mittler, R., Kim, Y., Song, L., Coutu, J., Coutu, A., Ciftci-Yilmaz, S., et al. (2006). Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. *FEBS Lett.* 580, 6537–6542.

Paschold, A., Halitschke, R., and Baldwin, I. T. (2007). Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *Plant J.* 51, 79–91.

Pauwels, L., Barbero, G. F., Geerinck, J., Tilleman, S., Grunewald, W., Pérez, A. C., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. *Nature* 464, 788–791.

Pauwels, L., and Goossens, A. (2011). The JAZ proteins: a crucial interface in the jasmonate signaling cascade. *Plant Cell* 23, 3089–3100.

Penninckx, I. A., Thomma, B. P. H. J., Buchala, A. J., Métraux, J. P., and Broekaert, W. F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10, 2103–2113.

Pieterse, C. M. J., der Does Van, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521.

Pré, M. M., Atallah, M. M., Champion, A. A., De Vos, M. M., Pieterse, C. M. J. C., and Memelink, J. J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol.* 147, 1347–1357.

Reymond, P., Bodenhausen, N., Van Poecke, R. M. P., Krishnamurthy, V., Dicke, M., and Farmer, E. E. (2004). A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16, 3132–3147.

Reymond, P., Weber, H., Damond, M., and Farmer, E. E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12, 707–720.

Rizhsky, L., Davletova, S., Liang, H., and Mittler, R. (2004). The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *J. Biol. Chem.* 279, 11736–11743.

Rowe, H. C., Walley, J. W., Corwin, J., Chan, E. K.-F., Dehesh, K., and Kliebenstein, D. J. (2010). Deficiencies in jasmonate-mediated plant defense reveal quantitative variation in *Botrytis cinerea* pathogenesis. *PLoS Pathog.* 6:e1000861. doi:10.1371/journal.ppat.1000861

Schoonhoven, L. M., van Loon, J. J. A., and Dicke, M. (2005). *Insect-Plant Biology*. Oxford: Oxford University Press.

Sclep, G., Allemeersch, J., Liechti, R., De Meyer, B., Beynon, J., Bhalerao, R., et al. (2007). CATMA, a comprehensive genome-scale resource for silencing and transcript profiling of *Arabidopsis* genes. *BMC Bioinformatics* 8:400. doi:10.1186/1471-2105-8-400

Shang, Y., Yan, L., Liu, Z.-Q., Cao, Z., Mei, C., Xin, Q., et al. (2010). The Mg-chelatase H subunit of *Arabidopsis* antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* 22, 1909–1935.

Sheard, L. B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T. R., et al. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* 468, 400–405.

Skibbe, M., Qu, N., Galis, I., and Baldwin, I. T. (2008). Induced plant defenses in the natural environment: *Nicotiana attenuata* WRKY3 and WRKY6 coordinate responses to herbivory. *Plant Cell* 20, 1984–2000.

Sønderby, I. E., Burow, M., Rowe, H. C., Kliebenstein, D. J., and Halkier, B. A. (2010). A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in *Arabidopsis*. *Plant Physiol.* 153, 348–363.

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E. E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12837–12842.

Thaler, J. S., Owen, B., and Higgins, V. J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. *Plant Physiol.* 135, 530–538.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448, 661–665.

Thomma, B. P. H. J., Eggermont, K., and Broekaert, W. F. (2000). Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. *Plant Physiol. Biochem.* 38, 421–427.

Tran, L.-S. P., Nakashima, K., Sakuma, Y., Simpson, S. D., Fujita, Y., Maruyama, K., et al. (2004). Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. *Plant Cell* 16, 2481–2498.

Verhage, A., Vlaardingerbroek, I., Raaijmakers, C., Van Dam, N., Dicke, M., Van Wees, S. C. M., et al. (2011). Rewiring of the jasmonate signaling pathway in *Arabidopsis* during insect gerbivory. *Front. Plant Sci.* 2:47. doi:10.3389/fpls.2011.00047

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Mol. Plant* 3, 2–20.

Walling, L. L. (2000). The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19, 195–216.

Wittstock, U., and Gershenzon, J. (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol.* 5, 300–307.

Xie, D., Feys, B. J., James, S., Nieto-Rostro, M., and Turner, J. G. (1998). COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science* 280, 1091–1094.

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* 18, 1310–1326.

Yan, Y., Stolz, S., Chételat, A., Reymond, P., Pagni, M., Dubugnon, L., et al. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. *Plant Cell* 19, 2470–2483.

Zheng, X.-Y., Spivey, N. W., Zeng, W., Liu, P.-P., Fu, Z. Q., Klessig, D. F., et al. (2012). Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596.

Zhu, Z., An, F., Feng, Y., Li, P., Xue, L. A. M., Jiang, Z., et al. (2011). Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12539–12544.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 December 2012; paper pending published: 11 January 2013; accepted: 18 January 2013; published online: 04 February 2013.

Citation: Schweizer F, Bodenhausen N, Lassueur S, Masclaux FG and Reymond P (2013) Differential contribution of transcription factors to Arabidopsis thaliana defense against Spodoptera littoralis. Front. Plant Sci. ***4****:13. doi: 10.3389/fpls.2013.00013*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Schweizer, Bodenhausen, Lassueur, Masclaux and Reymond. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 06 May 2013
doi: 10.3389/fpls.2013.00113

Arabidopsis redox status in response to caterpillar herbivory

Jamuna Paudel*†, *Tanya Copley*†, *Alexandre Amirizian, Alberto Prado and Jacqueline C. Bede*

Department of Plant Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Saskia C. M. Van Wees, Utrecht University, Netherlands
Igor Kovalchuk, University of Lethbridge, Canada

***Correspondence:**
Jacqueline C. Bede, Department of Plant Science, McGill University, 21,111 Lakeshore, Sainte-Anne-de-Bellevue, QC, Canada H9X 3V9.
e-mail: jacqueline.bede@mcgill.ca

†*Jamuna Paudel and Tanya Copley have contributed equally to this research.*

Plant responses to insect herbivory are regulated through complex, hormone-mediated interactions. Some caterpillar species have evolved strategies to manipulate this system by inducing specific pathways that suppress plant defense responses. Effectors in the labial saliva (LS) secretions of *Spodoptera exigua* caterpillars are believed to induce the salicylic acid (SA) pathway to interfere with the jasmonic acid (JA) defense pathway; however, the mechanism underlying this subversion is unknown. Since noctuid caterpillar LS contains enzymes that may affect cellular redox balance, this study investigated rapid changes in cellular redox metabolites within 45 min after herbivory. Caterpillar LS is involved in suppressing the increase in oxidative stress that was observed in plants fed upon by caterpillars with impaired LS secretions. To further understand the link between cellular redox balance and plant defense responses, marker genes of SA, JA and ethylene (ET) pathways were compared in wildtype, the glutathione-compromised *pad2-1* mutant and the *tga2/5/6* triple mutant plants. *AtPR1* and *AtPDF1.2* showed LS-dependent expression that was alleviated in the *pad2-1* and *tga2/5/6* triple mutants. In comparison, the ET-dependent genes *ERF1* expression showed LS-associated changes in both wildtype and *pad2-1* mutant plants and the ORA 59 marker *AtHEL* had increased expression in response to herbivory, but a LS-dependent difference was not noted. These data support the model that there are SA/NPR1-, glutathione-dependent and ET-, glutathione-independent mechanisms leading to LS-associated suppression of plant induced defenses.

Keywords: ***Arabidopsis thaliana*, caterpillar herbivory, cross-talk, induced defenses, signaling pathways, *Spodoptera exigua***

INTRODUCTION

As plants interact with multiple organisms, they need to prioritize their actions to respond appropriately. Plants manage this through synergistic or antagonistic interactions mediated through growth and defense hormones: a process known as cross-talk (Spoel and Dong, 2008; Robert-Seilaniantz et al., 2011). In plant–pathogen interactions, activation of the systemic acquired resistance (SAR) pathway by biotrophic pathogens may render the plant more susceptible to necrotrophic pathogens that elicit jasmonate (JA)- and ethylene (ET)-mediated responses (Glazebrook, 2005). Insect herbivores also exploit this plant hormone cross-talk to prevent the induction of defensive pathways (Felton and Korth, 2000); however, the mechanisms underlying this are not fully understood.

When tissues are damaged during caterpillar feeding, rapid changes in calcium signatures and the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), leads to the induction of the JA pathway and plant defense responses (Lou and Baldwin, 2006; Arimura et al., 2011). At low, regulated concentrations, H_2O_2 is an important signaling molecule, however, uncontrolled levels are destructive as H_2O_2 readily reacts with cellular components (Schröder and Eaton, 2008; Forman et al., 2010). ROS is generated by mechanical damage but also by enzymes, such as glucose oxidase (GOX), present in the caterpillar's labial saliva (LS; Eichenseer et al., 2010). In lima bean, the zone of H_2O_2 accumulation around the site of leaf damage is widened by ~500 μm by *Spodoptera littoralis* caterpillar herbivory compared to mechanical wounding (Maffei et al., 2006). This caterpillar LS-associated production of H_2O_2 is proposed to be a strategum of some insect species to interfere with induced plant defenses (Musser et al., 2002; Bede et al., 2006).

To avoid the detrimental effects of ROS, antioxidant proteins, such as superoxide dismutase, catalase, peroxidase, and the Halliwell–Asada (ascorbate/glutathione) cycle are activated to maintain cellular redox homeostasis (Noctor et al., 2012). The Halliwell–Asada cycle lowers cellular H_2O_2 levels through a series of redox reactions involving ascorbate and glutathione. Therefore, in response to stress, plants often alter the total glutathione pool or the ratio between oxidized to reduced glutathione (GSSG:GSH) to maintain low H_2O_2 levels. Recognition of biotrophic pathogen attack or salicylic acid (SA) mimic treatment may result in an increase in total glutathione levels (Fodor et al., 1997; Mou et al., 2003; Mateo et al., 2006; Mur et al., 2006). Infiltration of SA into *Arabidopsis* leaves initiates a transient oxidation of the glutathione

Abbreviations: ACT2, Actin2; ERF1, ethylene response factor1; ET, ethylene; GOX, glucose oxidase; HEL, hevein-like protein; H_2O_2, hydrogen peroxide; JA, jasmonic acid; LOX2, lipoxygenase2, LS, labial salivary; PDF1.2, defensin; PR1, pathogenesis-related1; SA, salicylic acid; SAP6, stress-associated protein6.

pool 6 h after the time of injection (Mou et al., 2003; Mateo et al., 2006). In response to mechanical damage, the ratio of GSSG/total glutathione increases, reflecting an oxidized cellular environment, with oxidized glutathione (GSSG) positively linked to JA signaling (Mhamdi et al., 2010; Gfeller et al., 2011). *Arabidopsis* glutathione mutants are more susceptible to microorganism and insect attack (Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008). *Arabidopsis pad2-1* mutant lacks γ-glutamylcysteine synthetase that catalyzes the first step in glutathione biosynthesis (Parisy et al., 2007); therefore, glutathione levels are approximately one-fifth wildtype levels. This line is more vulnerable to *S. littoralis* herbivory (Schlaeppi et al., 2008; Leon-Reyes et al., 2010; Mhamdi et al., 2010; Dubreuil-Mauriza et al., 2011). As well, as glutathione pools and ratio change, related processes, such as protein glutathionylation or *S*-nitrosylation that are also implicated in the regulation of defense against pathogens and herbivores, are affected (Wünsche et al., 2011; Espunya et al., 2012).

In response to caterpillar herbivory, the active form of JA, (+)-7-iso-jasmonoyl-L-isoleucine, bridges JA ZIM-domain (JAZ) proteins with the E3 ubiquitin ligase SCF^{COI1} complex, resulting in the proteasome-mediated degradation of JAZ and release of the basic helix-loop-helix transcription factor MYC2, responsible for the expression of JA-associated genes, such as *VSP2* and *LOX2* (Lorenzo et al., 2004; Dombrecht et al., 2007; Kazan and Manners, 2008; Fonseca et al., 2009; Robert-Seilaniantz et al., 2011). Caterpillar herbivory-related increases in ET biosynthesis may modulate these JA responses through cross-talk between the JA-dependent MYC2-branch and ET-dependent branches (Stotz et al., 2000; Bodenhausen and Reymond, 2007; Kazan and Manners, 2008; Diezel et al., 2009; Robert-Seilaniantz et al., 2011; Verhage et al., 2011). Two APETALA2/ERF transcription factors, ET response factor1 (ERF1) and ORA59 integrate ET cross-talk with the JA pathway (Penninckx et al., 1998; Lorenzo et al., 2003; Pré et al., 2008); though both these branches are induced by ET, evidence points to them being parallel and, perhaps, functionally redundant. Together, the MYC2 and ET pathways, ORA59/ERF1, act synergistically or antagonistically allowing the integration of temporal and spatial hormone concentrations and localization to generate a specific signal signature (Kazan and Manners, 2008; Robert-Seilaniantz et al., 2011).

Effectors in the caterpillar LS may also activate the SAR pathway leading to the attenuation of JA-dependent responses (Kazan and Manners, 2008; Weech et al., 2008; Leon-Reyes et al., 2010; Verhage et al., 2011). In recognition of attack by biotrophic pathogens, plants mount the systemic defense response, SAR, initiated by increases in cellular SA and H_2O_2 that positively impact each other's production (Rao et al., 1997; Glazebrook, 2005; Mateo et al., 2006). The resultant change in glutathione redox balance results in the activation of the non-expressor of PR-genes1 (NPR1) through thioredoxin-catalyzed reduction of the disulfide bridges, changing the protein from its cytosolic oligomer form to the monomer that enters the nucleus (Spoel et al., 2009; Noctor et al., 2012). Association of NPR1 with TGA transcription factors leads to the expression of pathogenesis-related genes, such as *PR1*. The mechanistic basis of the antagonism between SA and JA pathways is still debated (Lorenzo and Solano, 2005). Early evidence suggests that SA interferes directly with JA biosynthesis (Doares et al., 1995; Rayapuram and Baldwin, 2007). However, NPR1 has been shown to be interfere with JA signaling downstream of JA biosynthesis (Mou et al., 2003; Spoel et al., 2003; Ndamukong et al., 2007; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Tada et al., 2008; Leon-Reyes et al., 2010). This may reflect the observation that ET modifies SA/NPR1 inhibition of JA responses such that in the presence of ET, the attenuation of JA-dependent gene expression is NPR1-independent; however, in the absence of ET, NPR1 is necessary to interfere with these responses (Leon-Reyes et al., 2009). Weech et al. (2008) used *Arabidopsis* mutants to show that caterpillar LS interference of JA-dependent plant defenses by activation of the SAR pathway requires an active NPR1. In addition, Diezel et al. (2009) showed that damage of wild tobacco by caterpillars of the tobacco hornworm, *Manduca sexta*, result in an ET burst that attenuates the SA-mediated suppression of plant defense responses. Therefore, in plant–caterpillar interactions, there appears to be extensive interplay between JA, SA, and ET pathways.

The present research is designed to understand the potential role of cellular redox balance in the ability of caterpillar LS to interfere with host plant defense responses. Since caterpillar LS contains redox enzymes, such as GOX that generate H_2O_2, caterpillar saliva should perturb the redox state or balance even more than mere wounding (Eichenseer et al., 2010; Noctor et al., 2012). By using normal caterpillars with intact LS secretions or insects where LS secretions have been impaired by cauterization of the spinneret, one can tease out the effect of LS on the modulation of plant responses. Therefore, in response to herbivory by caterpillars with intact or impaired LS secretions, the redox metabolites glutathione and ascorbate were measured to identify the impact of LS on cellular redox balance. As well, transcript responses of JA-, ET-, and SA-dependent marker genes were compared in wildtype plants and two mutant lines, *pad2-1*, compromised in glutathione biosynthesis, and a *tga2/5/6* triple mutant that is deficient in the basic leucine zipper TGA transcription factors that interact with NPR1 (Zhang et al., 2003; Parisy et al., 2007).

MATERIALS AND METHODS

PLANTS

Arabidopsis seeds ecotype Col-0 (TAIR CS3749) and the *pad2-1* mutant (TAIR CS3804) were obtained from the Arabidopsis Biological Resource Centre (Ohio State University). Seeds of the *Arabidopsis tga2/5/6* triple mutant were a generous gift from Dr. Li (University of British Columbia).

For redox metabolite experiments, wildtype plants seeds were surface-sterilized by soaking them for 2 min in 70% ethanol, followed by 5 min in 50% bleach. Seeds were rinsed three times in sterile distilled water and sown in Premier Promix BS (Premier Horticulture Inc.). After cold treatment at 4°C for 3 days, seeds were transferred into a growth cabinet (light intensity 140 $\mu Em^{-2}s^{-1}$, 12:12 light:dark at 22°C). Plants were bottom-watered as needed, about three times per week with dilute 0.15 g/L N–P–K fertilizer.

For gene expression experiments, seeds were surface-sterilized as described above and germinated on half-strength Murashige and Skoog (MS) media with 1% agar. After cold treatment for 3 days at 4°C, seeds were placed in the growth cabinet

and transferred to Agro-Mix at germination. At 5 weeks post-germination, one plant from each genotype (Col-0, *pad2-1* and *tga2/5/6*) were transplanted into a 12.5 cm × 12 cm pot.

Approximately 6- to 7-week old plants in the late vegetative growth stage, between growth stages 3.7 and 3.9 according to Boyes et al. (2001), were used in redox metabolite or gene expression experiments.

CATERPILLARS

Beet armyworm, *Spodoptera exigua* (Hübner; Lepidoptera: Noctuidae), insects were reared for multiple generations from eggs purchased from Bio-Serv (Frenchtown, NJ, USA). Insects were reared under defined conditions in a growth cabinet (16:8 light:dark, RH 28-40%, temperature 28.5°C) and fed a wheat germ-based artificial diet (Bio-Serv). Adult moths were allowed to mate and the eggs collected to maintain the colony.

IMPAIRMENT OF CATERPILLAR LS SECRETIONS

Caterpillar LS is secreted through a specialized organ, the spinneret (Musser et al., 2002). To impair LS secretions, this spinneret was cauterized as previously described (Musser et al., 2002; Bede et al., 2006). Prior to the experiment, caterpillars were allowed to feed > 12 h on *Arabidopsis* plants to allow the insects to adjust to the plant diet.

MEASUREMENT OF REDOX METABOLITES

Leaf H_2O_2 levels were not measured directly due to the high variability associated with the instable nature of this compound and confounding effects by high leaf phenolic content and ascorbate (Queval et al., 2008). Therefore, other metabolites associated with the ascorbate/glutathione cycle were measured since they closely correlate with H_2O_2 levels (Ng et al., 2007). Six-week-old *Arabidopsis* plants were subject to one of three treatments: untouched (control) or subject to herbivory by 3 × 4th instar *S. exigua* caterpillars with intact or impaired salivary secretions. As *S. exigua* caterpillars feed most actively at night, experiments were performed during the dark to more accurately simulate an ecological scenario. Rosette leaves showing signs of herbivory were harvested at 5, 15, 25, 35, and 45 min and immediately frozen in N_2. The experiment was repeated thrice.

At each time point, ascorbate and glutathione were measured in three to four independent samples. Plant samples were finely ground in liquid nitrogen and extracted in 0.2 N HCl at a ratio of 100 mg leaf/mL acid. This was followed by neutralization with NaOH as described in Queval and Noctor (2007). Chemicals used in redox metabolite assays were purchased from Sigma Chemical Company.

Ascorbate

Total, oxidized and reduced ascorbate from the leaf extract supernatant were determined by measuring reduced ascorbate levels spectrophotometrically at A_{265} using an Infinite M200 Pro microplate reader (Tecan) according to Queval and Noctor (2007). Total ascorbate was measured by converting dehydroascorbate (DHA) to the reduced form by incubating the supernatant in dithiothreitol (0.4% v/v) in 67.2 mM sodium phosphate (NaH_2PO_4) buffer, pH 7.5 for 30 min at room temperature. Triplicates of each sample were incubated with ascorbate oxidase (0.2 U) and reduced ascorbate was measured after an 8 min incubation. Reduced ascorbate (ASc) levels were measured by adding ascorbate oxidase to the neutralized leaf extract supernatant in 0.1 M sodium phosphate buffer, pH 5.6, incubating at room temperature for 30 min and analyzing as above. Concentrations were determined from a six-point L-ascorbate standard curve (40–240 μM). Oxidized ascorbate levels (DHA) were calculated by subtracting reduced from total ascorbate.

Glutathione

Measurement of glutathione is based on a recycling assay (Rahman et al., 2006; Queval and Noctor, 2007); glutathione reductase, in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), catalyzes the reduction of GSSG to GSH that reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) forming 5-thio-2-nitrobenzoic acid (TNB) that can be measured spectrophotometrically at A_{412}. Total glutathione was measured by incubating leaf supernatant in 0.6 mM DTNB and glutathione reductase (0.015 U) in 0.1 M sodium phosphate (NaH_2PO_4) buffer, pH 7.5. After the addition of 0.5 mM β-NADPH, the TNB chromophore was monitored at A_{412} at 5 s intervals for the first 2 min. Total glutathione concentration was calculated based on triplicate eight-point standard curve (100 nM to 60 μM). Oxidized glutathione (GSSG) was measured by removing any reduced GSH from the sample by precipitation with 2-vinylpyridine followed by conversion of GSSG to GSH and measurement using the glutathione reductase/β-NADPH/DTNB method as described above (Griffith, 1980; Rahman et al., 2006; Queval and Noctor, 2007). Briefly, leaf supernatant was incubated with 1 μl 2-vinylpyridine (approximately 10-fold above GSH levels) for 30 min at room temperature. After centrifugation at 13,000 rpm for 5 min to remove excess 2-vinylpyridine, samples were diluted in 0.1 M sodium phosphate buffer, pH 7.5 and assayed in triplicate. GSSG levels were determined from a triplicate eight-point GSSG standard curve ranging from 100 nM to 3.2 μM. Reduced GSH was calculated by subtracting 2 × GSSG from total glutathione.

GENE EXPRESSION

Three days before the herbivory experiment, clear plastic bottles were placed around the plants with mesh covering the tops. *Arabidopsis* plants were subject to one of three treatments: untouched (control) or subject to herbivory for 36 h by 6 × 4th instar *S. exigua* caterpillars with intact or impaired salivary secretions. The experiment was repeated twice; at each time point, two independent samples were taken for gene expression analysis (total $n = 3$–4).

RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction

Plants were finely ground in liquid nitrogen and total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocols. After DNase treatment (Wipeout, QuantiTect Reverse Transcription kit; Qiagen), the absence of genomic contamination was confirmed using 5′-ATG GGT CGT CAT CAG ATT CAG AGC AGA TAA-3′ and 5′-CAT ATA AGA GGT GTG TTA GAG ACA ATA ATA-3′ primers which span an intron (Weech et al., 2008). One microgram of RNA was converted to

cDNA using a QuantiTect Reverse Transcription Kit following the manufacturer's instructions.

Gene-specific primers were identified from the literature or designed using Primer3 (**Table A1** in Appendix). Transcript expression was analyzed in duplicate using the Brilliant One-Step quantitative RT-PCR kit (Stratagene), according to the manufacturer's protocol, in a Mx3000p thermocycler (Stratagene). Gene amplicon products were verified by sequencing. Each 96-well plate, contained a standard curve of the gene-of-interest, a non-template control and each sample in duplicate. Each reaction contained 1× SYBR green I, 0.375 nM ROX, 100 nM of the forward and reverse primer, mastermix that contained dNTPs, $MgSO_4$ and *Taq* polymerase, and either water (non-template control), serial dilutions of PCR amplicon (standard curve) or 85 ng cDNA sample. Standard curves ensured an efficiency of between 90 and 110%. Thermocycler conditions are as follows: 95°C for 10 min; 40 cycles of denaturation at 95°C for 45 s, annealing for 1 min, and elongation at 70°C for 45 s. The annealing temperature was dependent on the primers used (**Table A1** in Appendix). Dissociation curves were performed to ensure amplicon purity. Two technical plate replicates were performed.

From the standard curve, gene copy numbers were estimated and normalized against the constitutive reference gene *AtACT2* (At3g18780). *Arabidopsis AtACT2* expression was not affected by osmotic stress or when plants were treated with viral pathogens or stress-related hormones, such as methyl JA or SA, or caterpillar herbivory (Stotz et al., 2000; Dufresne et al., 2008; Weech et al., 2008). In the current study, *AtACT* was stably expressed within a genotype and not affected by treatment [+/+: $F_{(2,9)} = 0.26$, $p = 0.77$; *pad2-1*: $F_{(2,9)} = 1.10$, $p = 0.37$; *tga2/5/6*: $F_{(2,7)} = 0.42$, $p = 0.68$; Brunner et al., 2004].

STATISTICAL ANALYSIS

For the redox experiment (repeated independently three times, $n = 5$–10), statistical differences ($p \leq 0.05$) in metabolite levels were determined using a two-way analysis of variance (ANOVA) using SPSS version 20 (SPSS Inc.). If a significant time × treatment factor was observed, a one-way ANOVA followed by a Tukey HSD (honestly significant difference) *post hoc* test was conducted to identify the significant difference. The gene expression experiment was repeated twice with two independent biological samples analyzed at each time (total $n = 3$–4). Within each genotype, transcript expression was analyzed by a one-way ANOVA. Statistical differences ($p < 0.05$) were determined using a Tukey HSD *post hoc* test (Rieu and Powers, 2009). Alternatively, because of the variation inherent with insect feeding studies, a greater than five-fold change in gene expression with respect to control plants was also considered significantly different. Results from statistical analyses are shown in **Table A2** in Appendix.

RESULTS AND DISCUSSION

ASCORBATE–GLUTATHIONE CYCLE

The ascorbate–glutathione cycle is critical to enable the plant to maintain cellular redox status during stresses, such as insect herbivory (Noctor et al., 2012). Oxidative stress, such as increased H_2O_2 levels, may result in either an increase in the levels of total glutathione (glutathione pool) or increased levels of GSSG relative to GSH (redox balance; Noctor et al., 2012). Total ascorbate levels were within the reported physiological range and did not change over the 45 min time course and was independent of treatment (**Figure 1A**; **Table A2** in Appendix; Queval and Noctor, 2007). Oxidized and reduced ascorbate levels and the ratio of oxidized ascorbate (DHA)/reduced ascorbate (ASc) did not change in response to caterpillar herbivory. Total glutathione levels were within the expected physiological range and affected by treatment (**Figure 1B**; **Table A2** in Appendix; Queval and Noctor, 2007). Caterpillar herbivory did not affect the oxidized GSSG/reduced GSH ratio but total glutathione levels are lower in plants infested with caterpillars with impaired salivary secretions compared to the control. This likely reflects the reduced glutathione levels found in this treatment. Caterpillar herbivory also had significantly lower oxidized GSSG levels at 35 min post-herbivory; this effect was not salivary-dependent.

Cellular glutathione–ascorbate metabolites levels and/or redox balance are involved in plant defense against pathogens or herbivores (Mou et al., 2003; Ball et al., 2004; Parisy et al., 2007; Schlaeppi et al., 2008; Wünsche et al., 2011; Espunya et al., 2012). The majority of experiments investigating changes in redox metabolites in response to stress (wound, herbivory, pathogens) characterize long-term changes in the cellular oxidative status (Fodor et al., 1997; Mou et al., 2003; Ball et al., 2004; Mateo et al., 2006; Schlaeppi et al., 2008; Gfeller et al., 2011). In this study, we are interested in early changes in cellular antioxidant levels or redox balance (ratio) to caterpillar herbivory that may lead to changes in gene expression. The difficulty in this short-term experiment is to synchronize the initiation and intensity of insect herbivory. Lou and Baldwin (2006) and this study monitored redox metabolites within the first 45 min after the initiation or simulation of herbivory. Lou and Baldwin (2006) noted an increase in H_2O_2 levels 30 min after wounding and application of *Manduca sexta* caterpillar regurgitant on *Nicotiana attenuata* leaves. In response to biotrophic pathogens, an increase in total or reduced glutathione levels leads to reduction and activation of NPR1 (Fodor et al., 1997; Mou et al., 2003; Fobert and Després, 2005; Mateo et al., 2006); even though SA injection into leaves shows a transient oxidation of the glutathione pool. In comparison, after wounding, the GSSG/total glutathione ratio increased leading to an activation of the JA pathway (Mhamdi et al., 2010; Gfeller et al., 2011).

Cellular redox changes occur in response to mechanical damage during insect feeding. However, noctuid caterpillar LS, that has been implicated as a stratagem to delay the induction of plant defenses, contains numerous enzymes that may affect cellular redox balance, most notably the H_2O_2-producing enzyme GOX (Musser et al., 2002; Weech et al., 2008; Eichenseer et al., 2010). Compared to controls, herbivory by caterpillars with intact salivary secretions did not affect cellular redox balance except for a transient decrease in oxidized GSSG at 35 min (**Figure 1B**). In comparison, reduced glutathione levels were lower in leaves subject to herbivory by caterpillars with impaired salivary secretion compared to controls, indicating oxidative stress. This suggests that the production of H_2O_2 by enzymes in the caterpillar LS may act to maintain cellular GSH levels so glutathione does not act further as a signaling molecule (Szalai et al., 2009).

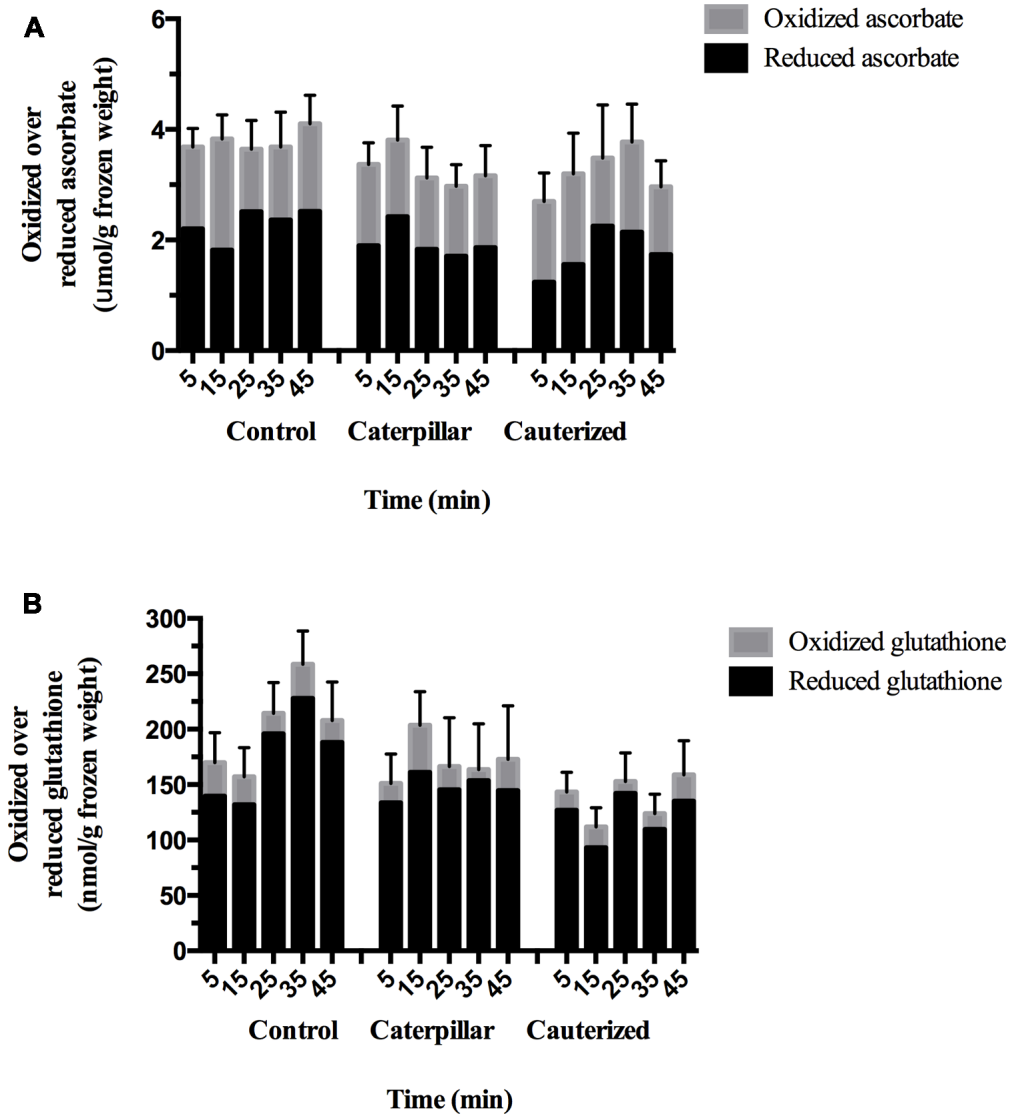


FIGURE 1 | Time course of redox metabolites in *Arabidopsis* plants subject to caterpillar herbivory. (A) Ascorbate levels. Foliar ascorbate levels in *Arabidopsis* plants subject to herbivory by caterpillars with normal (caterpillar) or impaired salivary secretions (cauterized) compared to control plants. Solid bars represent reduced ascorbate (ASc) levels. Open bars represent oxidized ascorbate (dehydroascorbate, DHA) levels. Values are given in μmol/g frozen weight (FW) and represent means ± SE of three to four independent biological replications. Significant differences in ascorbate were not observed in response to caterpillar herbivory. **(B)** Glutathione levels. Foliar glutathione levels in *Arabidopsis* plants subject to herbivory by caterpillars with normal (caterpillar) or impaired salivary secretions (cauterized) compared to control plants. Solid bars represent reduced glutathione (GSH) levels. Open bars represent oxidized glutathione (GSSG) levels. Values are given in nmol/g FW and represent means ± SE of three to four independent biological replications. Significant differences were determined by two-way ANOVA (**Table A2** in Appendix). At 35 min post-herbivory, a significant reduction in GSSG levels are observed in plants infested by caterpillars, both with normal or impaired salivary secretions, compared to controls. Total and reduced glutathione levels are significantly reduced in caterpillar with impaired salivary secretions compared to control levels.

TRANSCRIPT EXPRESSION IN RESPONSE TO CATERPILLAR HERBIVORY

To explore the link between cellular redox balance and plant responses to caterpillar LS, expression of JA-, ET-, and SA-dependent gene markers were analyzed in wildtype, *pad2-1* mutants, that contain only about 20% of normal glutathione levels, and the *tga2/5/6* triple mutant (Zhang et al., 2003; Parisy et al., 2007). Together with NPR1, TGA transcription factors are activated by a change in redox balance and responsible for SA-dependent gene expression (Després et al., 2003; Mou et al., 2003; Lindermayr et al., 2010). It must, however, be noted that the TGA transcription factors have also been shown to regulate a subset of oxylipin-dependent defensive gene expression (Mueller et al., 2008; Zander et al., 2010).

Jasmonate, SA, and ET play central roles in mediating the plant's response to caterpillar herbivory (**Figure 3**; Weech et al., 2008; Diezel et al., 2009; Onkokesung et al., 2010). Pré et al. (2008) recently suggested that the transcription factors ORA59 and ERF1 act in parallel pathways to integrate these JA/ET responses.

How caterpillar LS manages to manipulate these JA/ET pathways is unknown, but Weech et al. (2008) proposed that caterpillar LS requires an active SA/NPR1 pathway for this strategem. To further complicate issues, recent evidence suggests that ET potentiates SA antagonism with JA and renders it NPR1-independent (Leon-Reyes et al., 2009).

Pathogenesis-related 1 (*AtPR1*, At2g14610) is a SA-responsive, NPR1-dependent gene marker induced in response to biotrophic pathogen attack and aphid feeding (Glazebrook, 2005; Mur et al., 2006; Kusnierczyk et al., 2007; Walling, 2008; Zhang et al., 1999). In our study, *AtPR1* gene expression was greater than fivefold higher in plants infested by caterpillars with intact LS secretions compared to caterpillars with cauterized spinnerets and control plants, indicating that caterpillar LS secretions result in the activation of SA/NPR1-dependent gene expression (**Figure 2A**; **Table A2** in Appendix). Through activation of the SA pathway by effectors in their LS secretions, *S. exigua* caterpillars are believed to impair the plant's ability to fully mount a JA-dependent defense response (Weech et al., 2008). Mewis et al. (2006) also observed *AtPR1* expression in *Arabidopsis* response to herbivory by caterpillars of *P. rapae* and *S. exigua*; both these caterpillar LS glands contain redox enzymes, such as GOX (Eichenseer et al., 2010). The increase in *AtPR1* expression was alleviated in *pad2-1* and *tga2/5/6* mutant plants, in line with previous studies showing that glutathione and the TGA transcription factors are upstream signals in *AtPR1* expression (Després et al., 2003; Mou et al., 2003; Lindermayr et al., 2010).

Expression of the gene encoding plant defensin, *AtPDF1.2b* (At2g26020), is induced by treatment of plants with JA and ET working synergistically through ORA59 (Penninckx et al., 1998; Pré et al., 2008); however, antagonism between MYC2 and ERF1 regulation of *AtPDF1.2* is proposed to reflect MYC2 regulation of *ERF1* expression (Dombrecht et al., 2007). As well, SA-dependent suppression of *AtPDF1.2* expression

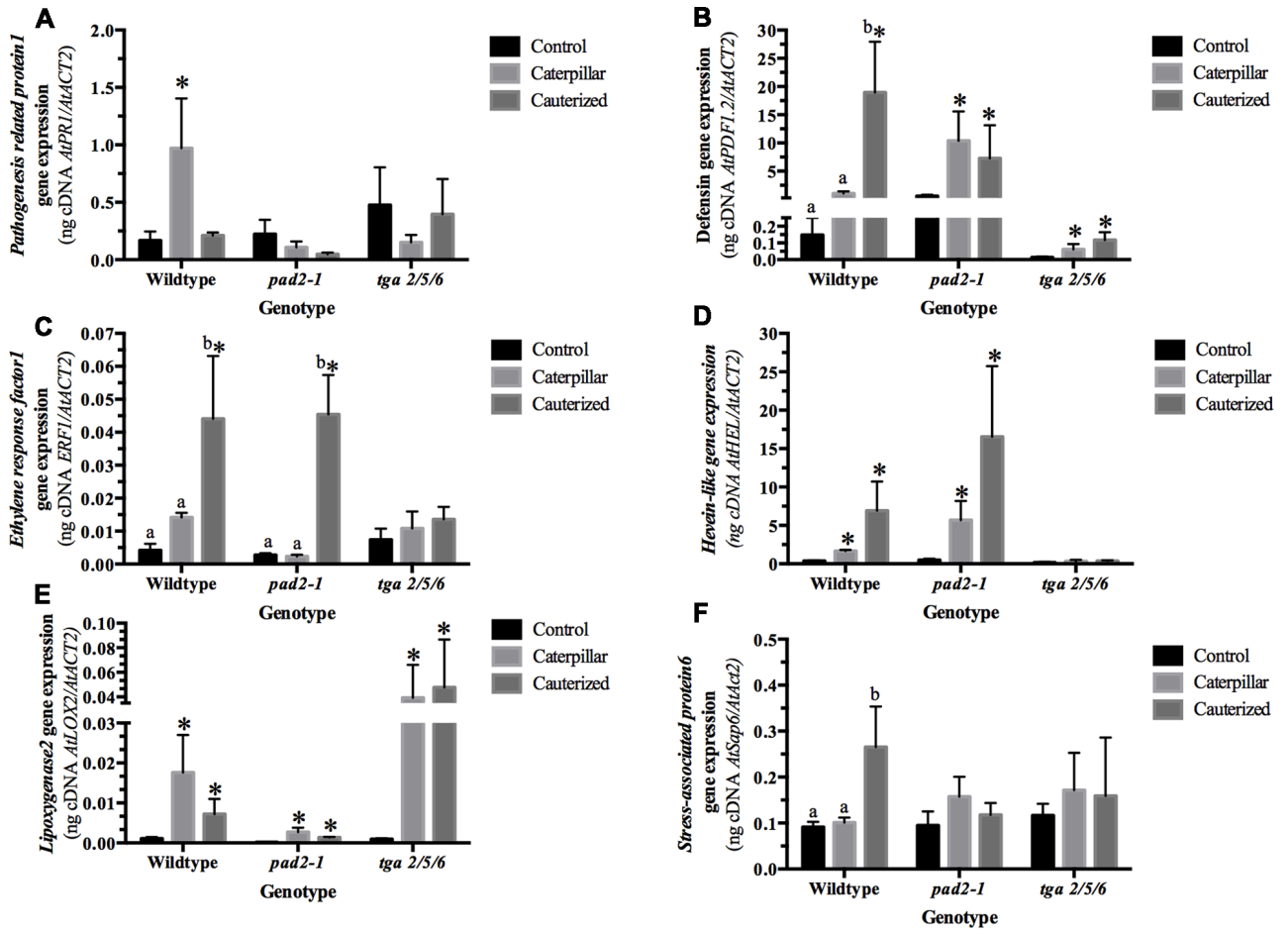


FIGURE 2 | *Arabidopsis* transcript expression in response to caterpillar herbivory analyzed by quantitative real-time reverse transcription PCR. Seven-week-old *Arabidopsis* plants (Col-0, +/+), *pad2-1* mutant (*pad2-1*), and *tga2/5/6* triple mutant (*tga*) were untreated (control) or subject to herbivory by caterpillars with normal (intact) or impaired (cauterized) salivary secretions for 36 h. From cDNA generated from total RNA, gene-specific primers were used to determine expression levels of **(A)** *AtPR1*, **(B)** *AtPDF1.2*, **(C)** *ERF1*, **(D)** *AtHEL*, **(E)** *AtLOX2*, **(F)** *AtSAP6*. Bars represent the mean values of three to four independent replicates normalized with the reference gene *AtACT2* ± SE. Within each genotype, lower case alphabetical letters indicate significant differences identified by ANOVA followed by a Tukey HSD ($p \leq 0.05$; n = 3–4; **Table A2** in Appendix). An asterisk denotes a fivefold or higher change in expression from control levels.

requires active NPR1 and TGA transcription factors (Spoel et al., 2003; Ndamukong et al., 2007; Koornneef et al., 2008). ET modulates this SA–JA antagonism; NPR1-dependent antagonism of the expression of JA-dependent genes, such as *AtPDF1.2*, becomes NPR1-independent in the presence of ET (Leon-Reyes et al., 2009).

In wildtype plants, an 18-fold increase in *AtPDF1.2* transcript expression is observed in response to herbivory by caterpillars with impaired salivary secretions compared to normal caterpillars or control plants, in agreement with previous studies that caterpillar LS suppresses JA-dependent plant defenses (**Figure 2B**; **Table A2** in Appendix; Musser et al., 2002; Weech et al., 2008). In *pad2-1* and *tga2/5/6* mutants, LS-mediated restraint of *AtPDF1.2* expression is not observed, indicating that glutathione and TGA transcription factors are required for the suppression of plant induced defenses by caterpillar herbivory. In *pad2-1* mutants, a 12.5-fold increase in *AtPDF1.2* levels is seen in plants infested by caterpillars compared to controls. The lower glutathione levels in the *pad2-1* mutant may impair the activation of a pathway, such as the reduction of NPR1 and/or TGA transcription factors, which are needed for the LS-mediated suppression of plant defenses (Mou et al., 2003; Fobert and Després, 2005). A fivefold increase in *AtPDF1.2* expression is seen in plants fed upon by caterpillars compared to controls in the *tga2/5/6* mutant plants. However, it must be noted that TGA transcription factors also regulate the late expression (∼48 h) of a subset of JA-dependent genes, such as *AtPDF1.2* (Zander et al., 2010). Perhaps, a strong difference in gene expression between normal and cauterized caterpillars is not observed because of the requirement for TGA transcription factors, although a fivefold increase in expression is observed in caterpillar-infested *tga2/5/6* mutants compared to controls. These results suggest that caterpillar LS-dependent suppression of JA-mediated activation of *AtPDF1.2* gene expression is dependent on glutathione levels and, perhaps, the activation of TGA transcription factors.

In wildtype plants, results correlate with previous observations that glutathione negatively regulates *AtPDF1.2* expression (Koornneef et al., 2008); we also observed that wildtype plants infested by caterpillars with impaired salivary secretions had lower reduced glutathione compared to controls and, consequently, higher *AtPDF1.2* expression (**Figures 1B** and **2B**). Also, the LS-associated negative regulation of *AtPDF1.2* is alleviated in the *pad2-1* mutant. Our observation that this LS-mediated suppression of *AtPDF1.2* is lessened in the *tga2/5/6* triple mutant supports observations that suppression of *AtPDF1.2* gene expression requires the interaction of glutaredoxin480 with TGA transcription factors (Ndamukong et al., 2007; Koornneef and Pieterse, 2008; Zander et al., 2011). ET also plays a role in modulating the mechanism of SA/NPR1 inhibition of JA-dependent responses (Leon-Reyes et al., 2009); in the presence of ET, this suppression becomes NPR1-independent. However, given the links to glutathione and, possibly TGA transcription factors, and previous research, our data points to a LS-mediated NPR1-dependent inhibition of *AtPDF1.2* gene expression (Weech et al., 2008).

Alternatively, current models propose that JA-dependent inhibition of *AtPDF1.2* expression may be mediated through the negative regulation of *ERF1* (At3g23240) by MYC2 (Dombrecht et al., 2007; (Zander et al., 2010). Therefore, *ERF1* expression was measured to determine if it was mirrored by *AtPDF1.2* expression. As seen with *AtPDF1.2*, a significant increase in *Arabidopsis ERF1* transcript expression is observed in response to herbivory by caterpillars with impaired LS secretions compared to normal caterpillars or control plants (**Figures 2B,C**; **Table A2** in Appendix); however, this LS-mediated suppression of ERF1 is also observed in the *pad2-1* mutants. The distinct patterns between *AtPDF1.2* and *ERF1* expression suggest LS-mediated regulation is likely not reflective of MYC2 antagonism of *ERF1*; however, they suggest that there may be LS-linked, an ET, glutathione-independent mechanism of suppression. LS-suppression of *ERF1* is alleviated in the *tga2/5/6* triple mutant. Zander et al. (2010) found that TGA transcription factors may suppress *ERF1* expression.

Hevein-like (*AtHEL*, PR4, At304720) gene expression is a marker of the ORA59 branch of the JA/ET signaling pathways (Potter et al., 1993; Dombrecht et al., 2007; Pré et al., 2008; Verhage et al., 2011; Zarei et al., 2011). In comparison to *AtPDF1.2*, suppression of JA-linked *AtHEL* expression by the SA pathway is NPR1-independent (Ndamukong et al., 2007). In wildtype and *pad2-1* mutant plants, over a fivefold increase in gene expression is observed in plants infested by caterpillars compared with controls (**Figure 2D**); however, a LS effect is not observed (**Table A2** in Appendix). These results support the argument that caterpillar LS-mediated suppression of induced plant defenses is glutathione- and NPR1-dependent. Unexpectedly, this caterpillar-mediated *AtHEL* expression was at basal levels in the *tga2/5/6* triple mutant plants, suggesting that these transcription factors may be involved in regulation of *AtHEL* expression.

The gene encoding *lipoxygenase2* (*AtLOX2*, At3g45410) is an early expression marker of the JA-responsive MYC2 branch (Bell et al., 1995; Dombrecht et al., 2007). As has been observed previously, *AtLOX2* levels are induced sevenfold in response to insect herbivory and a LS gland-specific difference in gene expression is not observed (**Figure 2E**; **Table A2** in Appendix; Weech et al., 2008). This same pattern was observed in *pad2-1* and *tga2/5/6* mutant plants. Though regulated by MYC2, the strong upregulation of this early gene occurs before SA/NPR1-mediated cross-talk (Mou et al., 2003; Spoel et al., 2003; Ndamukong et al., 2007; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Tada et al., 2008; Leon-Reyes et al., 2010). As well, LS-associated post-transcriptional modifications of LOX2 may regulate activity rather than gene expression (Thivierge et al., 2010).

The stress-associated *AtSAP6* (At3g52800) was induced in plants fed upon by caterpillars with impaired LS secretions compared to controls (**Figure 2F**; **Table A2** in Appendix). This difference was alleviated in the *pad2-1* and the *tga2/5/6* triple mutants indicating the possible involvement of glutathione and TGA transcription factors in the regulation of expression of this gene. *AtSAP6* is strongly induced in response to numerous stresses, such as wounding and herbivory by caterpillars of the specialist *P. rapae* (Reymond et al., 2004; Ströher et al., 2009); however, in response to herbivory, this transcript was induced in both the wildtype and the JA-perception impaired *coi1-1 gl1* mutant implying that JA signaling is not required for the expression of this gene.

CONCLUSION

Plant responses to insect herbivory are mediated through carefully regulated, complex hormone-mediated interactions. Herbivory by *S. exigua* caterpillars attenuate these JA-dependent plant defense responses; a mechanism believed to be related to LS-associated secretions (Musser et al., 2002; Weech et al., 2008). Given the presence of GOX in the LS of this caterpillar, the relationship between LS secretions and changes in cellular redox potential was investigated. Changes in cellular oxidative stress and, in particular, the GSSG/total glutathione ratio are signals for the induction of JA-dependent defenses (Szalai et al., 2009; Gfeller et al., 2011). Herbivory by caterpillars with intact salivary secretions did not affect cellular redox balance, except for a transient decrease in oxidized GSSG at 35 min (**Figure 3**). In comparison, herbivory by caterpillars with impaired salivary secretions resulted in an increase in cellular oxidative status through a decrease in reduced glutathione levels. In support of this, genes, such as *AtPR1* and *AtPDF1.2*, showed LS-dependent transcript expression that was alleviated in the *pad2-1* and *tga2/5/6* triple mutant (**Figures 2A,B** and **3**).

Increased expression of *AtPR1* by herbivory using caterpillars with intact salivary secretions support the notion that LS-mediated attenuation of JA responses acts through cross-talk with the SA/NPR1 pathway (**Figure 3**). As well, even though *AtPDF1.2* is a JA/ET marker, recent studies have shown that mid- to late-gene expression is regulated by TGA transcription factors (Zander et al., 2011). Therefore, suppression of *AtPDF1.2* gene expression by caterpillar LS may also reflect cross-talk between JA- and SA/NPR1 pathways. The LS-associated modulation of ET-dependent genes, *ERF1* and *AtHEL*, show disparate regulation since *ERF1* expression shows glutathione-independent, LS-associated suppression whereas a LS-dependent difference in *AtHEL* is not observed (**Figures 2C,D**). This may support recent evidence that the ET pathway is mediated through two distinct branches regulated by

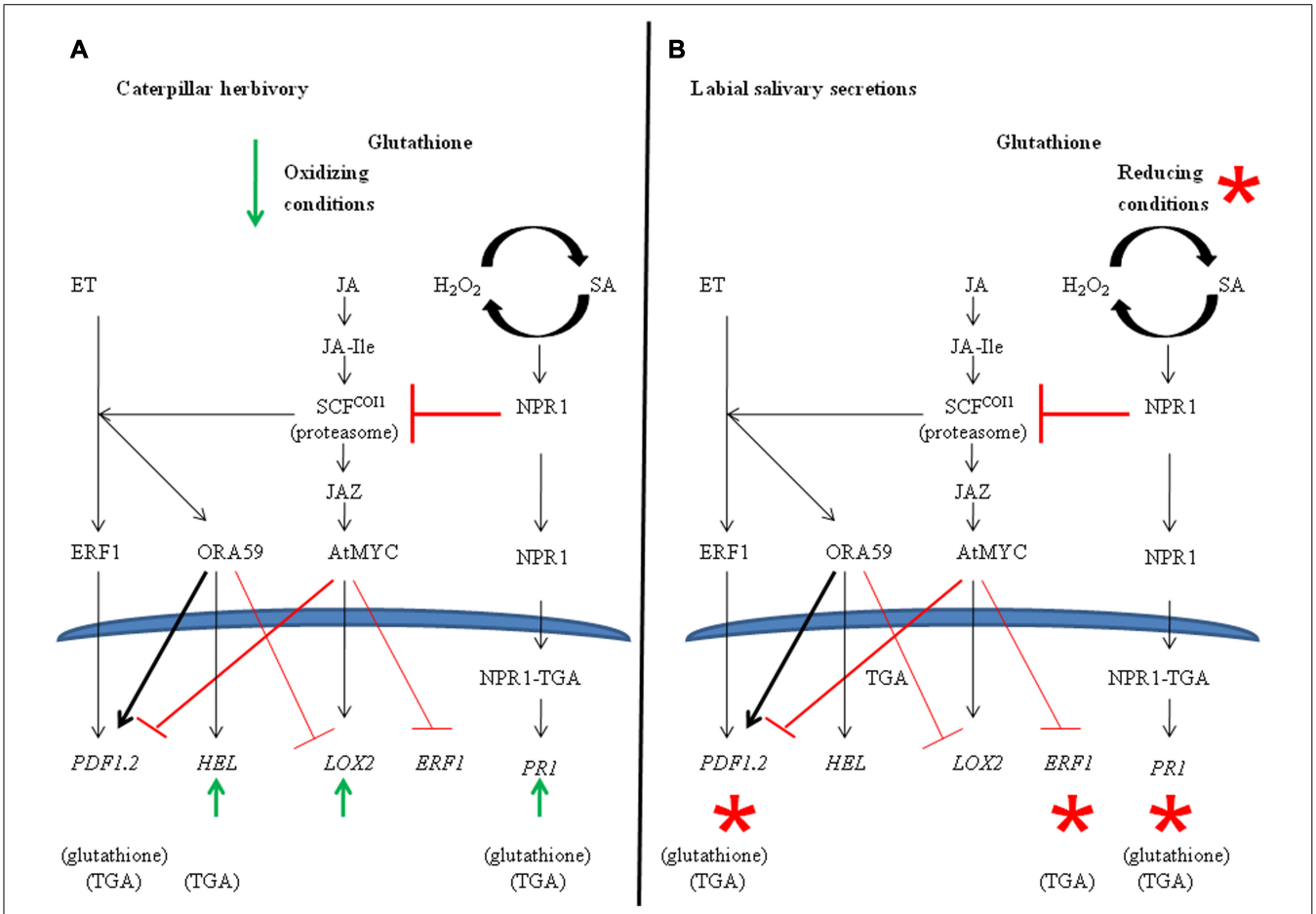


FIGURE 3 | Model of ethylene-, jasmonate-, and salicylate-dependent pathway illustrating major cross-talk signaling nodes and marker genes. **(A)** Changes in redox metabolites and gene expression in response to caterpillar herbivory. Illustrates caterpillar herbivory-dependent changes compared to control plants. Cellular GSSG, which is linked to the induction of JA-defenses, decrease transiently 35 min after caterpillar herbivory. Markers of the SA, ORA59, and AtMYC2 pathway, respectively *AtPR1*, *AtHEL*, and *AtLOX2*, are induced in response to herbivory. **(B)** Proposed model caterpillar labial saliva (LS) mediated suppression of jasmonate-dependent responses. Illustrates LS-associated changes between plants fed on by caterpillars with intact vs. impaired salivary secretions. Asterisks indicate LS-associated changes. Herbivory by caterpillars with impaired salivary secretions result in cellular oxidative stress (lower total and reduced cellular GSH levels) compared to controls. Induction of the *AtPR1* and suppression of *AtPDF1.2* and *ERF1* are LS-dependent. Involvement of glutathione or TGA transcription factors are indicated in brackets underneath the marker gene.

ORA59 or ERF1 transcription factors (Pré et al., 2008). In fact, *Pieris rapae* caterpillar oral secretions, which are a mixture of gut-derived regurgitant, secretions from the ventral eversible gland and salivary secretions from the mandibular and labial glands, specifically activate the ORA59 branch of the JA/ET pathway leading to the suppression of MYC2-dependent defenses (Felton, 2008; Hogenhout and Bos, 2011; Verhage et al., 2011; Zebelo and Maffei, 2012). These caterpillars also show a feeding preference for plants that overexpress ORA59.

The mechanism behind this LS-mediated cross-talk may be explained by the model recently proposed by Van der Does et al. (2013). In an elegant set of experiments, these authors systematically demonstrated that suppression of the JA-induced pathway by the SA/NPR1 pathway occurs downstream of SCF^{COI1}-mediated protein degradation. Instead, the SA/NPR1 pathway negatively regulates the expression of *AtPDF1.2* by affecting the accumulation of the ORA59 transcription factor. Our data from this and previous studies also show that caterpillar LS-mediated suppression of *AtPDF1.2* is SA/NPR1 pathway mediated and does not appear to involve cross-talk between the ERF1 pathway (Weech et al., 2008); therefore, further studies investigating ORA59 protein levels in this plant–insect system needs to be investigated.

ACKNOWLEDGMENTS

We are grateful to Drs Guillaume Queval, Graham Noctor, and Christine Foyer for valuable discussions and advice with the optimization of experimental techniques to analyze redox metabolites. We thank two reviewers for their constructive improvements to an earlier version of this manuscript. This research was supported by operating grants from the National Sciences and Engineering Research Council to Jacqueline C. Bede.

REFERENCES

Arimura, G., Ozawa, R., and Maffei, M. E. (2011). Recent advances in plant early signaling in response to herbivory. *Int. J. Mol. Sci.* 12, 3723–3739.

Ball, L., Accotto, G. P., Bechtold, U., Creissen, G., Funck, D., Jimenez, A., et al. (2004). Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. *Plant Cell* 16, 2448–2462.

Bede, J. C., Musser, R. O., Felton, G. W., and Korth, K. L. (2006). Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. *Plant Mol. Biol.* 60, 519–531.

Bell, E., Creelman, R. A., and Mullet, J. E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 8675–8679.

Bodenhausen, N., and Reymond, P. (2007). Signaling pathways controlling induced resistance to insect herbivores in *Arabidopsis*. *Mol. Plant Microbe Interact.* 20, 1406–1420.

Boyes, D. C., Zayed, A. M., Ascenzi, R., McCaskill, A. J., Hoffman, N. E., Davis, K. R., et al. (2001). Growth stage-based phenotypic analysis of *Arabidopsis*: a model for high throughput functional genomics in plants. *Plant Cell* 13, 1499–1510.

Brunner, A. M., Yakovlev, I. A., and Strauss, S. H. (2004). Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol.* 4:14. doi: 10.1186/1471-2229-4-14

Conn, V. M., Walker, A. R., and Franco, C. M. M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 21, 208–218.

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., et al. (2003). The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *Plant Cell* 15, 2181–2191.

Diezel, C., von Dahl, C. C., Gaquerel, E., and Baldwin, I. T. (2009). Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiol.* 150, 1576–1587.

Doares, S. H., Narvaez-Vasquez, J., Conconi, A., and Ryan, C. A. (1995). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108, 1741–1746.

Dombrecht, B., Xue, G. P., Sprague, S. J., Kirkegaard, J. A., Ross, J. J., Reid, J. B., et al. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* 19, 2225–2245.

Dubreuil-Mauriza, C., Vitecek, J., Marty, L., Branciard, L., Frettinger, P., Wendehenne, D., et al. (2011). Glutathione deficiency of the *Arabidopsis* mutant pad2-1 affects oxidative stress-related events, defense gene expression, and the hypersensitive response. *Plant Physiol.* 157, 2000–2012.

Dufresne, P. J., Ubalijoro, E., Fortin, M. G., and Laliberte, J.-F. (2008). *Arabidopsis thaliana* class II poly(A)-binding proteins are required for efficient multiplication of turnip mosaic virus. *J. Gen. Virol.* 89, 2339–2348.

Eichenseer, H., Mathews, M. C., Powell, J. S., and Felton, G. W. (2010). Survey of a salivary effector in caterpillars: glucose oxidase variation and correlation with host range. *J. Chem. Ecol.* 36, 885–897.

Espunya, M. C., De Michele, R., Gómez-Cadenas, A., and Carmen Martínez, M. C. (2012). S-nitrosoglutathione is a component of wound- and salicylic acid-induced systemic responses in *Arabidopsis thaliana*. *J. Exp. Bot.* 63, 3219–3227.

Felton, G. W. (2008). "Caterpillar secretions and induced plant responses," in *Induced Plant Resistance to Herbivory*, ed. A. Schaller (Berlin: Springer-Scientific), 369–387.

Felton, G. W., and Korth, K. L. (2000). Trade-offs between pathogen and herbivore resistance. *Curr. Opin. Plant Biol.* 3, 309–314.

Fobert, P. R., and Després, C. (2005). Redox control of systemic acquired resistance. *Curr. Opin. Plant Biol* 8, 378–382.

Fodor, J., Gullner, G., Adam, A. L., Barna, B., Komives, T., and Kiraly, Z. (1997). Local and systemic responses of antioxidants to tobacco mosaic virus infection and to salicylic acid to tobacco. *Plant Physiol.* 114, 1443–1451.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Prozel, A., Kramell, R., et al. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nat. Chem. Biol.* 5, 344–350.

Forman, H. J., Maiorino, M., and Ursini, F. (2010). Signaling functions of reactive oxygen species. *Biochemistry* 49, 835–842.

Gfeller, A., Baerenfaller, K., Loscos, J., Chételat, A., Baginsky, S., and Farmer, E. E. (2011). Jasmonate controls polypeptide patterning in undamaged tissue in wounded *Arabidopsis* leaves. *Plant Physiol.* 156, 1797–1807.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207–212.

Hogenhout, S. A., and Bos, J. I. B. (2011). Effector proteins that modulate plant–insect interactions. *Curr. Opin. Plant Biol.* 14, 422–428.

Jirage, D., Zhou, N., Cooper, B., Clarke, J. D., Dong, X., and Glazebrook, J. (2001). Constitutive salicylic acid-dependent signaling in cpr1 and cpr6 mutants requires PAD4. *Plant J.* 26, 395–407.

Kazan, K., and Manners, J. M. (2008). Jasmonate signaling: toward an integrated view. *Plant Physiol.* 146, 1459–1468.

Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F. C., Van Loon, L. C., et al. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiol.* 147, 1358–1368.

Koornneef, A., and Pieterse, C. M. J. (2008). Cross talk in defense signaling. *Plant Physiol.* 146, 839–844.

Kusnierczyk, A., Winge, P., Midelfart, H., Armbruster, W. S., Rossiter, J. T., and Bones, A. M. (2007). Transcriptional responses of *Arabidopsis thaliana* ecotypes with different glucosinolate profiles

after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae*. *J. Exp. Bot.* 58, 2537–2552.

Leon-Reyes, A., Spoel, S. H., De Lange, E. S., Abe, H., Kobayashi, M., Tsuda, S., et al. (2009). Ethylene mediates the role of NONEXPRESSOR of PATHOGENESIS RELATED GENES1 in cross talk between salicylate and jasmonate signaling. *Plant Physiol.* 149, 1797–1809.

Leon-Reyes, A., Van der Does, D., De Lange, E. S., Delker, C., Wasternack, C., Van Wees, S. C. M., et al. (2010). Salicylate-mediated suppression of jasmonate-responsive gene expression in *Arabidopsis* is targeted downstream of the jasmonate biosynthesis pathway. *Planta* 232, 1423–1432.

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907.

Lorenzo, O., Chico, J. M., Sánchez-Serrano, J. J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* 16, 1938–1950.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signaling from ethylene and jasmonate pathway in plant defense. *Plant Cell* 15, 165–178.

Lorenzo, O., and Solano, R. (2005). Molecular players regulating the jasmonate signalling network. *Curr. Opin. Plant Biol.* 8, 532–540.

Lou, Y., and Baldwin, I. T. (2006). Silencing of a germin-like gene in *Nicotiana attenuata* improves the performance of native herbivores. *Plant Physiol.* 140, 1126–1136.

Maffei, M. E., Mithöfer, A., Arimura, G., Uchtenhagen, H., Bossi, S., Bertea, C., et al. (2006). Effects of feeding *Spodoptera littoralis* on lima bean leaves. III Membrane depolarization and involvement of hydrogen peroxide. *Plant Physiol.* 140, 1022–1035.

Mateo, A., Funck, D., Mühlenbock, P., Kular, B., Mullineaux, P. M., and Karpinski, S. (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *J. Exp. Bot.* 57, 1795–1807.

Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C., and Gershenzon, J. (2006). Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67, 2450–2462.

Mhamdi, A., Hager, J., Chaouch, S., Queval, G., Han, Y., Taconnat, L., et al. (2010). *Arabidopsis* GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. *Plant Physiol.* 153, 1144–1160.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 113, 935–944.

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Muelle, M. J., et al. (2008). General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. *Plant Cell* 20, 768–785.

Mur, L. A. J., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006). The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* 140, 249–262.

Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J. B., et al. (2002). Caterpillar saliva beats plant defenses: a new weapon emerges in the coevolutionary race between plants and herbivores. *Nature* 416, 599–600.

Ndamukong, I., Abdallat, A. A., Thurow, C., Fode, B., Zander, M., Weigel, R., et al. (2007). SA-inducible *Arabidopsis* glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. *Plant J.* 50, 128–139.

Ng, C. F., Schafer, F. Q., Buettner, G. R., and Rodgers, V. G. J. (2007). The rate of cellular hydrogen peroxide removal shows dependency on GSH: mathematical insight into in vivo H2O2 and GPx concentrations. *Free Radic. Res.* 41, 1201–1211.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., et al. (2012). Glutathione in plants: an integrated overview. *Plant Cell Environ.* 35, 454–484.

Onkokesung, N., Baldwin, I. T., and Gális, I. (2010). The role of jasmonic acid and ethylene crosstalk in direct defense of *Nicotiana attenuata* plants against chewing herbivores. *Plant Signal. Behav.* 5, 1305–1307.

Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., and Mauch, F. (2007). Identification of PAD2 as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *Plant J.* 49, 159–172.

Penninckx, E. A. M. A., Thomma, B. P. H. J., Buchala, A., Metraux, J.-P., and Broekaert, W. F. (1998). Concomitant activation of jasmonate and ethylene response pathway is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10, 2103–2113.

Potter, S., Ukes, S., Lawton, K., Winter, A. M., Chandler, D., Di Maio, J., et al. (1993). Regulation of a hevein-like gene in *Arabidopsis*. *Mol. Plant Microbe Interact.* 6, 680–685.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C. M. J., and Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol.* 147, 1347–1357.

Queval, G., Hager, J., Gakière, B., and Noctor, G. (2008). Why are literature data for H2O2 contents so variable? A discussion of potential difficulties in the quantitative assay of leaf extracts. *J. Exp. Bot.* 59, 135–146.

Queval, G., and Noctor, G. (2007). A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: application to redox profiling during *Arabidopsis* rosette development. *Anal. Biochem.* 363, 58–69.

Rahman, I., Kode, A., and Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 1, 3159–3165.

Rao, M. V., Paliyath, G., Ormrod, D. P., Murr, D. P., and Watkins, C. B. (1997). Influence of salicylic acid on H2O2 production, oxidative stress and H2O2-metabolizing enzymes. *Plant Physiol.* 115, 137–149.

Rayapuram, C., and Baldwin, I. T. (2007). Increased SA in NPR1-silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked *Nicotiana attenuata* in nature. *Plant J.* 52, 700–715.

Reymond, P., Bodenhausen, N., Van Poecke, R. M., Krishnamurthy, V., Dicke, M., and Farmer, E. E. (2004). A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16, 3132–3147.

Rieu, I., and Powers, S. J. (2009). Real-time quantitative RT-PCR: design, calculations and statistics. *Plant Cell* 21, 1031–1033.

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. G. (2011). Hormone crosstalk in plant disease and defense: more than just JASMONATE-SALICYLATE antagonism. *Annu. Rev. Phytopathol.* 49, 317–343.

Schlaeppi, K., Bodenhausen, N., Buchala, A., Mauch, F., and Reymond, P. (2008). The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant J.* 55, 774–786.

Schröder, E., and Eaton, P. (2008). Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. *Curr. Opin. Pharmacol.* 8, 153–159.

Spoel, S. H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune response. *Cell Host Microbe* 3, 348–351.

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzellus, J. P., Van Pelt, J. A., Mueller, J. A., et al. (2003). NPR1 modulates crosstalk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytoplasm. *Plant Cell* 15, 760–770.

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcriptional activator NPR1 plays a dual role in regulating plant immunity. *Cell* 137, 860–872.

Stotz, H. U., Pittendrigh, B. R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., et al. (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. *Plant Physiol.* 124, 1007–1018.

Ströher, E., Wang, X.-J., Roloff, N., Klein, P., Husemann, A., and Dietz, K.-J. (2009). Redox-dependent regulation of the stress-induced zinc-finger protein SAP12 in *Arabidopsis thaliana*. *Mol. Plant* 2, 357–367.

Swarup, R., Perry, P., Hagenbeek, D., Van Der Straeten, D., Beemster, G. T. S., Sandberg, G., et al. (2007). Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. *Plant Cell* 19, 2186–2196.

Szalai, G., Kellös, T, Galiba, G., and Kocsy, G. (2009). Glutathione as an antioxidant and regulatory molecule in plants under abiotic stress conditions. *J. Plant Growth Regul.* 28, 66–80.

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational changes of NPR1 via *S*-nitrosylation and thioredoxins. *Science* 321, 952–956.

Thivierge, K., Prado, A., Driscoll, B. T., Bonneil, É., Thibault, P., and Bede, J. C. (2010). Caterpillar- and salivary-specific modification of plant proteins. *J. Proteome Res.* 9, 5887–5895.

Van der Does, D., Leon-Reyes, A., Koorneef, A., Van Verk, M. C., Rodenburg, N., Pauwels, L., et al. (2013). Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* 25, 744–761.

Verhage, A., Vlaardingerbroek, I., Raaymakers, C., Van Dam, N. M., Dicke, M., Van Wees, S. C. M., et al. (2011). Rewiring of the jasmonate signaling pathway in *Arabidopsis* during insect herbivory. *Front. Plant Sci.* 2:47. doi: 10.3389/fpls.2011.00047

Walling, L. L. (2008). Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiol.* 146, 859–866.

Weech, M.-H., Chapleau, M., Pan, L., Ide, C., and Bede, J. C. (2008). Caterpillar saliva interferes with induced *Arabidopsis thaliana* defence responses via the systemic acquired resistance pathway. *J. Exp. Bot.* 59, 2437–2448.

Wünsche, H., Baldwin, I. T., and Wu, J. (2011). *S*-nitrosoglutathione reductase (GSNOR) mediates the biosynthesis of jasmonic acid and ethylene is induced by feeding of the insect herbivore *Manduca sexta* and is important for jasmonate-elicited responses in *Nicotiana attenuata*. *J. Exp. Bot.* 62, 4605–4616.

Zander, M., Chen, S., Imkampe, J., Thurow, C., and Gatz, C. (2011). Repression of the *Arabidopsis thaliana* jasmonic acid/ethylene-induced defense pathway by TGA interacting glutaredoxins depends on their C-terminal ALWL. *Mol. Plant* 5, 831–840.

Zander, M., La Camera, S., Lamotte, O., Métraux, J. P., and Gatz, C. (2010) Arabidopsis thaliana class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. *Plant J.* 61, 200–210.

Zarei, A., Körbes, A. P., Younessi, P., Montiel, G., Champion, A., and Memelink, J. (2011). Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of PDF1.2 promoter in *Arabidopsis*. *Plant Mol. Biol.* 75, 321–331.

Zebelo, S. A., and Maffei, M. E. (2012). The ventral eversible gland (VEG) of *Spodoptera littoralis* triggers early responses to herbivory in *Arabidopsis thaliana*. *Arthropod Plant Interact.* 6, 543–551.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6523–6528.

Zhang, Y., Tessaro, M. J., Lassner, M., and Li, X. (2003). Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5 and TGA6 reveals their redundant and essential roles in Systemic Acquired Resistance. *Plant Cell* 15, 2647–2653.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 December 2012; accepted: 11 April 2013; published online: 06 May 2013.

Citation: Paudel J, Copley T, Amirizian A, Prado A and Bede JC (2013) Arabidopsis redox status in response to caterpillar herbivory. Front. Plant Sci. ***4****:113. doi: 10.3389/fpls.2013.00113*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Paudel, Copley, Amirizian, Prado and Bede. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

APPENDIX

Table A1 | Quantitative real-time polymerase chain reaction primers.

Gene	Accession number	Annealing temperature (°C)	Forward (5′–3′)	Reverse (5′–3′)	Reference
ERF1	At3g23240	62	GAC GGA GAA TGA CCA ATA AGA AG	CCC AAA TCC TCA AAG ACA ACT AC	Swarup et al. (2007)
AtHEL	At304720	57	CAA GTG TTT AAG GGT GAA GA	CGG TGT CTA TTT GAT TGA AC	Conn et al. (2008)
AtLOX2	At3g45410	57	GTC CTA CTT GCC TTC CCA AAC	ATT GTC AGG GTC ACC AAC ATC	Weech et al. (2008)
AtPDF1.2b	At2g26020	59	CGG CAA TGG TGG AAG CA	CAT GCA TTA CTG TTT CCG CAA	Jirage et al. (2001)
AtPR1	At2g14610	62	CAC TAC ACT CAA GTT GTT TGG A	CAT GCA TTA CTG TTT CCG CAA A	Primer3
AtSAP6	At3g52800	63	TCA ACG CAT CGA ACG GCT CTG A	GCG AAA GCG AAT CCG TTG GTG AAA	Primer3
AtACT2	At3g18780		ACC AGC TCT TCC ATC GAG AA	GAA CCA CCG ATC CAG ACA CT	Dufresne et al. (2008)

Table A2 | Statistical results of plant–insect experiments.

Ascorbate		
Total	Range: 2.69–3.98 μmol/g FW	Effect of treatment, $F_{(2,103)} = 1.33$, $p = 0.27$; effect of time, $F_{(4,103)} = 0.16$, $p = 0.96$; interaction, $F_{(8,103)} = 0.66$, $p = 0.73$
Oxidized (DHA)	Range: 1.22–1.83 μmol/g FW	Effect of treatment, $F_{(2,100)} = 0.41$, $p = 0.66$; effect of time, $F_{(4,100)} = 0.90$, $p = 0.47$; interaction, $F_{(8,100)} = 0.43$, $p = 0.90$
Reduced (Asc)	Range: 1.23–2.81 μmol/g FW	Effect of treatment, $F_{(2,105)} = 2.50$, $p = 0.09$; effect of time, $F_{(4,105)} = 0.58$, $p = 0.68$; interaction, $F_{(8,105)} = 0.97$, $p = 0.47$
Oxidized/reduced (DHA/Asc)		Effect of treatment, $F_{(2,100)} = 1.62$, $p = 0.21$; effect of time, $F_{(4,100)} = 0.1.30$, $p = 0.28$; interaction, $F_{(8,100)} = 1.11$, $p = 0.36$
Glutathione		
Total	Range: 151–226 nmol/g FW	Effect of treatment, $F_{(2,109)} = 3.35$, $p = 0.04$; effect of time, $F_{(4,109)} = 0.32$, $p = 0.86$; interaction, $F_{(8,109)} = 0.16$, $p = 0.99$; **Figure 2A**)
Oxidized (GSSG)	Range: 9.9–42.6 nmol/g FW	Effect of treatment, $F_{(2,89)} = 3.31$, $p = 0.04$; effect of time, $F_{(4,89)} = 2.10$, $p = 0.09$; interaction, $F_{(8,89)} = 2.24$, $p = 0.03$. Since interaction was significant, this was followed by a one-way ANOVA to determine the time point where there was a significant difference: 5 min: $F_{(2,19)} = 2.69$, $p = 0.09$; 15 min: $F_{(2,14)} = 1.91$, $p = 0.18$; 25 min: $F_{(2,19)} = 2.64$, $p = 0.10$; 35 min: $F_{(2,20)} = 3.76$, $p = 0.04$; 45 min: $F_{(2,19)} = 0.53$, $p = 0.60$
Reduced (GSH)	Range: 93.2–227.9 nmol/g FW	Effect of treatment, $F_{(2,87)} = 3.42$, $p = 0.04$; effect of time, $F_{(4,87)} = 0.67$, $p = 0.62$; interaction, $F_{(8,87)} = 0.56$, $p = 0.81$
Oxidized/reduced (GSSG/GSH)		Effect of treatment, $F_{(2,87)} = 0.99$, $p = 0.37$; effect of time, $F_{(4,87)} = 2.18$, $p = 0.08$; interaction, $F_{(8,87)} = 1.07$, $p = 0.39$

(Continued)

Table A2 | Continued

Gene expression	Genotype	
AtPR1	Wildtype	$F_{(2,8)} = 4.44$, $p = 0.05$; fivefold increase in gene expression in plants attacked by caterpillars with intact labial salivary secretions compared to control plants or between control plants or plants infested by caterpillars with impaired salivary secretions
	pad2-1	$F_{(2,9)} = 1.23$, $p = 0.32$
	tga2/5/6	$F_{(2,7)} = 0.35$, $p = 0.72$
AtPDF1.2	Wildtype	$F_{(2,8)} = 6.00$, $p = 0.03$; 18-fold increase in gene expression in response to herbivory by caterpillars with impaired salivary secretions compared to normal caterpillars or controls
	pad2-1	$F_{(2,8)} = 1.50$, $p = 0.28$; 12.5-fold increase in gene expression is seen between plants infested by caterpillars compared to controls
	tga2/5/6	$F_{(2,7)} = 3.31$, $p = 0.10$; fivefold increase in gene expression is seen in plants fed upon by caterpillars compared to controls
ERF1	Wildtype	$F_{(2,8)} = 5.07$, $p = 0.04$; 10-fold increase in gene expression seen in plants fed upon by caterpillars with impaired salivary secretions compared to control plants
	pad2-1	$F_{(2,9)} = 12.83$, $p = 0.002$; 20-fold increase in gene expression seen in plants fed upon by caterpillars with impaired salivary secretions compared to control plants or plants attacked by caterpillars with labial salivary secretions
	tga2/5/6	$F_{(2,7)} = 0.61$, $p = 0.57$
AtHEL	Wildtype	$F_{(2,9)} = 2.50$, $p = 0.14$; fivefold increase in gene expression is observed in plants infested by caterpillars compared with control
	pad2-1	$F_{(2,9)} = 2.22$, $p = 0.17$; 10-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
	tga2/5/6	$F_{(2,7)} = 3.31$, $p = 0.10$
AtLOX2	Wildtype	$F_{(2,7)} = 1.48$, $p = 0.29$; sevenfold increase in gene expression is observed in plants infested by caterpillars compared with controls
	pad2-1	$F_{(2,9)} = 3.68$, $p = 0.07$; sevenfold increase in gene expression is observed in plants infested by caterpillars compared with controls
	tga2/5/6	$F_{(2,7)} = 1.16$, $p = 0.37$; 40-fold increase in gene expression is observed in plants infested by caterpillars compared with controls
AtSAP6	Wildtype	$F_{(2,8)} = 5.02$, $p = 0.04$
	pad2-1	$F_{(2,9)} = 0.85$, $p = 0.46$
	tga2/5/6	$F_{(2,7)} = 0.14$, $p = 0.87$

A two-way ANOVA followed by a Tukey HSD was used to evaluate redox metabolite levels over a 45-min time course. A one-way ANOVA was used to evaluate differences in gene expression within each genotype. A fivefold or higher difference in gene expression is also indicated.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 27 June 2013
doi: 10.3389/fpls.2013.00222

Resistance to sap-sucking insects in modern-day agriculture

Arjen VanDoorn*[1,2] *and Martin de Vos*[1]*

[1] *Keygene NV, Wageningen, Netherlands*
[2] *Department of Plant Physiology, Swammerdam Institute of Life Sciences, University of Amsterdam, Amsterdam, Netherlands*

Edited by:
Mary Beth Mudgett, Stanford University, USA

Reviewed by:
Andrew Bent, University of Wisconsin – Madison, USA
Joop J. A. Van Loon, Wageningen University, Netherlands

****Correspondence:***
Martin de Vos, Keygene NV, Agro Business Park 90, 6708 PW Wageningen, Netherlands
e-mail: martin.de-vos@keygene.com

Plants and herbivores have co-evolved in their natural habitats for about 350 million years, but since the domestication of crops, plant resistance against insects has taken a different turn. With the onset of monoculture-driven modern agriculture, selective pressure on insects to overcome resistances has dramatically increased. Therefore plant breeders have resorted to high-tech tools to continuously create new insect-resistant crops. Efforts in the past 30 years have resulted in elucidation of mechanisms of many effective plant defenses against insect herbivores. Here, we critically appraise these efforts and – with a focus on sap-sucking insects – discuss how these findings have contributed to herbivore-resistant crops. Moreover, in this review we try to assess where future challenges and opportunities lay ahead. Of particular importance will be a mandatory reduction in systemic pesticide usage and thus a greater reliance on alternative methods, such as improved plant genetics for plant resistance to insect herbivores.

Keywords: phloem-feeding insects, crop pests, breeding, genetically modified crops, natural insecticides

EVOLUTIONARY PERSPECTIVE OF PLANT–INSECT INTERACTIONS

Around 350 m years ago, the first insects evolved to feed on plant material (Labandeira, 2007), after which plants evolved mechanisms to deter herbivores. These mechanisms include antibiosis, compounds toxic to insects, antixenosis, the deterrence of insect, physiological defensive properties, such as thorns and trichomes and tolerance (Smith and Clement, 2012). Insects on their part have evolved to detoxify or efficiently sequester these toxic metabolites. As early as 1888, Ernst Stahl elegantly demonstrated that extractable plant-based chemicals are responsible for defining host-specificity in plant–herbivore interactions (Stahl, 1888). It took until 1964 before the role of secondary metabolites in plants were again associated with insect host suitability, with Ehrlich and Raven (1964) in their landmark "plants and butterflies" paper. Here, the theory of co-evolution between plants and their herbivorous pests was laid out, and their paper was an important basis for subsequent plant–insect research.

CROP DOMESTICATION AND MODERN AGRICULTURE

Over 4000 years, humans have been domesticating a large variety of crops; primarily selecting for "easy" traits, such as fruit size and yield. Evidently, in for example strawberries, the wild ancestors have much smaller berries and a completely different taste than the currently cultivated big, juicy, and often very sweet strawberry varieties (Aharoni et al., 2004). During this selection process and before global spread and subsequent outbreaks of pests and diseases, little or no attention was given to resistance beyond those required for locally occurring biotic and abiotic conditions. Therefore, many naturally occurring resistances have probably been lost (de-selected) during the cultivation of our current staple crops.

During the last century's green revolution, crops were developed that are adapted to large-scale, high-input agriculture. This has driven an industrial-scale global agriculture and has, logically, resulted in industrial-sized seed production, for which a few suppliers in the EU and the USA provide seeds to a multitude of countries worldwide. The focus on high-input monocultures has advantages for industrial-sized agriculture, e.g., crops are easier to harvest, highly uniform, and produce predictably stable yields. However, such crop production also provides concerns and has drawbacks. Besides its high cost in energy input per unit arable land, one can also foresee that the use of these crop practices exert a tremendous selection pressure on pests and diseases, implying that resistances can easily be broken. In order to fight destructive herbivorous insects, humankind has heavily relied on the use of insecticides. However, in the last 15 years a large number of them, mostly systemic pesticides, have been banned because of their harmfulness toward consumers (e.g., parathion, dinitro-*o*-cresol), non-target organisms, or the environment [e.g., dichlorodiphenyltrichloroethane (DDT)]. More recently, neonicotinoids have come under fire because of harmful effects to non-target species such as bees and bumblebees (Henry et al., 2012; Whitehorn et al., 2012). Neonicotinoids are very effective pesticides as they are able to spread systemically throughout the plant, ensuring easy application and extending their usage in the formulations for seed coating. Overall, the EU and other countries worldwide have banned the use of many systemic pesticides[1] because; (i) concerns about insecticide retention in food crops; (ii) effects on off-target organisms; (iii) broader negative impact on ecosystems, and (iv) higher risk of insecticide resistance in key insect pests.

INSECT RESISTANCE IN MODERN-DAY BREEDING

With the current reduction in the range of pesticides that are available to farmers, efforts to find alternative methodologies

[1]EU directive 2009/128/EC for sustainable use of pesticides in plant protection. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0071:0086:en:PDF

for insect resistance have been on the rise. As a result, breeding for insect-resistant crops has received increased attention and many seed companies advertise their insect-resistant varieties. These insect resistance traits have come from a variety of sources, including plants and micro-organisms. For instance, broad resistance to Lepidoptera and Coleoptera is attained by the use of genetically modified (GM) plants, expressing a "Cry" toxin from *Bacillus thuringiensis* (Vaeck et al., 1987) in a number of important row crops, including corn, soybean, and cotton (Bohorova et al., 1996; Lambert et al., 1996; Stewart et al., 1996). Different Cry variants have been used in crops that exhibit differing spectra of efficacy against various groups of herbivorous insects, and are used widely in agriculture throughout the USA and other parts of the world[2]. Resistance or insensitivity to *Bt* in target insects has been observed in the laboratory (Meihls et al., 2012; Zhang et al., 2012) and the field (Gassmann et al., 2011). However, issues of insect resistance to *Bt* will be at least partly overcome in the latest generation *Bt*-crops, in which several Cry toxins, that do not show cross-resistance, are stacked or combined with other methodologies such as RNA interference (RNAi; Bhatia et al., 2012; Kumar et al., 2012; Tang et al., 2012) or the production of secondary metabolites. Evidently, the usage of *Bt*-crops has re-shaped the need for insecticide use, but has also allowed other, previously less economically important, insect pests to flourish. In particular, *Bt*-insensitive insects, such as aphids, whiteflies, and scale insects populations might increase in abundance. Hence, if GM strategies are to be used, these insect pests require other GM resistance strategies. GM approaches using plant-derived lectins, agglutinin, and protease inhibitors have been shown to provide high levels of resistance to aphids and other phloem-feeding insect species (Fitches et al., 2008; Alvarez-Alfageme et al., 2011; Carrillo et al., 2011). In addition, *in planta* expression of RNAi-vectors that target physiologically important insect transcripts for degradation, have been shown to result in crop protection against a number of insect pests, including phloem-feeding aphids (Price and Gatehouse, 2008; Upadhyay et al., 2011; Zha et al., 2011). Although potentially effective, none of these GM methodologies have been commercially marketed. A variety of reasons can underlie their lack of success on the market, these include (i) high risk of limited durability, particularly if less than 99% mortality is achieved; (ii) potential negative effects on non-target insects, ecosystems, or consumers; (iii) narrow target-specificity, i.e., high cost of deregulation of a GM does not pay off compared to the reduction in yield loss resulting from an economically minor pest or a niche market crop.

Hence, there is a strong incentive to develop alternative strategies against these pests. In that respect, combined approaches seem particularly attractive. For instance, the use of (non-GM) genetic crop resistance, combined with biological control using predatory insects or practical solutions that limit the build-up of high population densities of herbivorous pests will likely result in effective pest control.

[2]ISAAA Brief 43-2011. Global Status of Commercialized Biotech/GM Crops in 2011. Or summary at: http://www.isaaa.org/resources/publications/pocketk/16/default.asp

BENEFITTING FROM NATURAL VARIATION

An alternative to transgenic approaches is the use of wild relatives of crop plants, searching for desirable traits and then crossing those into the elite cultivars. This traditional way of plant breeding has been made substantially easier with the availability of novel sequence-based molecular approaches. For instance, genome-wide coverage of single nucleotide polymorphisms (SNPs; or other molecular markers) between wild and cultivated species are easily obtained and make marker assisted selection or marker assisted breeding for traits of interest feasible in many crops. Moreover, genome-wide association studies to identify SNPs linked to traits of interest and the subsequent use of novel breeding schemes (breeding by design) will further revolutionize crop breeding for insect resistance. All these methodologies are advanced by whole genome sequencing of crop plants, e.g., maize, rice, wheat, but also vegetable crops such as tomato, lettuce, and cabbage (Goff et al., 2002; Schnable et al., 2009; Brenchley et al., 2012; Sato et al., 2012), and re-sequencing of wild germplasm. However, a challenge remains when traits are polygenic, and the individual components have subtle effect. Moreover, the genetic background of elite cultivars might interfere with traits from wild relatives. There is a clear need to bridge the current gap in the understanding of these technological advances between bio-informaticians, bio-statisticians, entomologists, plant pathologists and (pre-) breeders. It is often overlooked that only their collective efforts will ensure important breakthroughs in pest and disease resistance in crops.

R-GENE-MEDIATED RESISTANCE TO INSECT PESTS

Although some resistances are effective against a broad range of pest species, most are highly herbivore-specific reactions. Exploitation of natural resistances, often found in wild relatives that are interbreedable with our current crops, is well-suited to combat pest species that consume a specific plant organ or tissue (e.g., aphids, whiteflies, and other phloem-feeding insects).

R-gene-based resistance relies on a "gene-for-gene" interaction, where a compound secreted by the insect is specifically recognized by the plant, thus enabling the plant to initiate a defense response.

Whereas R-gene-mediated resistance has not been established for tissue chewing insects (i.e., Lepidoptera and Coleoptera), several examples of strong monogenic natural resistance to phloem-feeding pests have been reported in literature. Only a few of these dominant R-genes – that provide resistance against phloem-feeders – have been cloned (e.g., Mi-1.2, VAT, and BPH16) and many more are extensively used in agricultural settings through the use of marker assisted breeding (for a recent review, see Broekgaarden et al., 2011; **Table 1**).

Therefore, it is tempting to draw a general conclusion about R-gene-mediated insect resistances found in nature: only those pests, such as phloem-feeding insects, that require an intimate relationship with their host plant to successfully colonize are likely to be contained using R-gene-mediated defenses.

Interestingly, even in crops where the R-gene is cloned and characterized, the mode-of-action of these resistances is unclear. It should involve attacker recognition and down-stream signal transduction leading to an effective defense response that results in the inability of phloem-feeding insects to establish prolonged feeding.

Table 1 | Overview of R-genes mediating insect resistance (adapted from Broekgaarden et al., 2011, with permission).

Plant species	Gene	Insect	Resistance broken	Reference
Triticum aestivum	*H genes*	*Mayetiola destructor*	yes	Wang et al. (2006); Yu et al. (2009), and Harris et al. (2012)
	Dn genes	*Diuraphis noxia*	yes	Liu et al. (2005), Peng et al. (2009), and Tolmay et al. (2007)
Oryza sativa	*Bph* genes	*Nilaparvata lugens*	yes	Du et al. (2009), Qiu et al. (2010), and Peñalver Cruz et al. (2011)
	Gm genes	*Gall midge*		Himabindu et al. (2010), and Kumar et al. (2009)
Solanum lycopersicum	*Mi-1.2*	*Macrosiphum euphorbiae, Bemisia tabaci*	yes	Rossi et al. (1998), Nombela et al. (2003), and Goggin et al. (2001)
Cucumis melo	*Vat*	*Aphis gossypii*	yes	Klingler et al. (2001) and Lombaert et al. (2009)
Medicago truncatula	*AIN*	*Acyrthosiphon kondoi*	yes	Klingler et al. (2009) and Humphries et al. (2010)
Glycine max	*Rag genes*	*Aphis glycines*	yes	Li et al. (2007); Zhang et al. (2009, 2010), and Kim et al. (2008)

Similarly to plant–pathogen interactions, the cloned insect resistance genes are members of the family of nucleotide-binding, leucine-rich repeat (NBS-LRR). Therefore, in analogy with pathogen recognition, it is expected that recognition of insect herbivores by NBS-LRR proteins takes place through direct or indirect binding of insect effector molecules (Dodds and Rathjen, 2010). Effector molecules of phloem-feeding insects are thought to be secreted into the host plant during probing (testing phase) or subsequent prolonged feeding (ingestion of phloem sap; Will et al., 2007; Moreno et al., 2011). Although several candidate effector molecules, e.g., secreted from the salivary glands of aphids, have been identified (Ramsey et al., 2007; Harmel et al., 2008; Carolan et al., 2009; Bos et al., 2010; Rodriguez and Bos, 2013), none of these have been associated with the binding by R-genes directly, or to known so-called virulence targets "guarded" by R-genes. It is expected that this field of research will take an enormous flight and shows a promise for plant breeding for insect-resistant crops.

The *Mi-1.2* gene in tomato, arguably most researched, is extensively used for control of root-knot nematodes [*Meloidogyne* species (Milligan et al., 1998; de Vos et al., 2008)], but also is effective against some clones of the tomato–potato aphid (*Macrosiphum euphorbiae*; Rossi et al., 1998), whiteflies (*Bemisia tabaci*; Nombela et al., 2003), and the potato psyllid (Casteel et al., 2006)]. This broad effectiveness of the Mi gene toward several tomato phloem-feeding pests is striking and suggests recognition of several species-specific effector molecules. As an alternative – and more likely – hypothesis one would expect these insect species use a similar gateway, guarded by Mi-1.2, to successfully colonize tomato. To date, no such effector from either of the insect, nor *Meloidogyne* species, has been identified that causes the hypersensitive response in *Mi1.2* tomato plants. Mi-mediated resistance to root-knot nematodes in characterized by a local hypersensitive response that takes place within 24 h upon feeding by *Meloidogyne* species. The Mi-mediated response to aphids is clone-specific and requires common signaling components characterized for pathogen defenses (Bhattarai et al., 2007, 2010; Atamian et al., 2012).

Over the past decades plant breeding companies have exploited natural variation for dominant monogenic insect resistance genes. The genes described above are extensively used in horticulture. Other dominant loci, such as those required for resistance against wheat against the Russian wheat aphid or Hessian flies have been extensively used in agricultural settings. The large-scale usage of these dominant loci has resulted in newly arisen insect populations (virulent biotypes). For example, aphid biotypes of *Nasonovia ribisnigri* have been identified in Europe that are able to feed from cultivated lettuce carrying a dominant monogenic resistance introgressed from *Lactuca virosa* (Thabuis et al., 2011). Other examples, include virulent biotypes of the Russian wheat aphid that break through Dn resistance in wheat (Haley et al., 2004).

Pyramiding of R-genes (similar to *Bt*-approaches), where more than one resistance trait is stacked, can possibly prevent, or at least delay, the formation of new insect biotypes that can evolve to feed on resistant crops and this strategy can contribute to increased durability of these resistances. This may be a more responsible use of the currently limited set of available resistance traits. Ultimately, the decision to pyramid resistance genes will depend on several, often economic, factors, including (i) the availability of natural germplasm; (ii) the current and future economic threat of a pest; (iii) the population characteristics of the pest and its ability to evolve counter measures that lead to insensitivity; (iv) the time-to-market for the crop at hand, and (v) the level of resistance in current (competitive) commercial varieties.

METABOLITE-MEDIATED RESISTANCE

As described above, R-gene-based defense can render strong species-specific resistance to a limited set of herbivores, but is certainly not effective against all herbivores. The constitutive or induced production of secondary metabolites can provide an alternative resistance strategy. These compounds, which may be specific for the plant genus or family, often accumulate in leaf tissue where they occur in specialized structures on the plant's surface or are compartmentalized within the host cell.

There is an incredible natural diversity of compounds present in plants (**Figure 1**). Whereas some of the biosynthetic pathways

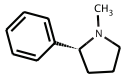
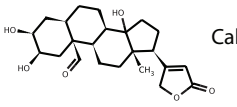
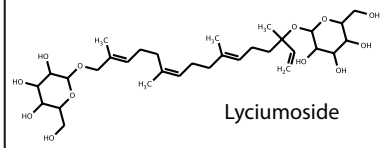
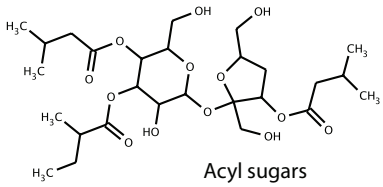
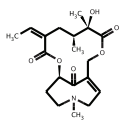
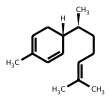
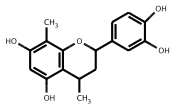
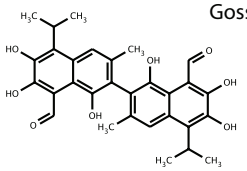
Structure		Plant species	Effective against	Reference
	Nicotine	*Nicotiana attenuata*	*Spodoptera exigua* *Manduca sexta*	Steppuhn et al., 2004
	Calotropagenin	*Asclepias humistrata*	*Danaus plexippus*	Zalucki et al., 2001
	Lyciumoside	*Nicotiana attenuata*	*Manduca sexta*	Heiling et al., 2011
	Acyl sugars	*Solanum pennellii*	*Bemisia tabaci*	Liedl et al., 1995
R — N = C = S	Isothiocyanides	Brassicaceae spp	*Otiorhynchus sulcatus*	Borek et al., 1998
	Senkirkine	*Senecio vernalis*	*Frankliniella occidentalis*	Macel et al., 2005
	7-epi zingiberene	*Solanum habrochaitus*	*Bemisia tabaci,* *Tetranychus urticae*	Bleeker et al., 2012
	Tannins	*Populus spp.*	*Lymantria dispar*	Bourchier and Nealis, 1993
	Gossypol	*Gossypium hirsutum*	*Heliothis zea*, *Heliothis virescens*	Shaver and Parrott, 1970

FIGURE 1 | Overview of insecticidal secondary metabolites and their plant origin.

are restricted to a certain family, others are spread throughout the plant kingdom. Examples of specialized plant metabolites are glucosinolates in *brassicaceae*, from which toxic and anti-feedant compounds are enzymatically formed as soon as the cells are ruptured by herbivore feeding (Lüthy and Matile, 1984). Moreover, a wide variety of alkaloids have been identified, such as the neurotoxin nicotine in *Nicotiana attenuata* (Steppuhn et al., 2004), saponin glycoalkaloids in tomato (Chan and Tam, 1985) and pyrrolizidine alkaloids in chrysanthemum (Macel et al., 2005) that are related to resistance to generalist insect pests.

On the contrary, compounds such as terpenes occur ubiquitously throughout the plant world, and are synthesized through

common pathways present in most plants. However, there is also immense structural variation in these terpenes themselves, with an estimated 30,000 different structures occurring in plants (Connolly and Hill, 1991; Degenhardt et al., 2009b; Pichersky and Lewinsohn, 2011). Small changes in the final biosynthetic enzymes (terpene synthases), the availability of substrates and the biosynthetic conditions in the cells play a defining role in determining which terpenes are produced (Degenhardt et al., 2009a). This has provided plants with an enormous evolutionary flexibility to fine-tune the chemical responses to herbivory.

Because many terpenes are volatile, and many plants induce their production when attacked by herbivores, they provide an opportunity for predators to locate herbivore-infested plants, and serve a role as semiochemicals (information-conveying chemicals; Turlings et al., 1990; Kessler and Baldwin, 2001). A very diverse set of terpenoids has been suggested to play a role in indirect defense, such as bergamotene in wild tobacco, and a blend of mono- and sesquiterpens in tomato (Kessler and Baldwin, 2001; Kant et al., 2004).

Terpenes have also been shown to act as direct toxins to a suite of insects and pathogens [e.g., 7-epi-zingiberene against whiteflies (Bleeker et al., 2011), resins against bark beetles in confiners (Phillips and Croteau, 1999), and terpenoid lactones against Colorado potato beetles (Szczepanik et al., 2005)], but are at high concentrations also toxic to the plant itself (Aharoni et al., 2003). Therefore, plants sequester and compartmentalize terpenes, transport them to the leaf surface, or produce and store terpenoids in trichomes. The latter allows a terpene coating toward the outside environment of the plant without the need to adapt to high intercellular concentrations of these compounds.

A major pest in commercial tomato cultivation is the whitefly *Bemisia tabaci*, mainly because it is a vector for *begomo* viruses , causing substantial losses in commercial vegetable cultivation (Navas-Castillo et al., 2011). Although some promising sources of resistance have been identified (Firdaus et al., 2013), to date no R-gene-based resistance has been identified for *Bemisia tabaci*, a highly polyphagous phloem-feeding insect with a host-range spanning over 100 plant species (Mound and Halsey, 1978). Although a whitefly population can quickly reach enormous numbers, their direct impact on crop yield is limited. In contrast, indirect damage from whitefly vectored viruses is a major threat to crop production. To prevent virus vectoring by sap-sucking pests one should ideally rely on a complete avoidance response of the insect toward the host plant. Volatile-mediated repellency of whiteflies might just provide such an opportunity in tomato, where tomato yellow leaf curl virus (TYLCV) is a major agricultural disease transmitted by *Bemisia tabaci*.

By screening a number of wild tomato plants for repellence against whiteflies, Bleeker et al. (2011) found that *Solanum habrochaites* showed strong repellency to whiteflies. Subsequently, the repellency was shown to be mediated by a sesquiterpene, namely 7-epi-zingiberene (Bleeker et al., 2011). 7-epi-zingiberene is exclusively produced in the glandular trichomes of *S. habrochaites* (Bleeker et al., 2012). In the offspring of interspecific crosses between *S. habrochaites* and cultivated tomato (*S. lycoperiscum*) were made, the F2 plants showed a strong correlation between 7-epi-zingiberene production and whitefly resistance. Surprisingly, this compound did not only confer resistance against whiteflies, but also against other herbivorous pests with entirely different modes of feeding, these include single-cell feeders (the spider mite *T. urticae*) and caterpillars (*Manduca sexta*; Bleeker et al., 2012). The above-described approach is very promising for multiple (vegetable) crop species. The repellent and toxic effects of such compounds produced at the plant–environment interface (e.g., in glandular trichomes) directly functions as an alarm bell that signals "inedible" to approaching herbivorous pests, but will be particularly important in fighting off virus vectoring insect species.

WHAT CHALLENGES ARE AHEAD

Preventing pre- and post-harvest damage caused by insects is a very challenging, but economically important, issue for plant breeders. Particularly, the proposed and partly implemented reductions in the use of systemic pesticides will further increase the need of genetic host resistance in the near future. GM approaches have been extremely successful in controlling some insect species, but their implementation, particularly in the EU, face heavy political opposition. Moreover, due to the de-regulatory process, GM introduction is expensive, thereby making it less feasible for the smaller vegetable crop markets, which are often locally tailored and also diversified to achieve specific consumer traits.

In order to have a chance against insect species that have multiple generations in a year, it is of crucial importance to widen our understanding of resistances in wild relatives of our current crops against insect herbivores. This will be an essential responsibility for plant pathologists, entomologists, breeders, and the entire research community. It has been estimated that for crops such as tomato, there is a multitude of gene-information "buried" in wild species that can be crossed with elite varieties. This genetic reservoir represents a largely untapped treasure for new or improved traits that could make our current crops significantly more productive. Because every day more genomic sequences are becoming available, this enables a quicker trait-to-gene path, thus providing a good academic opportunity to look beyond model plants and provide an insight in unique traits of wild species. Large efforts will need to be made to understand what genes are underlying the traits of future importance.

ACKNOWLEDGMENTS

We would like to thank the two reviewers for their valuable comments.

REFERENCES

Aharoni, A., Giri, A. P., Deuerlein, S., Griepink, F., De Kogel, W.-J., Verstappen, F. W. A., et al. (2003). Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell* 15, 2866–2884. doi: 10.1105/tpc.016253

Aharoni, A., Giri, A. P., Verstappen, F. W. A., Bertea, C. M., Sevenier, R., Sun, Z. K., et al. (2004). Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. *Plant Cell* 16, 3110–3131. doi: 10.1105/tpc.104.023895

Alvarez-Alfageme, F., Maharramov, J., Carrillo, L., Vandenabeele, S., Vercammen, D., Van Breusegem, F., et al. (2011). Potential use of a serpin from *Arabidopsis* for pest control. *PLoS ONE* 6:e20278. doi: 10.1371/journal.pone.0020278

Atamian, H. S., Eulgem, T., and Kaloshian, I. (2012). SlWRKY70

is required for Mi-1-mediated resistance to aphids and nematodes in tomato. *Planta* 235, 299–309. doi: 10.1007/s00425-011-1509-6

Bhatia, V., Bhattacharya, R., Uniyal, P. L., Singh, R., and Niranjan, R. S. (2012). Host generated siRNAs attenuate expression of serine protease gene in *Myzus persicae*. *PLoS ONE* 7:e46343. doi: 10.1371/journal.pone.0046343

Bhattarai, K. K., Atamian, H. S., Kaloshian, I., and Eulgem, T. (2010). WRKY72-type transcription factors contribute to basal immunity in tomato and *Arabidopsis* as well as gene-for-gene resistance mediated by the tomato R gene Mi-1. *Plant J.* 63, 229–240. doi: 10.1111/j.1365-313X.2010.04232.x

Bhattarai, K. K., Li, Q., Liu, Y., Dinesh-Kumar, S. P., and Kaloshian, I. (2007). The Mi-1-mediated pest resistance requires Hsp90 and Sgt1. *Plant Physiol.* 144, 312–323. doi: 10.1104/pp.107.097246

Bleeker, P. M., Diergaarde, P. J., Ament, K., Schütz, S., Johne, B., Dijkink, J., et al. (2011). Tomato-produced 7-epizingiberene and R-curcumene act as repellents to whiteflies. *Phytochemistry* 72, 68–73. doi: 10.1016/j.phytochem.2010.10.014

Bleeker, P. M., Mirabella, R., Diergaarde, P. J., Vandoorn, A., Tissier, A., Kant, M. R., et al. (2012). Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative. *Proc. Natl. Acad. Sci. U.S.A.* 109, 20124–20129. doi: 10.1073/pnas.1208756109

Bohorova, N., Maciel, A. M., Brito, R. M., Aguilart, L., Ibarra, J. E., and Hoisington, D. (1996). Selection and characterization of Mexican strains of *Bacillus thuringiensis* active against four major lepidopteran maize pests. *Entomophaga* 41, 153–165. doi: 10.1007/BF02764243

Borek, V., Elberson, L. R., Mccaffrey, J. P., and Morra, M. J. (1998). Toxicity of isothiocyanates produced by glucosinolates in *Brassicaceae species* to black vine weevil eggs. *J. Agric. Food Chem.* 46, 5318–5323. doi: 10.1021/jf9805754

Bos, J. I., Prince, D., Pitino, M., Maffei, M. E., Win, J., and Hogenhout, S. A. (2010). A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid). *PLoS Genet.* 6:e1001216. doi: 10.1371/journal.pgen.1001216

Bourchier, R. S., and Nealis, V. G. (1993). Development and growth of early-and late-instar gypsy moth (*Lepidoptera*: *Lymantriidae*) feeding on tannin-supplemented diets. *Environ. Entomol.* 22, 642–646. doi: 10.1007/BF00333222

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L. A., D'Amore, R., Allen, A. M., et al. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature* 491, 705–710. doi: 10.1038/nature11650

Broekgaarden, C., Snoeren, T. A., Dicke, M., and Vosman, B. (2011). Exploiting natural variation to identify insect-resistance genes. *Plant Biotechnol. J.* 9, 819–825. doi: 10.1111/j.1467-7652.2011.00635.x

Carolan, J. C., Fitzroy, C. I., Ashton, P. D., Douglas, A. E., and Wilkinson, T. L. (2009). The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. *Proteomics* 9, 2457–2467. doi: 10.1002/pmic.200800692

Carrillo, L., Martinez, M., Alvarez-Alfageme, F., Castanera, P., Smagghe, G., Diaz, I., et al. (2011). A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic *Arabidopsis* plants. *Transgenic Res.* 20, 305–319. doi: 10.1007/s11248-010-9417-2

Casteel, C. L., Walling, L. L., and Paine, T. D. (2006). Behavior and biology of the tomato psyllid, Bactericerca cockerelli, in response to the *Mi-1.2* gene. *Entomol. Exp. Appl.* 121, 67–72. doi: 10.1111/j.1570-8703.2006.00458.x

Chan, H. T., and Tam, S. Y. T. (1985). Toxicity of -tomatine to larvae of the Mediterranean fruit fly (*Diptera*: *Tephritidae*). *J. Econ. Entomol.* 78, 305–307. doi: 10.1653/024.095.0110

Connolly, J. D., and Hill, R. A. (1991). *Dictionary of Terpenoids.* London: Chapman and Hall. doi: 10.1002/ffj.2730070418

de Vos, M., Kriksunov, K. L., and Jander, G. (2008). Indole-3-acetonitrile production from indole glucosinolates deters oviposition by *Pieris rapae*. *Plant Physiol.* 146, 916–926. doi: 10.1104/pp.107.112185

Degenhardt, J., Köllner, T. G., and Gershenzon, J. (2009a). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70, 1621–1637. doi: 10.1016/j.phytochem.2009.07.030

Degenhardt, J., Köllner, T. G., and Gershenzon, J. (2009b). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70, 1621–1637. doi: 10.1016/j.phytochem.2009.07.030

Dodds, P. N., and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nat. Rev. Genet.* 11, 539–548. doi: 10.1038/nrg2812

Du, B., Zhang, W. L., Liu, B. F., Hu, J., Wei, Z., Shi, Z. Y., et al. (2009). Identification and characterization of Bph14, a gene conferring resistance to brown planthopper in rice. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22163–22168. doi: 10.1073/pnas.0912139106

Ehrlich, P. R., and Raven, P. H. (1964). Butterflies and plants: a study in coevolution. *Evolution* 18, 586–608. doi: 10.2307/2406212

Firdaus, S., Heusden, A., Hidayati, N., Supena, E., Mumm, R., Vos, R. H., et al. (2013). Identification and QTL mapping of whitefly resistance components in *Solanum galapagense*. *Theor. Appl. Genet.* 1–15. doi: 10.1007/s001220050803

Fitches, E., Wiles, D., Douglas, A. E., Hinchliffe, G., Audsley, N., and Gatehouse, J. A. (2008). The insecticidal activity of recombinant garlic lectins towards aphids. *Insect Biochem. Mol. Biol.* 38, 905–915. doi: 10.1016/j.ibmb.2008.07.002

Gassmann, A. J., Petzold-Maxwell, J. L., Keweshan, R. S., and Dunbar, M. W. (2011). Field-evolved resistance to Bt maize by western corn rootworm. *PLoS ONE* 6:e22629. doi: 10.1371/journal.pone.0022629

Goff, S. A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* 296, 92–100. doi: 10.1126/science.1068275

Goggin, F. L., Williamson, V. M., and Ullman, D. E. (2001). Variability in the response of *Macrosiphum euphorbiae* and *Myzus persicae* (*Hemiptera*: *Aphididae*) to the tomato resistance gene Mi. *Environ. Entomol.* 30, 101–106. doi: 10.1603/0046-225X-30.1.101

Haley, S. D., Peairs, F. B., Walker, C. B., Rudolph, J. B., and Randolph, T. L. (2004). Occurrence of a new Russian wheat aphid biotype in Colorado research supported through funding from Colorado Agric. Exp. Stn. Projects 795 and 646 and the Colorado Wheat Administrative Committee. *Crop Sci.* 44, 1589–1592. doi: 10.2135/cropsci2004.1589

Harmel, N., Létocart, E., Cherqui, A., Giordanengo, P., Mazzucchelli, G., Guillonneau, F., et al. (2008). Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. *Insect Mol. Biol.* 17, 165–174. doi: 10.1111/j.1365-2583.2008.00790.x

Harris, M. O., Freeman, T. P., Anderson, K. M., Harmon, J. P., Moore, J. A., Payne, S. A., et al. (2012). Hessian fly Avirulence gene loss-of-function defeats plant resistance without compromising the larva's ability to induce a gall tissue. *Entomol. Exp. Appl.* 145, 238–249. doi: 10.1111/eea.12010

Heiling, S., Schuman, M. C., Schoettner, M., Mukerjee, P., Berger, B., Schneider, B., et al. (2010). Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *Plant Cell* 22, 273–292. doi: 10.1105/tpc.109.071449

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., et al. (2012). A common pesticide decreases foraging success and survival in honey bees. *Science* 336, 348–350. doi: 10.1126/science.1215039

Himabindu, K., Suneetha, K., Sama, V., and Bentur, J. S. (2010). A new rice gall midge resistance gene in the breeding line CR57-MR1523, mapping with flanking markers and development of NILs. *Euphytica* 174, 179–187. doi: 10.1007/s10681-009-0106-2

Humphries, A., Peck, D., Robinson, S., Oldach, K., Glatz, R., and Howie, J. (2010). "A new highly virulent blue-green aphid causes severe damage in previously tolerant pasture and grain legumes," in *Food Security from Sustainable Agriculture. Proceedings of 15th Agronomy Conference 2010,* 15-18 November 2010, Lincoln, eds H. Dove and R. A. Culvenor (Gosford NSW: The Regional Institute Ltd).

Kant, M. R., Ament, K., Sabelis, M. W., Haring, M. A., and Schuurink, R. C. (2004). Differential timing of spider mite-induced direct and indirect defenses in tomato plants. *Plant Physiol.* 135, 483–495. doi: 10.1104/pp.103.038315

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144. doi: 10.1126/science.291.5511.2141

Kim, K.-S., Hill, C. B., Hartman, G. L., Mian, M., and Diers, B. W. (2008). Discovery of soybean aphid biotypes. *Crop Sci.* 48, 923–928. doi: 10.2135/cropsci2007.08.0447

Klingler, J., Kovalski, I., Silberstein, L., Thompson, G. A., and Perl-Treves,

R. (2001). Mapping of cotton-melon aphid resistance in melon. *J. Am. Soc. Hortic. Sci.* 126, 56–63. doi: 10.1007/s11032-006-9039-9

Klingler, J. P., Nair, R. M., Edwards, O. R., and Singh, K. B. (2009). A single gene, AIN, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid. *J. Exp. Bot.* 60, 4115–4127. doi: 10.1093/jxb/erp244

Kumar, A., Jain, A., Sahu, R. K., Shrivastava, M. N., Nair, S., and Mohan, M. (2005). Genetic analysis of resistance genes for the rice gall midge in two rice genotypes. *Crop Sci.* 45, 1631–1635. doi: 10.2135/cropsci2004.0406

Kumar, L. V., Chakravarthy, A., Patil, S., and Rajanna, D. (2009). Changing scenario of Asian rice gall midge biotypes at Mangalore, coastal Karnataka and computation of their growth rates. *J. Entomol. Res.* 33, 1–7.

Kumar, P., Pandit, S. S., and Baldwin, I. T. (2012). Tobacco rattle virus vector: a rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. *PLoS ONE* 7:e31347. doi: 10.1371/journal.pone.0031347

Labandeira, C. (2007). The origin of herbivory on land: initial patterns of plant tissue consumption by arthropods. *Insect Sci.* 14, 259–275. doi: 10.1111/j.1744-7917.2007.00141.x-i1

Lambert, B., Buysse, L., Decock, C., Jansens, S., Piens, C., Saey, B., et al. (1996). A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family noctuidae. *Appl. Environ. Microbiol.* 62, 80–86. doi: 10.1046/j.1365-2672.2002.01545.x

Li, Y., Hill, C. B., Carlson, S. R., Diers, B. W., and Hartman, G. L. (2007). Soybean aphid resistance genes in the soybean cultivars Dowling and Jackson map to linkage group M. *Mol. Breed.* 19, 25–34. doi: 10.1007/s11032-006-9039-9

Liedl, B. E., Lawson, D. M., White, K. K., Siiapiho, J. A., Cohen, D. E., Carson, W. G., et al. (1995). Acylsugars of wild tomato *Lycopersicon pennellii* alters settling and reduces oviposition of *Bemisia argentifolii* (Homoptera: Aleyrodidae). *J. Econ. Entomol.* 88, 742–748. doi: 10.1371/journal.pone.0033064

Liu, X. M., Smith, C. M., Friebe, B. R., and Gill, B. S. (2005). Molecular mapping and allelic relationships of Russian wheat aphid-resistance genes. *Crop Sci.* 45, 2273–2280. doi: 10.2135/cropsci2004.0704

Lombaert, E., Carletto, J. R. M., Piotte, C., Fauvergue, X., Lecoq, H., Vanlerberghe-Masutti, F., et al. (2009). Response of the melon aphid, *Aphis gossypii*, to host-plant resistance: evidence for high adaptive potential despite low genetic variability. *Entomol. Exp. Appl.* 133, 46–56. doi: 10.1111/j.1570-7458.2009.00904.x

Lüthy, B., and Matile, P. (1984). The mustard oil bomb: rectified analysis of the subcellular organisation of the myrosinase system. *Biochem. Physiol. Pflanz.* 179, 5–12. doi: 10.1016/S0015-3796(84)80059-1

Macel, M., Bruinsma, M., Dijkstra, S., Ooijendijk, T., Niemeyer, H., and Klinkhamer, P. L. (2005). Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *J. Chem. Ecol.* 31, 1493–1508. doi: 10.1007/s10886-005-5793-0

Meihls, L. N., Higdon, M. L., Ellersieck, M. R., Tabashnik, B. E., and Hibbard, B. E. (2012). Greenhouse-selected resistance to Cry3Bb1-producing corn in three western corn rootworm populations. *PLoS ONE* 7:e51055. doi: 10.1371/journal.pone.0051055

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. (1998). The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10, 1307–1319. doi: 10.1105/tpc.10.8.1307

Moreno, A., Garzo, E., Fernandez-Mata, G., Kassem, M., Aranda, M. A., and Fereres, A. (2011). Aphids secrete watery saliva into plant tissues from the onset of stylet penetration. *Entomol. Exp. Appl.* 139, 145–153. doi: 10.1111/j.1570-7458.2011.01117.x

Mound, L. A., and Halsey, S. H. (1978). *Whitefly of the World, A Systematic Catalog of the Aleyrodidae (Homoptera) with Host Plant and Natural Enemy Data*. New York: John Wiley & Sons.

Navas-Castillo, J., Fiallo-Olivé, E., and Sánchez-Campos, S. (2011). Emerging virus diseases transmitted by whiteflies. *Annu. Rev. Phytopathol.* 49, 219–248. doi: 10.1146/annurev-phyto-072910-095235

Nombela, G., Williamson, V. M., and Muñiz, M. (2003). The root-knot nematode resistance gene Mi-1.2 of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol. Plant Microbe Interact.* 16, 645–649. doi: 10.1094/MPMI.2003.16.7.645

Peñalver Cruz, A., Arida, A., Heong, K. L., and Horgan, F. G. (2011). Aspects of brown planthopper adaptation to resistant rice varieties with the Bph3 gene. *Entomol. Exp. Appl.* 141, 245–257. doi: 10.1111/j.1570-7458.2011.01193.x

Peng, J., Bai, Y., Haley, S., and Lapitan, N. (2009). Microsatellite-based molecular diversity of bread wheat germplasm and association mapping of wheat resistance to the Russian wheat aphid. *Genetica* 135, 95–122. doi: 10.1007/s10709-008-9262-x

Peng, J., Wang, H., Haley, S. D., Peairs, F. B., and Lapitan, N. L. V. (2007). Molecular mapping of the Russian wheat aphid resistance gene Dn2414 in wheat. *Crop Sci.* 47, 2418–2429. doi: 10.2135/cropsci2007.03.0137

Phillips, M. A., and Croteau, R. B. (1999). Resin-based defenses in conifers. *Trends Plant Sci.* 4, 184–190. doi: 10.1016/S1360-1385(99)01401-6

Pichersky, E., and Lewinsohn, E. (2011). Convergent evolution in plant specialized metabolism. *Annu. Rev. Plant Biol.* 62, 549–566. doi: 10.1146/annurev-arplant-042110-103814

Price, D. R., and Gatehouse, J. A. (2008). RNAi-mediated crop protection against insects. *Trends Biotechnol.* 26, 393–400. doi: 10.1016/j.tibtech.2008.04.004

Qiu, Y. F., Guo, J. P., Jing, S. L., Zhu, L. L., and He, G. C. (2010). High-resolution mapping of the brown planthopper resistance gene Bph6 in rice and characterizing its resistance in the 9311 and Nipponbare near isogenic backgrounds. *Theor. Appl. Genet.* 121, 1601–1611. doi: 10.1007/s00122-010-1413-7

Ramsey, J., Wilson, A., De Vos, M., Sun, Q., Tamborindeguy, C., Winfield, A., et al. (2007). Genomic resources for *Myzus persicae*: EST sequencing, SNP identification, and microarray design. *BMC Genomics* 8:423. doi: 10.1186/1471-2164-8-423

Rodriguez, P. A., and Bos, J. I. (2013). Toward understanding the role of aphid effectors in plant infestation. *Mol. Plant Microbe Interact.* 26, 25–30. doi: 10.1094/MPMI-05-12-0119-FI

Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., and Williamson, V. M. (1998). The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9750–9754. doi: 10.1073/pnas.95.17.9750

Sato, S., Tabata, S., Hirakawa, H., Asamizu, E., Shirasawa, K., Isobe, S., et al. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485, 635–641. doi: 10.1038/nature11119

Schnable, P. S., Ware, D., Fulton, R. S., Stein, J. C., Wei, F., Pasternak, S., et al. (2009). The B73 Maize genome: complexity, diversity, and dynamics. *Science* 326, 1112–1115. doi: 10.1126/science.1178534

Shaver, T. N., and Parrott, W. L. (1970). Relationship of larval age to toxicity of gossypol to bollworms, tobacco budworms, and pink bollworms. *J. Econ. Entomol.* 63, 1802–1804.

Smith, C. M., and Clement, S. L. (2012). Molecular bases of plant resistance to arthropods. *Annu. Rev. Entomol.* 57, 309–328. doi: 10.1146/annurev-ento-120710-100642

Stahl, E. (1888). *Pflanzen und Schnecken. Eine biologische Studie über die Schutzmittel der Pflanzen gegen Schneckenfraß*. Jena: G. Fischer.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R., and Baldwin, I. T. (2004). Nicotine's defensive function in nature. *PLoS Biol.* 2:e217. doi: 10.1371/journal.pbio.0020217

Stewart, C. N., Adang, M. J., All, J. N., Raymer, P. L., Ramachandran, S., and Parrott, W. A. (1996). Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis* crylAc gene. *Plant Physiol.* 112, 115–120. doi: 10.1104/pp.112.1.115

Szczepanik, M., Dams, I., and Wawrzenczyk, C. (2005). Feeding deterrent activity of terpenoid lactones with the p-menthane system against the Colorado potato beetle (*Coleoptera*: *Chrysomelidae*). *Environ. Entomol.* 34, 1433–1440. doi: 10.1603/0046-225X-34.6.1433

Tang, T., Zhao, C. Q., Feng, X. Y., Liu, X. Y., and Qiu, L. H. (2012). Knockdown of several components of cytochrome P450 enzyme systems by RNA interference enhances the susceptibility of *Helicoverpa armigera* to fenvalerate. *Pest Manag. Sci.* 68, 1501–1511. doi: 10.1002/ps.3336

Thabuis, A. P. P., Teekens, K. C., and Van Herwijnen, Z. O. (2011). *Lettuce That is Resistant to the Lettuce Aphid Nasonovia ribisnigri Biotype1*. WO Patent WO/2011/058,192.

Tolmay, V., Lindeque, R., and Prinsloo, G. (2007). Preliminary evidence of a resistance-breaking biotype of the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov)(Homoptera: Aphididae), in South Africa. *Afr. Entomol.* 15, 228. doi: 10.4001/1021-3589-15.1.228

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*

250, 1251–1253. doi: 10.1126/science.250.4985.1251

Upadhyay, S. K., Chandrashekar, K., Thakur, N., Verma, P. C., Borgio, J. F., Singh, P. K., et al. (2011). RNA interference for the control of whiteflies (*Bemisia tabaci*) by oral route. *J. Biosci.* 36, 153–161. doi: 10.1007/s12038-011-9009-1

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., Debeuckeleer, M., Dean, C., et al. (1987). Trangenic plants protected from insect attack. *Nature* 328, 33–37. doi: 10.1038/328033a0

Wang, T., Xu, S. S., Harris, M. O., Hu, J. G., Liu, L. W., and Cai, X. W. (2006). Genetic characterization and molecular mapping of *Hessian fly* resistance genes derived from *Aegilops tauschii* in synthetic wheat. *Theor. Appl. Genet.* 113, 611–618. doi: 10.1007/s00122-006-0325-z

Whitehorn, P. R., O'Connor, S., Wackers, F. L., and Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* 336, 351–352. doi: 10.1126/science.1215025

Will, T., Tjallingii, W. F., Thönnessen, A., and Van Bel, A. J. (2007). Molecular sabotage of plant defense by aphid saliva. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10536–10541. doi: 10.1371/journal.pone.0046903

Yu, G. T., Cai, X. W., Harris, M. O., Gu, Y. Q., Luo, M. C., and Xu, S. S. (2009). Saturation and comparative mapping of the genomic region harboring *Hessian fly* resistance gene H26 in wheat. *Theor. Appl. Genet.* 118, 1589–1599. doi: 10.1007/s00122-009-1006-5

Zalucki, M. P., Brower, L. P., and Alonso-M, A. (2001). Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae *Danaus plexippus* feeding on the sandhill milkweed *Asclepias humistrata*. *Ecol. Entomol.* 26, 212–224. doi: 10.1046/j.1365-2311.2001.00313.x

Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., and He, G. (2011). Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. *PLoS ONE* 6:e20504. doi: 10.1371/journal.pone.0020504

Zhang, G. R., Gu, C. H., and Wang, D. C. (2009). Molecular mapping of soybean aphid resistance genes in PI 567541B. *Theor. Appl. Genet.* 118, 473–482. doi: 10.1007/s00122-008-0914-0

Zhang, G. R., Gu, C. H., and Wang, D. C. (2010). A novel locus for soybean aphid resistance. *Theor. Appl. Genet.* 120, 1183–1191. doi: 10.1007/s00122-009-1245-5

Zhang, X., Tiewsiri, K., Kain, W., Huang, L. H., and Wang, P. (2012). Resistance of *Trichoplusia ni* to *Bacillus thuringiensis* toxin Cry1Ac is independent of alteration of the Cadherin-like receptor for cry toxins. *PLoS ONE* 7:e35991. doi: 10.1371/journal.pone.0035991

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2013; accepted: 08 June 2013; published online: 27 June 2013.

*Citation: VanDoorn A and de Vos M (2013) Resistance to sap-sucking insects in modern-day agriculture. Front. Plant Sci. **4**:222. doi: 10.3389/fpls.2013.00222*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 VanDoorn and de Vos. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 11 June 2013
doi: 10.3389/fpls.2013.00185

Where do herbivore-induced plant volatiles go?

Jarmo K. Holopainen* and James D. Blande

Department of Environmental Science, University of Eastern Finland, Kuopio, Finland

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Martin Heil, Centro de Investigación y de Estudios Avanzados del I.P.N.—Unidad Irapuato, Mexico
Matthias Erb, Max Planck Institute for Chemical Ecology, Germany

****Correspondence:***
Jarmo K. Holopainen, Department of Environmental Science, University of Eastern Finland, Yliopistonranta 1, PO Box 1627, FI-70211 Kuopio, Finland
e-mail: jarmo.holopainen@uef.fi

Herbivore induced plant volatiles (HIPVs) are specific volatile organic compounds (VOC) that a plant produces in response to herbivory. Some HIPVs are only produced after damage, while others are also produced by intact plants, but in lower quantities. Among the known functions of HIPVs are within plant volatile signaling to activate systemic plant defenses, the priming and activation of defenses in neighboring plants and the attraction of natural enemies of herbivores. When released into the atmosphere a plant's control over the produced compounds ends. However, many of the HIPVs are highly reactive with atmospheric oxidants and their atmospheric life times could be relatively short, often only a few minutes. We summarise the potential ecological and atmospheric processes that involve the reaction products of HIPVs in their gaseous, liquid and solid secondary organic aerosol (SOA) forms, both in the atmosphere and after deposition on plant surfaces. A potential negative feedback loop, based on the reactions forming SOA from HIPVs and the associated stimulation of sun screening cloud formation is presented. This hypothesis is based on recent field surveys in the geographical areas facing the greatest degree of global warming and insect outbreaks. Furthermore, we discuss how these processes could benefit the individual plant or conspecifics that originally released the HIPVs into the atmosphere. Further ecological studies should aim to elucidate the possible reasons for biosynthesis of short-lived volatile compounds to have evolved as a response to external biotic damage to plants.

Keywords: terpenoids, monoterpenes, green leaf volatiles, semivolatiles, secondary aerosols

INTRODUCTION

Most vascular plants constitutively emit volatile organic compounds (VOCs), but emissions may substantially increase and diversify under conditions of abiotic and biotic stress (Holopainen and Gershenzon, 2010). Feeding by herbivores was found to induce the emission of novel volatile compounds often referred to as herbivore-induced plant volatiles (HIPVs) (Hare, 2011) that attract natural enemies of the herbivores. This was shown for the first time in seminal studies conducted with spider mites and predatory mites by Dicke and Sabelis (1988) and with moth larvae and parasitic wasps by Turlings et al. (1990). Since the first studies of plant volatiles, interest in the synthesis and control of volatiles by plants and their ecological and atmospheric functions have increased substantially (Dicke and Loreto, 2010) and several other ecophysiological and ecological effects of constitutive and inducible plant volatiles have been described (Laothawornkitkul et al., 2009; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Peñuelas and Staudt, 2010).

HIPVs are often expected to increase the fitness of the emitting plant either directly or indirectly (Dicke, 2009). Direct defense reduces herbivore approach and attack or decreases the herbivore's consumption rate, but the roles of HIPVs could be complicated. For instance, the ratios of compounds in the typical HIPV profiles of *Quercus robur* trees correlate with the tree's susceptibility to herbivore damage (Ghirardo et al., 2012). Trees representive of an herbivore-resistant phenotype emitted HIPVs that included the sesquiterpenes α-farnesene and germacrene D and were avoided by females of the defoliating moth *Tortrix viridian* (Ghirardo et al., 2012). However, in the same outbreak area trees emitting other typical HIPVs including the monoterpene β-ocimene and homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) were susceptible and were largely defoliated (Ghirardo et al., 2012).

Indirect defense involves the recruitment of natural enemies of herbivores to increase predation or parasitism rates and eventually reduce damage. Many laboratory reports have given support to this hypothesis (Hare, 2011). However, there is scarce field-based -evidence that attraction of natural enemies by HIPVs actually reduces herbivore populations (Kessler and Baldwin, 2001). Furthermore, studies to show improved Darwinian fitness in plants emitting HIPVs, i.e., more offspring in the next generation, are lacking (Hare, 2011). A current ecological view of the role of HIPVs is as components of a wider infochemical web, including e.g., pollinators and root synergists, that overlay the food webs of a community rather than simply defending against attackers or attracting carnivores (Dicke and Baldwin, 2010). Ghirardo et al. (2012) concluded that for *Q. robur*, the strategy of emitting herbivore-repellent rather than natural enemy attracting HIPVs, appears to be the better mechanism for avoiding defoliation. However, when plants are influenced by a diverse community of chewing and sucking herbivores, a single HIPV compound could be an efficient repellent against one herbivore, but act as an attractant of another herbivore and many of the community's predators and parasitoids (Xiao et al., 2012).

HIPVs are not only emitted by aboveground parts of plants. Many plant species have an extensive root system where HIPV-releasing resins are stored (Kivimaenpää et al., 2012) or HIPVs synthesized (Degenhardt et al., 2009) and released in the soil air space and eventually to the atmosphere. Root feeding by herbivores induces the emission of HIPVs by the root system, which act as belowground attractants for parasitic nematodes (van Tol et al., 2001; Rasmann et al., 2005). Aboveground herbivory also has a systemic impact on belowground HIPV production and vice versa (Erb et al., 2009). Defoliation of *Pinus syvestris* by diprionid sawflies induced substantial HIPV emissions from the shoots, but resulted in significant reduction in monoterpene and sesquiterpene emissions from the root system (Ghimire et al., 2013). This was expected to be related to reduced carbon allocation to below-ground parts after defoliation. Arbuscular mycorrhizal (AM) infection of bean plant roots affected the HIPV composition emitted by foliage by making it less attractive to predators (Schausberger et al., 2012), whereas an ectomycorrhizal (EM) root symbiont did not affect the terpene pool of pine needles (Manninen et al., 1998). These studies highlight the complex and systemic nature of HIPVs and the need for a holistic view of a plant's volatile emissions and their various related roles.

HIPV compounds typically have relatively short atmospheric lifetimes after release from plants, which may limit the efficiency with which they attract natural enemies of herbivores and mediate other ecological interactions (Yuan et al., 2009). However, reactive VOCs have various functions in the atmospheric processes, such as formation of ozone in NO_x polluted atmospheres (Atkinson and Arey, 2003), formation of OH-radicals (Mentel et al., 2009), formation of organic nitrates (Pratt et al., 2012) and formation of secondary aerosols (SOA) (Joutsensaari et al., 2005; Kiendler-Scharr et al., 2009; Mentel et al., 2009; Virtanen et al., 2010). Laothawornkitkul et al. (2009) divided the various functions of plant VOCs into three broad categories; biological, chemical and physical.

In this review we focus on the different roles and fate of inducible VOC molecules after release from the VOC synthesizing plant including biological, chemical and physical aspects. Furthermore, we discuss why biosynthesis and emission of short-lived volatile compounds has evolved as a general response to external biotic damage to plants. We will pay attention to the biological and ecological role of VOCs post-emission and their atmospheric reaction products. These effects may take place in their gaseous, liquid and solid organic particulate forms in the atmosphere, but probably also after deposition on plant surfaces. We also discuss how the post-emission reaction products of VOCs may improve plant fitness. Finally we try to present the potential routes that the carbon fixed by a plant and bound in VOC molecules will ultimately take.

MAJOR GROUPS OF HIPVs

The majority of the typically documented herbivore-induced plant volatiles can be classified in three major chemical groups based on their biosynthesis pathways or their known within-plant functions (Holopainen and Gershenzon, 2010). First, and the dominating group of constitutively emitted VOCs and HIPVs in many plant species, are the terpenoids, which are produced by two separate pathways, one active in plastids (MEP) and one (MVA) in the cytosol (Loreto and Schnitzler, 2010; Maffei, 2010). The volatile terpenoids (**Figure 1**) include the five carbon (C5) isoprene molecule and a range of molecules comprising various multiples of this basic C5 unit, including monoterpenes (C10), homoterpenes (C11 or C16) and sesquiterpenes (C15). The second group is the C6 lipoxygenase (LOX) products better known as Green Leaf Volatiles (GLVs). GLVs, such as (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate are compounds released after mechanical or other destructive damage to cell membranes (Maffei, 2010; Holopainen, 2011). The third group is the volatile aromatic compounds such as methyl salicylate and indole produced by the shikimate pathway and containing an aromatic ring (Maffei, 2010). In addition to these three main groups there are a multitude of other volatile compounds that are specific to varying degrees such as to an order, genus or species. The volatile plant hormone ethylene has often been considered an inducible volatile and has several functions in plant physiological processes

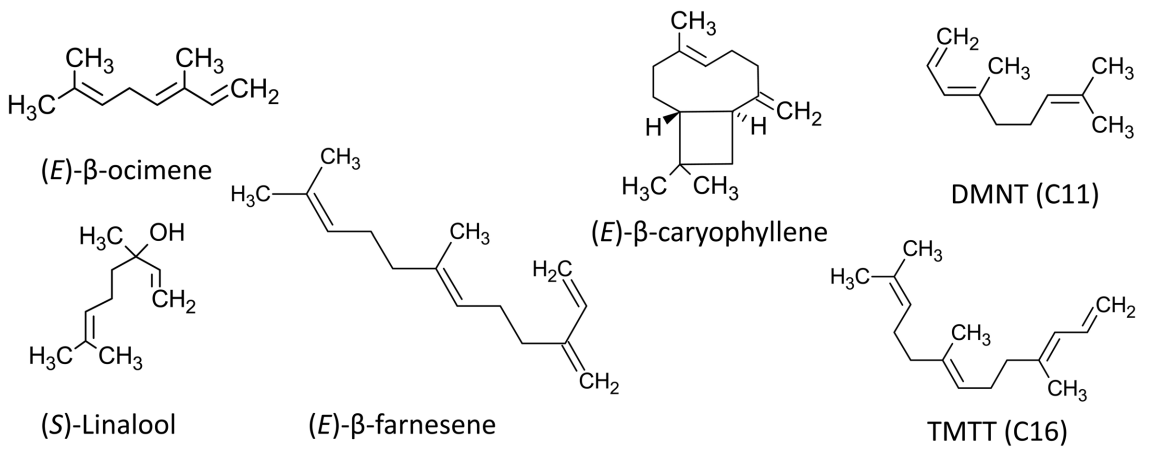


FIGURE 1 | Examples of molecular structures of isoprenoid HIPVs showing double bonds.

and growth, while membrane-bound ethylene receptors are well known (Holopainen and Gershenzon, 2010).

HIPVs can also be classified based on their volatility or the atmospheric life times of the compounds in atmospheres with standardized levels of reactive scavengers such as ozone (O_3), nitrate (NO_3) or hydroxyl (OH^-) radicals (see Holopainen, 2011; Holopainen et al., 2013 and **Table 1**). These scavengers appear in higher concentrations in polluted air, but they are common in ambient air and are involved in important chemical and physical processes in the atmosphere. Significantly, plant VOCs also participate in their formation (Hallquist et al., 2009). A greater number of C-C double bonds in the VOC molecules (**Figure 1**) will make them more prone to reactions with atmospheric radicals, degrade faster and form more particles (Hoffmann et al., 1997; Atkinson and Arey, 2003; Pinto et al., 2010).

SUCCESSION OF INDUCED VOCS

When a plant is attacked by a herbivore, there is a succession of different inducible volatile compounds appearing in the emission bouquet. Inducible defenses of herbivore-attacked plants involve herbivore perception, transcriptional responses, protein formation and biosynthetic responses (Dicke, 2009). In plant species storing a constitutively synthesized volatile mixture, these compounds will volatilize very rapidly upon rupture of storage structures such as glandular trichomes and constitute the first response to external damage (Jansen et al., 2011). In this case the emission is the result of mechanical injury, which will occur before the attacker induces the biosynthesis of volatiles in growing plant tissues. The GLV emissions have a time lag between herbivore-feeding and compound emission of from just a few seconds to several minutes and these compounds show very rapid response to mechanical or biological damage to cell membranes. The C18 fatty acids of membranes are cleaved to C12 and C6 compounds by hydroperoxide lyases with 3-*Z*-hexenal (aldehyde) being the first C6 GLV compound synthesized by the lipoxygenase (LOX)/lyase pathway (Maffei, 2010). This compound is then converted to other common C6 GLVs such as (*E*)-2-hexenal (aldehyde), 3-hexenol (alcohol) and (*Z*)-3-hexenyl acetate (ester) (Shiojiri et al., 2006). Geometrid moth feeding induces emissions of GLVs, which peak soon after larval feeding starts on deciduous tree foliage (Blande et al., 2007), while on-line monitoring of HIPVs shows that GLV peaks can even reveal the timing of larval feeding periods (Schaub et al., 2010).

Continuous mechanical injury (Mithofer et al., 2005), oral secretions of herbivores (Turlings et al., 1990), plant cell membrane damage by biotrophic fungal leaf pathogens (Toome et al., 2010; Jansen et al., 2011) or bacterial pathogens (Yi et al., 2009) elicit signal transduction pathways that are mediated by phytohormones such as jasmonic acid (JA) and ethylene in the case of chewing herbivores, salicylic acid (SA) and ethylene in the case of fungal pathogens and result in the synthesis of typical HIPV terpenoids and aromatic compounds (Jansen et al., 2011). There is variability in the succession of different herbivore-induced terpenoid emissions, which could be partly due to variable allocation of precursors into the different biosynthesis pathways. Biosynthesis of the homoterpene (*E*)-DMNT, for example, could originate predominantly from the MVA-pathway in herbivore-stressed plants, while the fungal elicitor alamethicin stimulates the biosynthesis of (*E*)-DMNT via the MEP-pathway (Bartram et al., 2006). In *Alnus* foliage damaged by geometrid moths,

Table 1 | Examples of typical herbivore induced plant volatiles (HIPV) and their estimated atmospheric life times in the detected concentrations of three major reactive air pollutants in less polluted areas.

	HIPV compounds and their estimated atmospheric life times			
Atmospheric pollutants	**<10 min**	**10 min–1 h**	**1 h–24 h**	**<24 h**
Ozone (O_3)	**β-Caryophyllene**	*cis*-/*trans*-Ocimene, Linalool, DMNT, TMTT **β-Farnesene**	α-Pinene, β-Phellandrene, Limonene *cis*-3-Hexenyl acetate, *cis*-3-Hexen-1-ol, *cis*-3-Hexenal[1)]	METHYL SALICYLATE
Hydroxyl radical (OH)		*cis*-/*trans*-Ocimene, β-Phellandrene, Limonene, Linalool, **β-Caryophyllene**, DMNT, TMTT	α-Pinene, *cis*-3-Hexenyl acetate, *cis*-3-Hexen-1-ol *cis*-3-Hexenal	METHYL SALICYLATE
Nitrate radical (NO_3)	α-Pinene, *cis*-/*trans*-Ocimene, β-Phellandrene, Limonene Linalool, **β-Caryophyllene** DMNT, TMTT,	**β-Farnesene** METHYL SALICYLATE	*cis*-3-Hexenyl acetate, *cis*-3-Hexen-1-ol	

Monoterpenes (normal font), sesquiterpenes (bold font), homoterpenes (capital font), GLVs (underlined) and aromatic compounds (small caps)

Data is compiled from the following sources: Atkinson and Arey (2003), Arneth and Niinemets (2010), Roger Atkinson, personal communication., Ulo Niinemets, personal communication, Canosa-Mas et al. (2002), Holopainen et al. (2013).

Pollutant concentrations used were O3: 30 ppb 24-h average, OH: 0.074 pmol mol^{-1} 12-h average, NO_3: 9.3 pmol mol^{-1}, 24-h average. OH concentrations can be measured mostly in day time and NO_3 only in night time, due to solar UV-radiation (Holopainen et al., 2013).

emissions of (*E*)-β-ocimene and (*E*)-DMNT peaked on day 3 (Copolovici et al., 2011). In the same study the emission kinetics of the sesquiterpene (*E*,*E*)-α-farnesene tended to be biphasic with peaks on days 2 and 4 after the start of larval feeding. Emission rates of the induced LOX products, (*E*)-β-ocimene and (*E*,*E*)-α-farnesene were positively correlated with the number of larvae feeding (Copolovici et al., 2011).

Variation in the feeding strategies of herbivores can result in profound variation in the volatiles emitted by damaged plants. Herbivores that feed via stylets, such as aphids, inflict apparently minor mechanical damage, but still induce the emission of a rich blend of volatiles including both GLVs and terpenoids (Gosset et al., 2009). Sustained feeding by aphids and colony growth can also result in large increases in emission of methyl salicylate (Blande et al., 2010), which can take several days to start appearing in emission bouquets. The volatiles induced by chewing herbivores can vary with the life stage of the herbivore, with early instars (first to fourth) of *Pseudaletia separata* larvae inducing different volatile bouquets to larger more advanced larvae (fifth to sixth instars) (Takabayashi et al., 1995). In this case, foraging parasitoids are able to distinguish between the volatile blends induced by potential host larvae (the younger instars) and larvae that are too old to be used as hosts and may actually constitute a threat through their aggressive defensive behaviors (Takabayashi et al., 1995). In an alternative system, with *Pieris brassicae* feeding on Brussels sprouts, *Cotesia glomerata* parasitoids do not appear to determine larval instar through volatile emissions, but can determine presence of suitable hosts through other cues on infested leaves, without necessarily contacting the host itself (Mattiacci and Dicke, 1995). Elicitors in the saliva of the herbivores are responsible for alterations in the herbivore-induced blend, the specificity and range of which can vary (Mattiacci and Dicke, 1995; Takabayashi et al., 1995; Roda et al., 2004). It has also been shown that deposition of eggs by the Brassica specialising Lepidopteran *Pieris brassicae* can induce changes in the expression of hundreds of genes (Little et al., 2007; Fatouros et al., 2008) and emission of volatiles that are attractive to its parasitic wasps (Fatouros et al., 2012).

In *Salix* hybrid plantlets infected with *Melampsora epitea* leaf rust fungi, the total monoterpene emissions did not change although a stress-signaling compound (*Z*)-β-ocimene showed an increase in infected plants on several days. The infection also increased the emission of sesquiterpenes and LOX products by factors of 175-fold and 10-fold, respectively (Toome et al., 2010). The induced VOCs showed two clear peaks during the experiment; at 6–7 and 12 days post-infection, whereby the relative volatile emission signal increased to about 6-fold that of uninfected plants. Peak emission periods were directly connected to rust infection with day 6 corresponding with the appearance of the first rust pustules on the leaves and day 12 corresponding with necrosis developing around several pustules (Toome et al., 2010).

Isoprene -a major biogenic VOC released from vegetation— and some monoterpenes are constitutively emitted, but are induced by elevated temperatures, which can greatly enhance the overall emission of these compounds (Loreto and Schnitzler, 2010). Of these compounds, isoprene in particular is not induced by fungal pathogens or insect feeding. Toome et al. (2010) reported that isoprene emissions from *Salix* hybrids with rust-infected leaves decreased 3-fold compared to controls, Ghirardo et al. (2012) found that *Tortrix viridiana* larval feeding did not affect isoprene emissions from *Quercus robur* and Blande et al. (2007) found that emission of isoprene in *Populus* hybrids did not respond significantly to geometrid moth or leaf weevil feeding.

Several studies have implicated the blend of volatiles emitted by plants either constitutively, or after herbivore-damage, to play an important role in the behavior of foraging insects. Aphids in particular have been shown to utilize blends of volatiles emitted by undamaged plants as host location cues (Bruce and Pickett, 2011; Webster, 2012), while parasitoids have also been shown to utilize chemical blends to locate their hosts (Pareja et al., 2009; Clavijo McCormick et al., 2012). It is clear that the blend emitted by plants can evolve as the degree of herbivore-induced stress changes. Differences in degradation rates of certain chemicals could result in rapid changes to the blend, by reducing the proportions of compounds relative to each other (Pinto et al., 2007a), which could render the blend less effective as a cue for foraging insects (Pinto et al., 2007b).

KNOWN FUNCTIONS AND EFFECTS OF HIPVs

HIPVs AND PLANT ADAPTATION TO ABIOTIC STRESSES

Emissions of plant volatiles are strongly dependent on physical conditions and the changes in these conditions could rapidly "induce" emissions or alter emission dynamics. Ambient temperature and light conditions affect synthesis and emissions of terpenoids particularly strongly (Niinemets et al., 2004). Emissions of many HIPVs are also induced by a range of abiotic factors such as drought, CO_2 level and ozone (Vuorinen et al., 2004; Dicke and Loreto, 2010; Peñuelas and Staudt, 2010). However, inducibility of HIPVs can be affected by environmental conditions during attack by herbivores (Gouinguene and Turlings, 2002; Holopainen and Gershenzon, 2010). In plant leaves isoprene and monoterpenes have been shown to protect the photosynthetic apparatus of plants from damage under high temperature episodes and maintain the photosynthetic capacity under temperature increase (Behnke et al., 2007; Loreto and Schnitzler, 2010).

WITHIN PLANT SIGNALS

If HIPVs are considered to improve the fitness of a unitary plant, their role in signaling between vascularly separate parts of an individual could be one of their primary functions (Karban et al., 2006; Frost et al., 2007; Rodriguez-Saona et al., 2009; Shiojiri et al., 2009). Such observations, whereby volatile signaling between herbivore-damaged and intact branches results in unwounded branches being better protected against subsequent herbivore-attack, have been made in several plant species, including lima bean, blueberry, sagebrush and hybrid poplar (Karban et al., 2006; Frost et al., 2007; Heil and Silva Bueno, 2007; Rodriguez-Saona et al., 2009). The relevant signals might be mixtures of HIPVs (Rodriguez-Saona et al., 2009) or single compounds such as the GLV (*Z*)-3-hexenyl acetate (Frost et al., 2008). When distant parts of a plant are exposed to a HIPV signal, priming of defenses might occur. This involves expression of defense genes being primed upon receipt of a volatile signal and

plants subsequently responding more vigorously to herbivore-attack than non-primed plants (Engelberth et al., 2004; Heil and Kost, 2006; Kessler et al., 2006; Heil and Silva Bueno, 2007; Frost et al., 2008). The advantage of volatile signaling is that it functions between vascularly disconnected plant parts, but also acts as a more rapid method of communication than vascular signals (Frost et al., 2007).

HIPVs IN PLANT TO PLANT SIGNALLING

HIPVs that elicit defense responses within-plant may also prime or induce defenses in neighboring plants (Karban et al., 2003, 2006; Heil and Kost, 2006). This process has been observed to occur between conspecific (Karban and Shiojiri, 2009) and heterospecific individuals (Heil and Karban, 2010). Interestingly, VOCs from undamaged plants have also been shown to have an impact on the defenses of their neighbors, which indicates that it is not always the specific HIPVs that are responsible for inducing defenses (Glinwood et al., 2004, 2009). The mechanisms involved in volatile mediated plant-plant interactions have yet to be fully elucidated, although we are now seeing regular demonstrations of the complexity of the process. In sagebrush, plant-plant signaling has been shown to be more efficient between clonal cuttings of the same plant than between non-clonal conspecifics (Karban and Shiojiri, 2009; Karban et al., 2013). This indicates a degree of self or kin recognition to occur in receiver plants. It was also recently shown that hybrid aspen exposed to damaged neighbors temporally regulate two indirect defense responses, the emission of VOCs and the secretion of extra-floral nectar (EFN). EFN secretion was induced by the exposure, but not primed, whereas the emission of HIPVs was primed but not immediately induced by the exposure (Li et al., 2012), which further indicates complexity in the responses of plants to volatile signals.

The mechanisms of volatile-mediated interactions between plants require further elucidation, but there is some knowledge about the relevant signaling compounds. As for within-plant signaling, the GLVs and particularly the compound (*Z*)-3-hexenyl acetate have been implicated as providing a key signal (Kost and Heil, 2006). Other GLVs can also induce defenses in receiver plants, but in lima bean they reduce in efficiency as a signal the more they differ from (*Z*)-3-hexenyl acetate, which is the main GLV emitted by that species (Heil et al., 2008). A recent study of early responses to volatile signals by tomato receiver plants has shown that a range of volatile compounds induce depolarization of plasma membranes and cytosolic calcium flux, with green-leaf volatiles and low molecular weight molecules having a stronger impact on these responses than higher molecular weight compounds such as monoterpenes and sesquiterpenes (Zebelo et al., 2012). These early plant responses combined with the accumulating evidence in support of gene transcriptional changes in response to volatile signals could be essential in understanding the mechanisms of plant-plant signaling.

A longer exposure period and greater accumulation of HIPV compounds from damaged neighboring plants results in a greater degree of resistance against bacterial plant pathogens (Angeles Lopez et al., 2012; Giron-Calva et al., 2012) and herbivorous mites (Choh et al., 2004). This suggests that the occurrence of plant-plant signaling under natural conditions will be largely dependent on the quantity of volatiles emitted by damaged plants, the proximity of the receiver to the emitter, the sensitivity of the receiving plant and the suitability of the environment for transfer of signals. Indeed, under natural conditions the distances over which plant-plant signaling occurs are rather short, usually across distances of less than a meter (Dolch and Tscharntke, 2000; Karban et al., 2003, 2006; Heil and Adame-Álvarez, 2010). Under laboratory conditions the presence of ozone has been shown to significantly reduce the distance of signaling in lima bean (Blande et al., 2010).

VOCs IN DIRECT DEFENCE AGAINST HERBIVORES AND PATHOGENS

Direct defenses could affect behavior, performance or fecundity of the attacker. In many plant species specific VOCs produced by plants give them direct protection against feeding herbivores by repelling them from attacking or by deterring feeding (Egigu et al., 2011). Herbivore preference is often based on the relative proportions of constitutive volatile compounds such as the ratios of α-pinene and β-pinene (Evans et al., 1991). A whole blend of compounds that individually elicit negative responses can be attractive to aphids searching for a host plant (Webster et al., 2010). Feeding by a herbivore affects the proportions of various constitutive VOCs (e.g., Blande et al., 2007) and may thus influence the impact of HIPVs on herbivores. Specific VOCs induced by biotic stress also have specific effects on the attacking organisms, including, microbial pathogens or various herbivores. HIPVs may also have a signaling effect, whereby they repel conspecific individuals. It has been shown that female moths restrict themselves from laying eggs on plants damaged by conspecific larvae and that this decision is based on the recognition of HIPVs (De Moraes et al., 2001). Such behavior has likely evolved to hinder overcrowding, but the opposite has been observed whereby a mixture of major HIPVs is highly attractive to host seeking oligophagous moth females (Sun et al., 2012). These traits could have coevolved whereby egg induced volatiles attract herbivore females over a longer distances and indicate that suitable host plants and mating males are available in the habitat, but the final oviposition decision involves avoidance of the actual HIPV emitting plant in favor of neighbors. Utilization of foraging cues in this way could involve a number of steps such as host habitat location, host location, host recognition, host acceptance, host suitability, host (regulation and) consumption, which is similar to the classical six step framework for successful foraging by parasitoids (Vinson, 1976).

Interactions between VOCs and pathogens have not been studied extensively, but there is indication that VOCs can reduce pathogen growth. Monoterpenes (e.g., Tsao and Zhou, 2000) and GLVs (Shiojiri et al., 2006) inhibit the growth of common fungal leaf and fruit pathogens, while the sesquiterpene (*E*)-β-caryophyllene has been shown to offer *Arabidopsis thaliana* flowers a degree of defense against a bacterial pathogen (Huang et al., 2012). Exposure to the GLV (Z)-3-hexenal, resulted in significantly reduced lesions on *Botrytis cinerea* infected Arabidopsis plants (Shiojiri et al., 2006). Repellent effects of HIPVs on plant virus vectors such as aphids may result in lower infection rates and reduce the spread of aphid-transmitted plant virus diseases. However, HIPV traits can also be "hijacked" by some

parasites. Cucumber mosaic virus is such a pathogen. Virus-infected plants are poor food for virus transmitting aphids, but the spread of viruses in a plant population requires feeding by virus-transmitting vector insects. HIPV emissions make infected plants attractive to two virus-vector aphid species. Brief feeding periods on poor quality plants is enough for aphids to receive the virus and then spread it onto healthy plants (Mauck et al., 2010).

HIPVs IN INDIRECT PLANT DEFENCE—ATTRACTION OF NATURAL ENEMIES OF HERBIVORES

The importance of HIPVs in attracting natural enemies of herbivores has been shown in numerous studies, mainly under laboratory conditions. There are substantial differences in composition of HIPVs from the same plant depending on the type of herbivore making the damage. For example, feeding by aphids has been shown to induce emission of methyl salicylate more distinctively than feeding by chewing herbivores (Blande et al., 2010). Feeding by the generalist feeding spider mite *Tetranychus urticae*, induced very distinctive HIPV blends from a range of different host plants (Van den Boom et al., 2002), but the predatory mite *Phytoseiulus persimilis* was still attracted to HIPVs from all of the spider mite-damaged plant species (Van den Boom et al., 2004). Generalist and specialist *Cotesia* spp. parasitoids of Brassicaceous plants seem to lack specificity at the herbivore level, whereas on the plant species level differences in HIPV attractiveness to parasitoids have been found (Geervliet et al., 1994).

OTHER POTENTIAL FUNCTIONAL ROUTES OF HIPVs

The ecological functions of HIPVs described above have all been established experimentally. All rely mechanistically on the responses of a receiver organism to volatile emissions from a plant that has been subjected to a degree of stress or stimulation. While the volatile compounds remain intact they theoretically constitute a signal that can be detected and potentially perceived by organisms of the surrounding community. However, as soon as the chemicals leave the plant there are a range of potential fates or roles that could be played out. In the following section those potential adaptive roles have been classified as roles for intact HIPVs and for reaction products of HIPVs (**Figure 2**). In practice, in the atmosphere HIPVs and constitutively emitted VOCs cannot in most cases be separated from each other and the following description concerns the mixture of both. The following fates of VOCs and the corresponding functions are suggested:

1. Intact volatiles travel in air currents and facilitate the interactions detailed above
2. Intact volatiles, under certain environmental conditions, such as cooling temperatures, adsorb to the surfaces of surrounding vegetation including the emitting plant itself
3. Volatiles react in the atmosphere with ozone or other oxidants and thus form degradation products which could give a spatial and/or temporal dimension to the volatile signal
4. Volatiles react in the presence of NO_x and sunlight to produce ozone plus other degradation products, which could have lower volatility than the original VOCs
5. Degradation products of HIPVs can adsorb to plant surfaces or nucleate in the atmosphere to form secondary organic aerosol (SOA)
6. SOA particles could deposit on plant surfaces and have further ecological effects.

ADSORPTION OF HIPVs ON NEIGBOURING PLANT SURFACES

It has been shown that species specific semivolatile VOCs emitted constitutively by plants can be adsorbed to the surfaces of neighboring plants (Himanen et al., 2010). There is also evidence that constitutively emitted VOCs, particularly monoterpenes, can be taken up through the stomata of neighboring plants (Noe et al., 2008; Bamberger et al., 2011). So far the ecological and ecophysiological functions of these "borrowed" VOCs are not well known, although there is evidence that the adsorbed compounds can protect receiver plants against herbivores (Himanen et al., 2010) or improve indirect defense against herbivores by attracting predators (Choh et al., 2004). Furthermore, we do not know what proportions of VOCs released by plants are adsorbed onto the surfaces of their own foliage. During the day time leaf surfaces are often warmer than the surrounding air, thus some of the semivolatile compounds will possibly 'bounce' between the warmer leaf surface and the colder air before condensation takes place on the colder leaf surface at night.

There is evidence that during colder night-time temperatures semi-volatile sesquiterpenoids condensate on neighboring plant surfaces. In the following morning these compounds were emitted at higher rates than any endogenous VOC compound (Himanen et al., 2010). In the afternoon the concentration of adsorbed compounds on leaf surfaces was rapidly decreased, due to evaporation from the surface as the temperature warmed. This temperature dependent behavior of sesquiterpenoids makes them an ecologically very interesting group of VOCs, because after adsorption to neighboring plant surfaces they may give protection against herbivores which attack during the late evening, night and early morning. Another important group of HIPVs, GLVs, did not show any evidence of accumulation on tested glass surfaces at +12°C or higher temperatures (Schaub et al., 2010).

ATMOSPHERIC REACTIONS OF HIPVs AND THEIR CHEMICAL TRANSFORMATION

After release from the plant leaf tissue, either to be adsorbed to the leaf surface or disperse into the atmosphere, HIPVs are exposed to UV-radiation and various reactive gases which constrain their lifetimes (Kroll and Seinfeld, 2008). Therefore, the ecological functions of the original compounds synthesized by plants will only be active for a limited time, which depends on the dispersal and reactivity of the compound. There could be further potential ecological roles of HIPVs that are related to their relatively high reactivity and the properties of the rapidly formed reaction products, which include various gaseous compounds with lower volatility (Kroll and Seinfeld, 2008) and formation of solid nanoparticles (Joutsensaari et al., 2005; Virtanen et al., 2010) in secondary organic aerosols (SOA). Many of the isoprenoid oxidation products are known to be unpleasant smelling and tasting aldehydes, ketones and organic acids in gaseous or particulate form. For example, smaller SOA nanoparticles

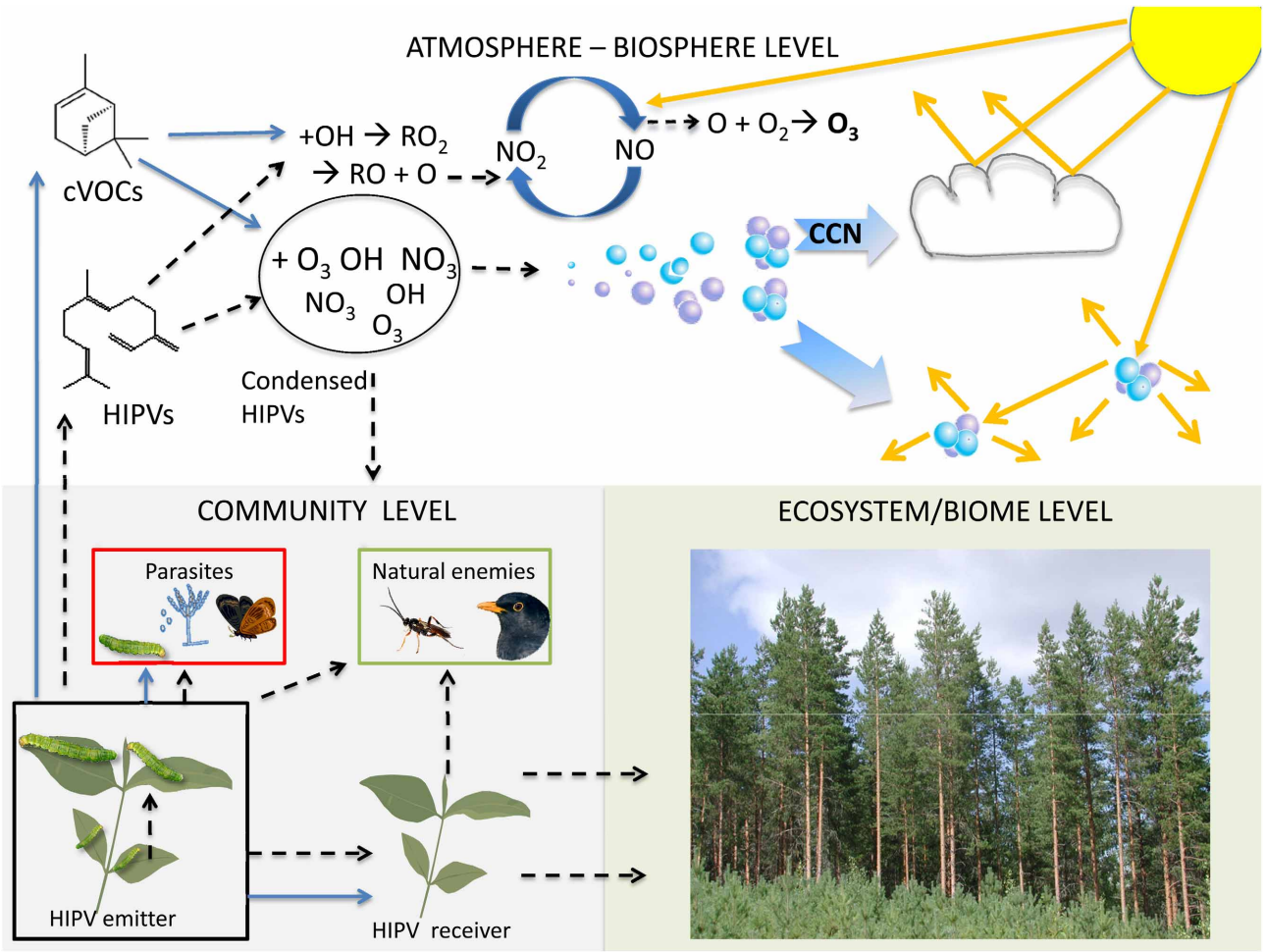


FIGURE 2 | The functions and fates of VOCs are depicted at three levels; community, ecosystem/biome and atmosphere-biosphere. The passages of HIPVs are indicated by broken black arrows, while the passages of constitutively emitted VOCs (cVOCs) are indicated by solid blue arrows. At the community level, the functions of HIPVs include signaling from herbivore-damaged plants to plant parasites, natural enemies of the parasites and neighboring plants. Signalling within-plant via HIPVs from older leaves to younger leaves is also indicated. cVOCs are also known to be involved in host location behaviors of various plant parasites and in signaling between plants. These interactions can generally be considered as mediated by intact volatile compounds emitted by the damaged plants. After emission from plants cVOCs and HIPVs enter the atmosphere-biosphere level where they undergo various reactions that see them either re-enter the community level or have consequences on the ecosystem/biome level. In the atmosphere VOCs are influenced by ozone (O_3), hydroxyl radical (OH) or nitrate radical (NO_3). VOCs may lose their volatility in colder night temperatures and become sticky compounds, which may re-enter the community level as either condensed HIPVs, which adsorb to plant surfaces with effects on various community members, or as reaction products of volatiles and secondary organic aerosol (SOA) particles, which also adsorb to plant surfaces with largely unknown functions. In areas with NOx pollution the oxidation of cVOCs and HIPVs is triggered by hydroxyl radicals (OH) and results in several alkyl peroxy (RO_2) radicals which lead to the conversion of nitric oxide (NO) to nitrogen dioxide (NO_2). In the presence of solar radiation (yellow arrows) reactions are reversible releasing excited O atoms, which can lead to ozone formation. Ozone can then react with other VOCs in the atmosphere to form degradation products and SOA via ozonolysis. Particle growth and formation of cloud condensation nuclei (CCN) will then result in formation of cloud cover leading to enhanced albedo and reduced solar radiation at the ground level. SOA in the lower atmosphere may add diffusion of solar radiation and improve light penetration in canopies. This atmospheric interaction will feed into the ecosystem level through improving photosynthesis efficiency, but also by facilitating a net cooling effect.

(10–20 nm) originating from α-pinene ozonolysis contain carboxylic acids, while larger particles (40 nm) have been shown to have higher concentrations of carbonyl-containing compounds and low molecular weight organic acids (Winkler et al., 2012). Earlier studies have shown that carboxylic acids and organic acids are repellent to aphids (Glinwood et al., 2003). It has also been shown that very low concentrations of aldehydes and ketones can be repellent to pollinating insects such as honey bees, (Mishra and Sihag, 2009), while the precursor monoterpenes and sesquiterpenes emitted by flowers are major floral attractants for bees (Nieuwenhuizen et al., 2009). This indicates that the functional role of plant emitted volatiles can be altered dramatically by degradation in the atmosphere. It is also known that many of the more volatile plant VOCs become less volatile in reactions with

oxidants and atmospheric radicals (Kroll and Seinfeld, 2008). This may also reduce their diffusion and drift away from leaf boundary layers and increase their accumulation on the surfaces of the releasing plant.

FORMATION OF OZONE FROM HIPVs AND OTHER VOCs

"Trees cause more pollution than automobiles do," a famous quotation from USA President Ronald Reagan in 1981, indeed is partially right. When NO and NO_2 (collectively, NOx) levels in the atmosphere are high, as occurs in environments contaminated by smoke and exhaust gases from fossil fuel combustion, VOC oxidation increases ozone levels (Lerdau and Slobodkin, 2002). Solar UV-radiation triggers the oxidation of VOCs by hydroxyl radicals (OH·), which results in several alkyl peroxy (RO_2·) and hydroperoxy (HO_2·) radicals. This will lead to the conversion of NO to NO_2, and thus, promotes O_3 accumulation and the efficient regeneration of the OH radical. Both O_3 and OH radicals can react with other VOCs in the atmosphere (Pinto et al., 2010). In environments with cleaner air and low levels of atmospheric NOx, oxidation of biogenic VOCs removes ozone from the troposphere and promotes secondary aerosol formation by ozonolysis (Lerdau and Slobodkin, 2002; Virtanen et al., 2010). In forest environments, organic nitrates ($RONO_2$) are formed via reactions of isoprene, monoterpenes and sesquiterpenes with NO_3 radicals in the presence of OH radicals and NO. Organic nitrates are removed from the air during precipitation events and thus plant VOCs help to remove NOx from the lower troposphere (Lerdau and Slobodkin, 2002). The depositions of organic nitrogen may influence nitrogen availability from soil to vegetation and hence affect vegetation succession. On the other hand, organic nitrates could also act as atmospheric reservoirs of NOx leading to later formation of ozone and secondary organic aerosols (Pratt et al., 2012).

The ability of plants to control and even promote the formation of phytotoxic ozone in the lower troposphere may benefit plants e.g., by eliciting defense reactions which provide better plant resistance against fungal pathogens and herbivores (Sandermann et al., 1998). Cui et al. (2012) have shown that exposure of tomato plants to elevated O_3 reduced the fecundity and prolonged the developmental time of whiteflies (*Bemisia tabaci*). Reduced performance of whiteflies was related to up-regulated pathogenesis-related protein (PR1) genes, increased phenylalanine ammonia-lyase enzyme activity and elevated concentrations of salicylic acid (SA), phenolics and condensed tannins (Cui et al., 2012). Elevated atmospheric O_3 levels may cause slight reduction in plant growth, but plant fitness could be improved by the reduced impact of aggressive plant pathogenic fungi such as *Drechslera teres* on barley (Plessl et al., 2005). Furthermore, elevated O_3 limits hyphal growth, sporulation and germination of conidia in many other plant pathogenic and saprophytic fungi (Tzortzakis et al., 2008; Ozkan et al., 2011).

SECONDARY ORGANIC AEROSOLS (SOA)

In the atmosphere, nano-scale aerosol particles are typically formed during the late morning and then grow throughout the day with growth rates of 1–20 nm h^{-1} (Kulmala, 2003). The smallest observed nucleating particles are 1 nm in diameter (Riipinen et al., 2012; Kulmala et al., 2013). Nucleation may be ion-induced or involve sulphuric acid or ammonia mixtures with water for the growth of nanoparticles less than 5 nm in diameter. The condensation of organic vapors in the particle size range 20–50 nm—mostly the oxidation products of plant VOCs—on particle surfaces will have increasing importance to particle size growth and several theories have been presented (Riipinen et al., 2012). The chemistry of these processes is extremely complicated. For example, an intact Scots pine seedling can emit 20 different monoterpenes (Heijari et al., 2011) and a single monoterpene of this blend, limonene, can form nearly 1200 different organic compounds in atmospheric ozonolysis reactions (Kundu et al., 2012). Finally, cloud condensation nuclei (CCN), are formed when the particle diameter reaches 30–100 nm (Riipinen et al., 2012) through addition of organic and sulphuric acid molecules.

Chamber experiments (Joutsensaari et al., 2005; Hao et al., 2009; Virtanen et al., 2010) have shown that HIPVs emitted after induction by chemical elicitors or mechanical damage can efficiently react with O_3 and OH leading to formation and growth of atmospheric SOA particles. Insect damage to pine saplings can increase the emission rates of reactive monoterpenes and sesquiterpenes by up to 10-fold after bark feeding (Heijari et al., 2011) and up to 16-fold after needle defoliation (Ghimire et al., 2013). Recently there have been observations that in forest pest outbreak areas HIPV emissions dominate and emission rates of biogenic VOCs can increase by at least 4-fold in bark beetle outbreak areas compared to intact forests (Amin et al., 2012). This substantial increase in reactive HIPVs may significantly increase the atmospheric SOA concentrations in the affected areas (Berg et al., 2012).

Earlier studies (Mercado et al., 2009) have shown that diffusion of light by the higher particle concentrations in the atmosphere may affect photosynthesis efficiency of global vegetation. In forests the impact is caused by the better penetration of photosynthetically active light inside the tree canopy (Roderick et al., 2001). Although, there is not any direct experimental evidence that reactive HIPVs can increase light interception and improve photosynthesis through SOA formed from reactive HIPVs, this could potentially occur in insect outbreak areas. Enhanced photosynthesis rates will ultimately increase fitness of a plant, but in plant communities the emissions of an individual plant will affect the whole plant community or due to atmospheric drift of HIPVs and SOA particles, most probably conspecific plant individuals in other plant communities.

Some of the constitutively emitted plant VOCs may have much longer life times in ozone-rich atmospheres than some HIPVs that have life times of only a few minutes (Holopainen, 2011). Such compounds include e.g., isoprene (1.3 d), the monoterpenes camphene (18 d) and 1,8 cineole (110 d), and the sesquiterpene longifolene (>33d) (Atkinson and Arey, 2003). The consequence of this difference is that these constitutively emitted compounds will drift longer distances than the HIPVs compounds. Therefore, they cannot act as nucleation centers as easily as HIPV and high concentrations of HIPVs will rapidly lead to nucleation and formation of SOA particles (Joutsensaari et al., 2005; Virtanen et al., 2010), and possibly stimulate CCN formation rates locally (Riipinen et al., 2012).

The behavior of SOA particles inside a canopy and their deposition on plants and other surfaces is not yet sufficiently understood (Holopainen, 2011; Carslaw et al., 2012). Semivolatile and easily condensable HIPVs and other VOCs will condense on external plant surfaces at temperatures of +12°C and lower (Himanen et al., 2010; Schaub et al., 2010). Leaf surfaces could be a good site for ozonolysis reactions and for formation of secondary organic particles, which may also detoxify ozone before stomatal uptake Tuzet et al. (2011). The soil nitrate pool has been found to be an important source of atmospheric nitric oxide (NO) and nitrous acid (HONO) leading to atmospheric hydroxyl radical (OH) production (Su et al., 2011). This observation suggests that reactions of VOCs with OH inside the shady canopies and on leaf surfaces of smaller plants closer to the soil surface could be more common than earlier expected. These reactions may lead to formation of SOA particles (Virtanen et al., 2010; Riipinen et al., 2012) even in the boundary layer of plant leaves and stems.

PLANT EVOLUTION, HIPVs AND ATMOSPHERIC PROCESSES

Production of HIPVs, many of which are highly reactive in the atmosphere, is an evolved response in many plant species under biotic stress. When considering the chemical and physical properties of the HIPV compounds, a longer atmospheric life time of the compounds could possibly attract natural enemies over longer distances. However, the molecular concentration in the atmosphere will in any case be rapidly reduced due to increased distance from the point source of a damaged plant. The selection of highly reactive compounds as attractive signals in indirect plant defense does not necessarily maximize the attraction capacity of HIPVs (McFrederick et al., 2009) or improve plant fitness (Hare, 2011). Therefore, additional potential traits which can be linked to production of reactive HIPVs may give an alternative explanation for the type of HIPV compounds that evolved. Conversely, Peñuelas and Llusia (2004) proposed that an increase in biological complexity of plant physiological processes during evolution is one of the causes of the diversity of VOC emissions and their emission is just an unavoidable trait as a result of their volatility. Volk (2003) also noted that feedback loops in the biosphere contain segments based solely upon by-products of organisms' metabolisms. According to Volk (2003) these were not metabolically evolved by organisms to be sent out into the environment for altering its chemistry and do not represent a trait that was selected during evolution by natural selection.

Increased emission of reactive HIPV compounds from plants under biotic stress will inevitably lead to SOA formation as shown experimentally (Joutsensaari et al., 2005) and in a modeling study in insect outbreak areas (Berg et al., 2012). SOA particles can grow by aggregation and by VOC vapor uptake to form cloud condensation nuclei (CCN), which will have an impact on light quality reaching vegetation, influence precipitation and affect the amount of sunlight reflected to space (Riipinen et al., 2012). The importance of SOA and other aerosols in controlling global temperature and radiation balance has been convincingly shown (Arneth et al., 2009). Recently, the results from large scale measurement campaigns (Paasonen et al., 2013) suggested that VOCs emitted from vegetation substantially increase sun-screening SOA formation under climate warming. This biogenic VOC based growth mechanism produces roughly 50% of particles at the size of cloud condensation nuclei across Europe (Paasonen et al., 2013). An important question that remains to be answered, is whether HIPV have evolved to act as a part of a biosphere-atmosphere feedback system that improves the abiotic growth conditions of the plants under attack? This question prompts a further question of whether a biogenic SOA related change in environmental conditions to indirectly better defend against biotic attackers comes anywhere close to the expenditure in terms of VOCs and carbon used to create the change? If the costs of a HIPV based biosphere-atmosphere feedback system are higher than the improvement in fitness of the HIPV emitting individuals, selection of the trait does not fall in line with natural selection in a strict Darwinian sense (Moody, 2012), except perhaps in clonal plants with a population distributed across large areas (De Woody et al., 2009).

Recent evolutionary models incorporate phenotypes expressed in the external environment; however, there is still debate whether such traits generate dynamics that alter evolution (Bailey, 2012). Such models of extended phenotypes predict that the individual carrying genes for the trait, which has an impact on the physical environment, may have effects on conspecifics including an emitting individual's own offspring or siblings, but also other species (Bailey, 2012). These kinds of extended effects are particularly created by species known as ecosystem engineers (Jones et al., 1994, 2010; Hastings et al., 2007) which by niche construction modify their own niche and those of other organisms e.g., through behavior or metabolic processes.

We suggest that high HIPV emission rate capacity of Boreal conifer forest trees could be such a trait and that it may have an impact on SOA formation in insect outbreak areas (Berg et al., 2012). Higher atmospheric SOA density and associated cooling effect could provide a stabilizing feedback (Lenton, 1998; Paasonen et al., 2013) to protect conifer ecosystems against factors such as global warming, insect outbreaks and spread of invasive deciduous tree species related to warming (Kellomaki et al., 2001). It is expected that current global warming will increase the frequency of forest pest outbreaks at higher latitudes (Niemelä et al., 2001), and warmer temperatures could substantially increase the HIPV emission rates of affected conifer trees (Heijari et al., 2011; Amin et al., 2012). However, it has not yet been shown if there really is a negative feedback loop (Lenton, 1998; Lovelock, 2003) related to insect outbreak areas, i.e., the cooling effect of HIPVs through enhanced albedo by HIPV-induced SOA formation (Berg et al., 2012), CCN formation (Paasonen et al., 2013) and finally by improved cloud albedo. This cooling feedback loop would reduce the frequency of forest pest outbreaks and relieve heat stress of vegetation. More efficiently dispersed light may improve photosynthesis, but cooling would counteract the light effect, thus reducing photosynthesis rate and additionally reducing VOC production and the protective role of HIPVs against pests and pathogens.

WHERE DOES THE CARBON OF HIPVs GO?

To return to our original question; in this review we have tried to demonstrate that the carbon fixed by a plant and then, particularly under biotic stress, released back to the atmosphere as

volatile organic compounds, will have important roles in chemical, physical and biological processes during their life time. These may be facilitated by the HIPV compounds originally synthesized and emitted by a plant, or in the form of other chemical compounds after atmospheric reactions of the HIPVs. So far, we know only a fraction of the highly diverse potential routes and functions that the VOCs emitted by plants may have. For example, the carbon of highly volatile C10 monoterpenes and semivolatile C15 sesquiterpenes in the atmosphere could be bound to freshly nucleated SOA particles as a result of ozonolysis during the day time or oxidized by reactions with NO_3 at night. The secondary organic aerosol particles formed from the same HIPV may have different chemical composition and different biological functions depending on the time of day during SOA formation. The size of SOA particles in the atmosphere may also grow by adsorption of other organic vapors to their surface (Kroll and Seinfeld, 2008) or the particles could be influenced by solar UV irradiation leading to the photolysis and formation of several oxygenated C1–C3 compounds (Pan et al., 2009). The reactions could also be reversible and low molecular mass carbon compounds may react again to form larger carbon-based molecules. Finally the carbon bound to the HIPV compounds will be oxidized to CO or CO_2 in the atmosphere (Kroll and Seinfeld, 2008) and may again be utilized by plants in the process of photosynthesis, while it could alternatively end up as organic polymers in the sediments of terrestrial or aquatic ecosystems.

ACKNOWLEDGMENTS

We acknowledge the financial support from the Academy of Finland (project nos. 133322, 251898 and 141053) and the UEF spearhead project CABI. James D. Blande acknowledges the European Science Foundation EUROCORES Programme EuroVOL, which was supported by funds from the Academy of Finland.

REFERENCES

Amin, H., Atkins, P. T., Russo, R. S., Brown, A. W., Sive, B., Hallar, A. G., et al. (2012). Effect of bark beetle infestation on secondary organic aerosol precursor emissions. *Environ. Sci. Technol.* 46, 5696–5703. doi: 10.1021/es204205m

Angeles Lopez, Y. I., Angelica Martinez-Gallardo, N., Ramirez-Romero, R., Lopez, M. G., Sanchez-Hernandez, C., and Paul Delano-Frier, J. (2012). Cross-kingdom effects of plant-plant signaling via volatile organic compounds emitted by tomato (*Solanum lycopersicum*) plants infested by the greenhouse whitefly (*Trialeurodes vaporariorum*). *J. Chem. Ecol.* 38, 1376–1386. doi: 10.1007/s10886-012-0201-z

Arneth, A., and Niinemets, U. (2010). Induced BVOCs: how to bug our models? *Trends Plant Sci.* 15, 118–125. doi: 10.1016/j.tplants.2009.12.004

Arneth, A., Unger, N., Kulmala, M., and Andreae, M. O. (2009). Clean the air, heat the planet? *Science* 326, 672–673. doi: 10.1126/science.1181568

Atkinson, R., and Arey, J. (2003). Gas-phase tropospheric chemistry of biogenic volatile organic compounds: a review. *Atmos. Environ.* 37, S197–S219. doi: 10.1016/S1352-231000391-1

Bailey, N. W. (2012). Evolutionary models of extended phenotypes. *Trends Ecol. Evol.* 27, 561–569. doi: 10.1016/j.tree.2012.05.011

Bamberger, I., Hoertnagl, L., Ruuskanen, T. M., Schnitzhofer, R., Mueller, M., Graus, M., et al. (2011). Deposition fluxes of terpenes over grassland. *J. Geophys. Res. Atmos.* 116:D14305. doi: 10.1029/2010JD015457

Bartram, S., Jux, A., Gleixner, G., and Boland, W. (2006). Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves. *Phytochemistry* 67, 1661–1672. doi: 10.1016/j.phytochem.2006.02.004

Behnke, K., Ehlting, B., Teuber, M., Bauerfeind, M., Louis, S., Hasch, R., et al. (2007). Transgenic, non-isoprene emitting poplars don't like it hot. *Plant J.* 51, 485–499. doi: 10.1111/j.1365-313X.2007.03157.x

Berg, A. R., Heald, C. L., Huff-Hartz, K. E., Hallar, A. G., Meddens, A. J. H., Hicke, J. A., et al. (2012). The impact of bark beetle infestation on monoterpene emissions and secondary organic aerosol formation in Western North America. *Atmos. Chem. Phys. Disc.* 12, 29763–29800. doi: 10.5194/acp-13-3149-2013

Blande, J. D., Holopainen, J. K., and Li, T. (2010). Air pollution impedes plant-to-plant communication by volatiles. *Ecol. Lett.* 13, 1172–1181. doi: 10.1111/j.1461-0248.2010.01510.x

Blande, J. D., Korjus, M., and Holopainen, J. K. (2010). Foliar methyl salicylate emissions indicate prolonged aphid infestation on silver birch and black alder. *Tree Physiol.* 30, 404–416. doi: 10.1093/treephys/tpp124

Blande, J. D., Tiiva, P., Oksanen, E., and Holopainen, J. K. (2007). Emission of herbivore-induced volatile terpenoids from two hybrid aspen (*Populus tremula* x tremuloides) clones under ambient and elevated ozone concentrations in the field. *Global Change Biol.* 13, 2538–2550. doi: 10.1111/j.1365-2486.2007.01453.x

Bruce, T. J. A., and Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry* 72, 1605–1611. doi: 10.1016/j.phytochem.2011.04.011

Canosa-Mas, C., Duffy, J., King, M., Thompson, K., and Wayne, R. (2002). The atmospheric chemistry of methyl salicylate - reactions with atomic chlorine and with ozone. *Atmos. Environ.* 36, 2201–2205. doi: 10.1016/S1352-2310(02)00173-5

Carslaw, N., Mota, T., Jenkin, M. E., Barley, M. H., and McFiggans, G. (2012). A significant role for nitrate and peroxide groups on indoor secondary organic aerosol. *Environ. Sci. Technol.* 46, 9290–9298. doi: 10.1021/es301350x

Clavijo McCormick, A., Unsicker, S. B., and Gershenzon, J. (2012). The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. *Trends Plant Sci.* 17, 303–310. doi: 10.1016/j.tplants.2012.03.012

Choh, Y., Shimoda, T., Ozawa, R., Dicke, M., and Takabayashi, J. (2004). Exposure of lima bean leaves to volatiles from herbivore-induced conspecific plants results in emission of carnivore attractants: active or passive process? *J. Chem. Ecol.* 30, 1305–1317. doi: 10.1023/B:JOEC.0000037741.13402.19

Copolovici, L., Kaennaste, A., Remmel, T., Vislap, V., and Niinemets, U. (2011). Volatile emissions from *Alnus glutionosa* induced by herbivory are quantitatively related to the extent of damage. *J. Chem. Ecol.* 37, 18–28. doi: 10.1007/s10886-010-9897-9

Cui, H., Sun, Y., Su, J., Ren, Q., Li, C., and Ge, F. (2012). Elevated O_3 reduces the fitness of *Bemisia tabaci* via enhancement of the SA-dependent defense of the tomato plant. *Arthropod-Plant Interact.* 6, 425–437. doi: 10.1007/s11829-012-9189-0

De Woody, J., Rickman, T. H., Jones, B. E., and Hipkins, V. D. (2009). Allozyme and microsatellite data reveal small clone size and high genetic diversity in aspen in the southern Cascade Mountains. *For. Ecol. Manage.* 258, 687–696. doi: 10.1016/j.foreco.2009.05.006

Degenhardt, J., Hiltpold, I., Koellner, T. G., Frey, M., Gierl, A., Gershenzon, J., et al. (2009). Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13213–13218. doi: 10.1073/pnas.0906365106

De Moraes, C. M., Mescher, M. C., and Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410, 577–580. doi: 10.1038/35069058

Dicke, M. (2009). Behavioural and community ecology of plants that cry for help. *Plant Cell Environ.* 32, 654–665. doi: 10.1111/j.1365-3040.2008.01913.x

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends Plant Sci.* 15, 167–175. doi: 10.1016/j.tplants.2009.12.002

Dicke, M., and Loreto, F. (2010). Induced plant volatiles: from genes to climate change. *Trends Plant Sci.* 15, 115–117. doi: 10.1016/j.tplants.2010.01.007

Dicke, M., and Sabelis, M. (1988). How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38, 148–165. doi: 10.1163/156854288X00111

Dolch, R., and Tscharntke, T. (2000). Defoliation of alders (*Alnus glutinosa*) affects herbivory by leaf beetles on undamaged neighbours. *Oecologia* 125, 504–511. doi: 10.1007/s004420000482

Egigu, M. C., Ibrahim, M. A., Yahya, A., and Holopainen, J. K. (2011). *Cordeauxia edulis* and *Rhododendron tomentosum* extracts disturb orientation and feeding behavior of Hylobius abietis and *Phyllodecta laticollis*. *Entomol. Exp. Appl.* 138, 162–174. doi: 10.1111/j.1570-7458.2010.01082.x

Engelberth, J., Alborn, H. T., Schmelz, E. A. and Tumlinson, J. H. (2004). Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1781–1785. doi: 10.1073/pnas.0308037100

Erb, M., Lenk, C., Degenhardt, J., and Turlings, T. C. J. (2009). The underestimated role of roots in defense against leaf attackers. *Trends Plant Sci.* 14, 653–659. doi: 10.1016/j.tplants.2009.08.006

Evans, H., Stoakley, J., Leather, S., and Watt, A. (1991). Development of an integrated approach to control of pine beauty moth in Scotland. *For. Ecol. Manage.* 39, 19–28. doi: 10.1016/0378-1127(91)90158-R

Fatouros, N. E., Broekgaarden, C., Bukovinszkine'Kiss, G., van Loon, J. J. A., Mumm, R., Huigens, M. E., et al. (2008). Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10033–10038. doi: 10.1073/pnas.0707809105

Fatouros, N. E., Lucas-Barbosa, D., Weldegergis, B. T., Pashalidou, F. G., van Loon, J. J. A., Dicke, M., et al. (2012). Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. *PLoS ONE* 7:e43607. doi: 10.1371/journal.pone.0043607

Frost, C. J., Appel, M., Carlson, J. E., De Moraes, C. M., Mescher, M. C., and Schultz, J. C. (2007). Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores. *Ecol. Lett.* 10, 490–498. doi: 10.1111/j.1461-0248.2007.01043.x

Frost, C. J., Mescher, M. C., Dervinis, C., Davis, J. M., Carlson, J. E., and De Moraes, C. M. (2008). Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. *New Phytol.* 180, 722–733. doi: 10.1111/j.1469-8137.2008.02599.x

Geervliet, J., Vet, L., and Dicke, M. (1994). Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid cotesia rubecula. *Entomol. Exp. Appl.* 73, 289–297. doi: 10.1111/j.1570-7458.1994.tb01866.x

Ghimire, R., Markkanen, J. M., Kivimäenpää, M., Lyytikäinen-Saarenmaa, P., and Holopainen, J. K. (2013). Needle removal by pine sawfly larvae increases branch-level VOC emissions and reduces below-ground emissions of Scots pine. *Environ. Sci. Technol.* 47, 4325–4332. doi: 10.1021/es4006064

Ghirardo, A., Heller, W., Fladung, M., Schnitzler, J., and Schroeder, H. (2012). Function of defensive volatiles in pedunculate oak (Quercus robur) is tricked by the moth *Tortrix viridana*. *Plant Cell Environ.* 35, 2192–2207. doi: 10.1111/j.1365-3040.2012.02545.x

Giron-Calva, P. S., Molina-Torres, J., and Heil, M. (2012). Volatile dose and exposure time impact perception in neighboring plants. *J. Chem. Ecol.* 38, 226–228. doi: 10.1007/s10886-012-0072-3

Glinwood, R., Ahmed, E., Qvarfordt, E., Ninkovic, V., and Pettersson, J. (2009). Airborne interactions between undamaged plants of different cultivars affect insect herbivores and natural enemies. *Arthropod. Plant Interact.* 3, 215–224. doi: 10.1007/s11829-009-9072-9

Glinwood, R., Ninkovic, V., Pettersson, J., and Ahmed, E. (2004). Barley exposed to aerial allelopathy from thistles (Cirsium spp.) becomes less acceptable to aphids. *Ecol. Entomol.* 29, 188–195. doi: 10.1111/j.0307-6946.2004.00582.x

Glinwood, R., Pettersson, J., Ahmed, E., Ninkovic, V., Birkett, M., and Pickett, J. (2003). Change in acceptability of barley plants to aphids after exposure to allelochemicals from couch-grass (*Elytrigia repens*). *J. Chem. Ecol.* 29, 261–274. doi: 10.1023/A:1022687025416

Gosset, V., Harmel, N., Göbel, C., Francis, F., Haubruge, E., Wathelet, J.-P., et al. (2009). Attacks by a piercing-sucking insect (*Myzus persicae* Sultzer) or a chewing insect (*Leptinotarsa decemlineata* Say) on potato plants (*Solanum tuberosum* L.) induce differential changes in volatile compound release and oxylipin synthesis. *J. Exp. Bot.* 60, 1231–1240. doi: 10.1093/jxb/erp015

Gouinguene, S. P., and Turlings, T. C. J. (2002). The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiol.* 129, 1296–1307. doi: 10.1104/pp.001941

Hallquist, M., Wenger, J. C., Baltensperger, U., Rudich, Y., Simpson, D., Claeys, M., et al. (2009). The formation, properties and impact of secondary organic aerosol: current and emerging issues. *Atmos. Chem. Phys.* 9, 5155–5236. doi: 10.5194/acp-9-5155-2009

Hao, L. Q., Yli-Pirila, P., Tiitta, P., Romakkaniemi, S., Vaattovaara, P., Kajos, M. K., et al. (2009). New particle formation from the oxidation of direct emissions of pine seedlings. *Atmosph. Chem. Phys.* 9, 8121–8137. doi: 10.5194/acp-9-8121-2009

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annu. Rev. Entomol.* 56, 161–180. doi: 10.1146/annurev-ento-120709-144753

Hastings, A., Byers, J. E., Crooks, J. A., Cuddington, K., Jones, C. G., Lambrinos, J. G., et al. (2007). Ecosystem engineering in space and time. *Ecol. Lett.* 10, 153–164. doi: 10.1111/j.1461-0248.2006.00997.x

Heijari, J., Blande, J. D., and Holopainen, J. K. (2011). Feeding of large pine weevil on Scots pine stem triggers localised bark and systemic shoot emission of volatile organic compounds. *Environ. Exp. Bot.* 71, 390–398. doi: 10.1016/j.envexpbot.2011.02.008

Heil, M., and Adame-Álvarez, R. M. (2010). Short signalling distances make plant communication a soliloquy. *Biol. Lett.* 6, 843–845. doi: 10.1098/rsbl.2010.0440

Heil, M., and Karban, R. (2010). Explaining evolution of plant communication by airborne signals. *Trends Ecol. Evol.* 25, 137–144. doi: 10.1016/j.tree.2009.09.010

Heil, M., and Kost, C. (2006). Priming of indirect defences. *Ecol. Lett.* 9, 813–817. doi: 10.1111/j.1461-0248.2006.00932.x

Heil, M., Lion, U., and Boland, W. (2008). Defence-inducing volatiles: in search for the active motif. *J. Chem. Ecol.* 34, 601–604. doi: 10.1007/s10886-008-9464-9

Heil, M., and Silva Bueno, J. C. (2007). Within-plant signalling by volatiles leads to induction and priming of an indirect defense in nature. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5467–5472. doi: 10.1073/pnas.0610266104

Himanen, S. J., Blande, J. D., Klemola, T., Pulkkinen, J., Heijari, J., and Holopainen, J. K. (2010). Birch (*Betula spp.*) leaves adsorb and re-release volatiles specific to neighbouring plants - a mechanism for associational herbivore resistance? *New Phytol.* 186, 722–732. doi: 10.1111/j.1469-8137.2010.03220.x

Hoffmann, T., Odum, J., Bowman, F., Collins, D., Klockow, D., Flagan, R., et al. (1997). Formation of organic aerosols from the oxidation of biogenic hydrocarbons. *J. Atmos. Chem.* 26, 189–222. doi: 10.1023/A:1005734301837

Holopainen, J. K. (2011). Can forest trees compensate for stress-generated growth losses by induced production of volatile compounds? *Tree Physiol.* 31, 1356–1377. doi: 10.1093/treephys/tpr111

Holopainen, J. K., and Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.* 15, 176–184. doi: 10.1016/j.tplants.2010.01.006

Holopainen, J. K., Nerg, A.-M., and Blande, J. D. (2013). "Multitrophic signaling in polluted atmospheres," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Tree Physiology, eds Ü. Niinemets and R. K. Monson (Springer).

Huang, M., Sanchez-Moreiras, A. M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., et al. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytol.* 193, 997–1008. doi: 10.1111/j.1469-8137.2011.04001.x

Jansen, R. M. C., Wildt, J., Kappers, I. F., Bouwmeester, H. J., Hofstee, J. W., and van Henten, E. J. (2011). Detection of diseased plants by analysis of volatile organic compound emission. *Annu. Rev. Phytopathol.* 49, 157–174. doi: 10.1146/annurev-phyto-072910-095227

Jones, C. G., Gutierrez, J. L., Byers, J. E., Crooks, J. A., Lambrinos, J. G., and Talley, T. S. (2010). A framework for understanding physical ecosystem engineering by organisms. *Oikos* 119, 1862–1869. doi: 10.1111/j.1600-0706.2010.18782.x

Jones, C. G., Lawton, J. H., and Shachak, M. (1994). Organisms as ecosystem engineers. *Oikos* 69, 373–386.

Joutsensaari, J., Loivamaki, M., Vuorinen, T., Miettinen, P., Nerg, A. M., Holopainen, J. K., et al. (2005). Nanoparticle formation by ozonolysis of inducible plant volatiles. *Atmosph. Chem. Phys.* 5, 1489–1495. doi: 10.5194/acp-5-1489-2005

Karban, R., Maron, J., Felton, G. W., Ervin, G., and Eichenseer, H. (2003). Herbivore damage to sagebrush induces resistance in wild tobacco: evidence for eavesdropping between plants. *Oikos* 100, 325–332. doi: 10.1034/j.1600-0706.2003.12075.x

Karban, R., Shiojiri, K., Huntzinger, M., and McCall, A. C. (2006). Damage-induced resistance in sagebrush: volatiles are key to intra- and interplant communication. *Ecology* 87, 922–930. doi: 10.1890/0012-965887[922:DRISVA]2.0.CO;2

Karban, R., and Shiojiri, K. (2009). Self-recognition affects plant communication and defense. *Ecol. Lett.* 12, 502–506. doi: 10.1111/j.1461-0248.2009.01313.x

Karban, R., Shiojiri, K., Ishizaki, S., Wetzel, W. C., and Evans, R. Y. (2013). Kin recognition affects plant communication and defence. *Proc. R. Soc. B Biol. Sci.* 280, 1471–2954. doi: 10.1098/rspb.2012.3062

Kellomaki, S., Rouvinen, I., Peltola, H., Strandman, H., and Steinbrecher, R. (2001). Impact of global warming on the tree species composition of boreal forests in Finland and effects on emissions of isoprenoids. *Global Change Biol.* 7, 531–544.

Kessler, A., Halitschke, R., Diezel, C., and Baldwin, I. T. (2006). Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*. *Oecologia* 148, 280–292. doi: 10.1007/s00442-006-0365-8

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144. doi: 10.1126/science.291.5511.2141

Kiendler-Scharr, A., Wildt, J., Dal Maso, M., Hohaus, T., Kleist, E., Mentel, T.F., et al. (2009). New particle formation in forests inhibited by isoprene emissions. *Nature* 461, 381–384. doi: 10.1038/nature08292

Kivimaenpää, M., Magsarjav, N., Ghimire, R., Markkanen, J., Heijari, J., Vuorinen, M., et al. (2012). Influence of tree provenance on biogenic VOC emissions of Scots pine (*Pinus sylvestris*) stumps. *Atmos. Environ.* 60, 477–485. doi: 10.1016/j.atmosenv.2012.07.018

Kost, C., and Heil, M. (2006). Herbivore-induced plant volatiles induce an indirect defence in neighbouring plants. *J. Ecol.* 94, 619–628. doi: 10.1016/j.bbalip.2005.03.001

Kroll, J. H., and Seinfeld, J. H. (2008). Chemistry of secondary organic aerosol: formation and evolution of low-volatility organics in the atmosphere. *Atmos. Environ.* 42, 3593–3624. doi: 10.1016/j.atmosenv.2008.01.003

Kulmala, M. (2003). How particles nucleate and grow. *Science* 302, 1000–1001. doi: 10.1126/science.1090848

Kulmala, M., Kontkanen, J., Junninen, H., Lehtipalo, K., Manninen, H. E., Nieminen, T., et al. (2013). Direct observations of atmospheric aerosol nucleation. *Science* 339, 943–946. doi: 10.1126/science.1227385

Kundu, S., Fisseha, R., Putman, A. L., Rahn, T. A., and Mazzoleni, L. R. (2012). High molecular weight SOA formation during limonene ozonolysis: insights from ultrahigh-resolution FT-ICR mass spectrometry characterization. *Atmos. Chem. Phys.* 12, 5523–5536. doi: 10.5194/acpd-12-2167-2012

Laothawornkitkul, J., Taylor, J. E., Paul, N. D., and Hewitt, C. N. (2009). Biogenic volatile organic compounds in the Earth system. *New Phytol.* 183, 27–51. doi: 10.1111/j.1469-8137.2009.02859.x

Lenton, T. (1998). Gaia and natural selection. *Nature* 394, 439–447. doi: 10.1038/28792

Lerdau, M., and Slobodkin, K. (2002). Trace gas emissions and species-dependent ecosystem services. *Trends Ecol. Evol.* 17, 309–312. doi: 10.1016/S0169-534702535-1

Li, T., Holopainen, J. K., Kokko, H., Tervahauta, A. I., and Blande, J. D. (2012). Herbivore-induced aspen volatiles temporally regulate two different indirect defences in neighbouring plants. *Func. Ecol.* 26, 1176–1185. doi: 10.1111/j.1365-2435.2012.01984.x

Little, D., Gouhier-Darimont, C., Bruessow, F., and Reymond, P. (2007). Oviposition by Pierid butterflies triggers defense responses in Arabidopsis. *Plant Physiol.* 143, 784–800. doi: 10.1104/pp.106.090837

Loreto, F., and Schnitzler, J. P. (2010). Abiotic stresses and induced BVOCs. *Trends Plant Sci.* 15, 154–166. doi: 10.1016/j.tplants.2009.12.006

Lovelock, J. (2003). The living Earth. *Nature* 426, 769–770. doi: 10.1038/426769a

Maffei, M. E. (2010). Sites of synthesis, biochemistry and functional role of plant volatiles. *South Afr. J. Bot.* 76, 612–631. doi: 10.1016/j.sajb.2010.03.003

Manninen, A., Holopainen, T., and Holopainen, J. (1998). Susceptibility of ectomycorrhizal and nonmycorrhizal Scots pine (*Pinus sylvestris*) seedlings to a generalist insect herbivore, *Lygus rugulipennis*, at two nitrogen availability levels. *New Phytol.* 140, 55–63. doi: 10.1046/j.1469-8137.1998.00246.x

Mattiacci, L., and Dicke, M. (1995). Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. *Entomol. Exp. Appl.* 76, 37–48. doi: 10.1111/j.1570-7458.1995.tb01944.x

Mauck, K. E., De Moraes, C. M., and Mescher, M. C. (2010). Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3600–3605. doi: 10.1073/pnas.0907191107

McFrederick, Q. S., Fuentes, J. D., Roulston, T., Kathilankal, J. C., and Lerdau, M. (2009). Effects of air pollution on biogenic volatiles and ecological interactions. *Oecologia* 160, 411–420. doi: 10.1007/s00442-009-1318-9

Mentel, T. F., Wildt, J., Kiendler-Scharr, A., Kleist, E., Tillmann, R., Dal Maso, M., et al. (2009). Photochemical production of aerosols from real plant emissions. *Atmos. Chem. Phys.* 9, 4387–4406. doi: 10.5194/acpd-9-3041-2009

Mercado, L. M., Bellouin, N., Sitch, S., Boucher, O., Huntingford, C., Wild, M., et al. (2009). Impact of changes in diffuse radiation on the global land carbon sink. *Nature* 458, 1014–U87. doi: 10.1038/nature07949

Mishra, S., and Sihag, R. C. (2009). Efficacy of some chemicals as repellents against two honey bee species, *Apis mellifera* L. and *Apis florea* F. in semi-field trials. *J. Apic. Sci.* 53, 53–66.

Mithofer, A., Wanner, G., and Boland, W. (2005). Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137, 1160–1168. doi: 10.1104/pp.104.054460

Moody, D. E. (2012). Seven misconceptions regarding the Gaia hypothesis. *Clim. Change* 113, 277–284. doi: 10.1007/s10584-011-0382-4

Niemelä, P., Chapin, F., Danell, K., and Bryant, J. (2001). Herbivory-mediated responses of selected boreal forests to climatic change. *Clim. Change* 48, 427–440. doi: 10.1023/A:1010787714349

Nieuwenhuizen, N. J., Wang, M. Y., Matich, A. J., Green, S. A., Chen, X., Yauk, Y., et al. (2009). Two terpene synthases are responsible for the major sesquiterpenes emitted from the flowers of kiwifruit (*Actinidia deliciosa*). *J. Exp. Bot.* 60, 3203–3219. doi: 10.1093/jxb/erp162

Niinemets, U., Loreto, F., and Reichstein, M. (2004). Physiological and physicochemical controls on foliar volatile organic compound emissions. *Trends Plant Sci.* 9, 180–186. doi: 10.1016/j.tplants.2004.02.006

Noe, S. M., Copolovici, L., Niinemets, U., and Vaino, E. (2008). Foliar limonene uptake scales positively with leaf lipid content: "non-emitting" species absorb and release monoterpenes. *Plant Biol.* 10, 129–137. doi: 10.1055/s-2007-965239

Ozkan, R., Smilanick, J. L., and Karabulut, O. A. (2011). Toxicity of ozone gas to conidia of *Penicillium digitatum, Penicillium italicum*, and *Botrytis cinerea* and control of gray mold on table grapes. *Postharvest Biol. Technol.* 60, 47–51. doi: 10.1016/j.postharvbio.2010.12.004

Paasonen, P., Asmi, A., Petäjä, T., Kajos, M. K., Äijälä, M., Junninen, H., et al. (2013). Warming-induced increase in aerosol number concentration likely to moderate climate change. *Nature. Geosci.* 6, 438–442. doi: 10.1038/ngeo1800

Pan, X., Underwood, J. S., Xing, J.-., Mang, S. A., and Nizkorodov, S. A. (2009). Photodegradation of secondary organic aerosol generated from limonene oxidation by ozone studied with chemical ionization mass spectrometry. *Atmos. Chem. Phys.* 9, 3851–3865. doi: 10.5194/acp-9-3851-2009

Pareja, M., Mohib, A., Birkett, M. A., Dufour, S., and Glinwood, R. T. (2009). Multivariate statistics coupled to generalized linear models reveal complex use of chemical cues by a parasitoid. *Animal Behav.* 77, 901–909. doi: 10.1016/j.anbehav.2008.12.016

Peñuelas, J., and Llusia, J. (2004). Plant VOC emissions: making use of the unavoidable. *Trends Ecol. Evol.* 19, 402–404. doi: 10.1016/j.tree.2004.06.002

Peñuelas, J., and Staudt, M. (2010). BVOCs and global change. *Trends in Plant Science* 15, 133–144. doi: 10.1016/j.tplants.2009.12.005

Pinto, D. M., Blande, J. D., Nykänen, R., Dong, W. X., Nerg, A.-M., and Holopainen, J. K. (2007a). Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids? *J. Chem. Ecol.* 33, 683–684. doi: 10.1007/s10886-007-9255-8

Pinto, D. M., Nerg, A.-M., and Holopainen, J. K. (2007b). The role of ozone-reactive compounds, terpenes, and green leaf volatiles (GLVs), in the orientation of *Cotesia plutellae*. *J. Chem. Ecol.* 33, 2218–2228. doi: 10.1007/s10886-007-9376-0

Pinto, D. M., Blande, J. D., Souza, S. R., Nerg, A., and Holopainen, J. K. (2010). Plant volatile organic compounds (VOCs) in ozone (O_3) polluted atmospheres: the ecological effects. *J. Chem. Ecol.* 36, 22–34. doi: 10.1007/s10886-009-9732-3

Plessl, M., Heller, W., Payer, H., Elstner, E., Habermeyer, J., and Heiser, I. (2005). Growth parameters and resistance against Drechslera teres of spring barley (*Hordeum vulgare* L. cv. Scarlett) grown at elevated ozone and carbon dioxide concentrations. *Plant Biol.* 7, 694–705. doi: 10.1055/s-2005-873002

Pratt, K. A., Mielke, L. H., Shepson, P. B., Bryan, A. M., Steiner, A. L., Ortega, J., et al. (2012). Contributions of individual reactive biogenic volatile organic compounds to organic nitrates above a mixed forest. *Atmos. Chem. Phys.* 12, 10125–10143. doi: 10.5194/acp-12-10125-2012

Rasmann, S., Kollner, T., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., et al. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434, 732–737. doi: 10.1038/nature03451

Riipinen, I., Yli-Juuti, T., Pierce, J. R., Petäjä, T., Worsnop, D. R., Kulmala, M., et al. (2012). The contribution of organics to atmospheric nanoparticle growth. *Nat. Geosci.* 5, 453–458. doi: 10.1038/ngeo1499

Roda, A., Halitschke, R., Steppuhn, A., and Baldwin, I. T. (2004). Individual variability in herbivore-specific elicitors from the plant's perspective. *Molecular Ecology* 13, 2421–2433. doi: 10.1111/j.1365-294X.2004.02260.x

Roderick, M. L., Farquhar, G. D., Berry, S. L., and Noble, I. R. (2001). On the direct effect of clouds and atmospheric particles on the productivity and structure of vegetation. *Oecologia* 129, 21–30. doi: 10.1007/s004420100760

Rodriguez-Saona, C. R., Rodriguez-Saona, L. E., and Frost, C. J. (2009). Herbivore-induced volatiles in the perennial shrub, *Vaccinium corymbosum*, and their role in inter-branch signaling. *J. Chem. Ecol.* 35, 163–175. doi: 10.1007/s10886-008-9579-z

Sandermann, H., Ernst, D., Heller, W., and Langebartels, C. (1998). Ozone: an abiotic elicitor of plant defence reactions. *Trends Plant Sci.* 3, 47–50. doi: 10.1016/S1360-138501162-X

Schaub, A., Blande, J. D., Graus, M., Oksanen, E., Holopainen, J. K., and Hansel, A. (2010). Real-time monitoring of herbivore induced volatile emissions in the field. *Physiol. Plantarum* 138, 123–133. doi: 10.1111/j.1399-3054.2009.01322.x

Schausberger, P., Peneder, S., Juerschik, S., and Hoffmann, D. (2012). Mycorrhiza changes plant volatiles to attract spider mite enemies. *Funct. Ecol.* 26, 441–449. doi: 10.1111/j.1365-2435.2011.01947.x

Shiojiri, K., Ozawa, R., Matsui, K., Kishimoto, K., Kugimaya, S., and Takabayashi, J. (2006). Role of the lipoxygenase/lyase pathway of host-food plants in the host searching behavior of two parasitoid species, *Cotesia glomerata* and *Cotesia plutellae*. *J. Chem. Ecol.* 32, 969–979. doi: 10.1007/s10886-006-9047-6

Shiojiri, K., Karban, R., and Ishizaki, S. (2009). Volatile communication among sagebrush branches affects herbivory: timing of active cues. *Arthropod. Plant Interact.* 3, 99–104. doi: 10.1007/s11829-009-9060-0

Su, H., Cheng, Y., Oswald, R., Behrendt, T., Trebs, I., Meixner, F. X., et al. (2011). Soil nitrite as a source of atmospheric HONO and OH radicals. *Science* 333, 1616–1618. doi: 10.1126/science.1207687

Sun, J., Huang, L., and Wang, C. (2012). Electrophysiological and behavioral responses of *Helicoverpa assulta* (Lepidoptera: Noctuidae) to tobacco volatiles. *Arthropod. Plant Interact.* 6, 375–384. doi: 10.1007/s11829-012-9190-7

Takabayashi, J., Takahashi, S., Dicke, M., and Posthumus, M. A. (1995). Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol.* 21, 273–287. doi: 10.1007/BF02036717

Toome, M., Randjarv, P., Copolovici, L., Niinemets, U., Heinsoo, K., Luik, A., et al. (2010). Leaf rust induced volatile organic compounds signalling in willow during the infection. *Planta* 232, 235–243. doi: 10.1007/s00425-010-1169-y

Tsao, R., and Zhou, T. (2000). Antifungal activity of monoterpenoids against postharvest pathogens *Botrytis cinerea* and *Monilinia fructicola*. *J. Essent. Oil Res.* 12, 113–121. doi: 10.1080/10412905.2000.9712057

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253. doi: 10.1126/science.250.4985.1251

Tuzet, A., Perrier, A., Loubet, B., and Cellier, P. (2011). Modelling ozone deposition fluxes: the relative roles of deposition and detoxification processes. *Agric. For. Meteorol.* 151, 480–492. doi: 10.1016/j.agrformet.2010.12.004

Tzortzakis, N., Singleton, I., and Barnes, J. (2008). Impact of low-level atmospheric ozone-enrichment on black spot and anthracnose rot of tomato fruit. *Postharvest Biol. Technol.* 47, 1–9. doi: 10.1016/j.postharvbio.2007.06.004

Van den Boom, C., van Beek, T., and Dicke, M. (2002). Attraction of *Phytoseiulus persimilis* (Acari: Phytoseiidae) towards volatiles from various *Tetranychus urticae*-infested plant species. *Bull. Entomol. Res.* 92, 539–546. doi: 10.1079/BER2002193

Van den Boom, C., van Beek, T., Posthumus, M., De Groot, A., and Dicke, M. (2004). Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. *J. Chem. Ecol.* 30, 69–89. doi: 10.1023/B:JOEC.0000013183.72915.99

van Tol, R., van der Sommen, A., Boff, M., van Bezooijen, J., Sabelis, M., and Smits, P. (2001). Plants protect their roots by alerting the enemies of grubs. *Ecol. Lett.* 4, 292–294.

Vinson, S. B. (1976). Host selection by insect parasitoids. *Annu. Rev. Entomol.* 21, 109–133. doi: 10.1146/annurev.en.21.010176.000545

Virtanen, A., Joutsensaari, J., Koop, T., Kannosto, J., Yli-Pirilä, P., Leskinen, J., et al. (2010). An amorphous solid state of biogenic secondary organic aerosol particles. *Nature* 467, 824–827. doi: 10.1038/nature09455

Volk, T. (2003). Natural selection, Gaia, and inadvertent by-products - a reply to Lenton and Wilkinson's response. *Clim. Change* 58, 13–19. doi: 10.1023/A:1023463510624

Vuorinen, T., Nerg, A., Ibrahim, M., Reddy, G., and Holopainen, J. (2004). Emission of *Plutella xylostella*-induced compounds from cabbages grown at elevated CO2 and orientation behavior of the natural enemies. *Plant Physiol.* 135, 1984–1992. doi: 10.1104/pp.104.047084

Webster, B. (2012). The role of olfaction in aphid host location. *Physiol. Entomol.* 37, 10–18. doi: 10.1111/j.1365-3032.2011.00791.x

Webster, B., Bruce, T., Pickett, J., and Hardie, J. (2010). Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Anim. Behav.* 79, 451–457. doi: 10.1016/j.anbehav.2009.11.028

Winkler, P. M., Ortega, J., Karl, T., Cappellin, L., Friedli, H. R., Barsanti, K., et al. (2012). Identification of the biogenic compounds responsible for size-dependent nanoparticle growth. *Geophys. Res. Lett.* 39, L20815. doi: 10.1029/2012GL053253

Xiao, Y., Wang, Q., Erb, M., Turlings, T. C. J., Ge, L., Hu, L., et al. (2012). Specific herbivore-induced volatiles defend plants and determine insect community composition in the field. *Ecol. Lett.* 15, 1130–1139. doi: 10.1111/j.1461-0248.2012.01835.x

Yi, H., Heil, M., Adame-Alvarez, R. M., Ballhorn, D. J., and Ryu, C. (2009). Airborne induction and priming of plant defenses against a bacterial pathogen. *Plant Physiol.* 151, 2152–2161. doi: 10.1104/pp.109.144782

Yuan, J. S., Himanen, S. J., Holopainen, J. K., Chen, F., and Stewart, C. N. (2009). Smelling global climate change: mitigation of function for plant volatile organic compounds. *Trends Ecol. Evol.* 24, 323–331. doi: 10.1016/j.tree.2009.01.012

Zebelo, S. A., Matsui, K., Ozawa, R., and Maffei, M. E. (2012). Plasma membrane potential depolarization and cytosolic calcium flux are early events involved in tomato (*Solanum lycopersicon*) plant-to-plant communication. *Plant Sci.* 196, 93–100. doi: 10.1016/j.plantsci.2012.08.006.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2013; accepted: 22 May 2013; published online: 11 June 2013.

*Citation: Holopainen JK and Blande JD (2013) Where do herbivore-induced plant volatiles go? Front. Plant Sci. **4**:185. doi: 10.3389/fpls.2013.00185*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Holopainen and Blande. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 28 June 2013
doi: 10.3389/fpls.2013.00209

Herbivore-induced maize leaf volatiles affect attraction and feeding behavior of *Spodoptera littoralis* caterpillars

Georg E. von Mérey, Nathalie Veyrat, Marco D'Alessandro and Ted C. J. Turlings*

Laboratory for Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Anne M. Cortesero, University of Rennes1, France
James Blande, University of Eastern Finland, Finland

Correspondence:
Ted C. J. Turlings, Laboratory for Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Emile-Argand 11, C.P. 158, CH-2009 Neuchâtel, Switzerland
e-mail: ted.turlings@unine.ch

Plants under herbivore attack emit volatile organic compounds (VOCs) that can serve as foraging cues for natural enemies. Adult females of Lepidoptera, when foraging for host plants to deposit eggs, are commonly repelled by herbivore-induced VOCs, probably to avoid competition and natural enemies. Their larval stages, on the other hand, have been shown to be attracted to inducible VOCs. We speculate that this contradicting behavior of lepidopteran larvae is due to a need to quickly find a new suitable host plant if they have fallen to the ground. However, once they are on a plant they might avoid the sites with fresh damage to limit competition and risk of cannibalism by conspecifics, as well as exposure to natural enemies. To test this we studied the effect of herbivore-induced VOCs on the attraction of larvae of the moth *Spodoptera littoralis* and on their feeding behavior. The experiments further considered the importance of previous feeding experience on the responses of the larvae. It was confirmed that herbivore-induced VOCs emitted by maize plants are attractive to the larvae, but exposure to the volatiles decreased the growth rate of caterpillars at early developmental stages. Larvae that had fed on maize previously were more attracted by VOCs of induced maize than larvae that had fed on artificial diet. At relatively high concentrations synthetic green leaf volatiles, indicative of fresh damage, also negatively affected the growth rate of caterpillars, but not at low concentrations. In all cases, feeding by the later stages of the larvae was not affected by the VOCs. The results are discussed in the context of larval foraging behavior under natural conditions, where there may be a trade-off between using available host plant signals and avoiding competitors and natural enemies.

Keywords: *Spodoptera littoralis*, green leaf volatiles, maize, larval foraging behavior, host plant suitability

INTRODUCTION

Maize plants attacked by herbivorous insects emit volatile organic compounds (VOCs) that attract natural enemies of herbivores (Dicke et al., 1990; Turlings et al., 1990; Turlings and Wäckers, 2004; Arimura et al., 2009). In the case of maize plants, the blend of VOCs emitted by caterpillar-damaged plants is typically composed of green leaf volatiles (GLVs, C-6 aldehydes, alcohols, and their esters), nitrogenous, and aromatic compounds, as well as mono, homo and sesquiterpenes (Paré and Tumlinson, 1999; D'Alessandro and Turlings, 2006). Among the VOCs that have been identified in these blends, GLVs have received particular attention. They are emitted upon mechanical damage, immediately after feeding on the maize plant begins (Turlings et al., 1998), and have been considered important for the innate attraction of parasitoids, as they are emitted in higher amounts by freshly damaged plants than by plants with only old damage (Whitman and Eller, 1990; Hoballah and Turlings, 2005). Commonly, insect herbivores are repelled by inducible plant volatiles (Bernasconi et al., 1998; De Moraes et al., 2001; Rostas and Hilker, 2002). This is particularly evident for Lepidoptera (De Moraes et al., 2001), but this is not true for all herbivores. In particular coleopterans are known to be attracted to previously infested plants (Bolter et al., 1997; Landolt et al., 1999) and they may be attracted to GLVs as was found for scarab (Hansson et al., 1999) and buprestid beetles (de Groot et al., 2008), and flea beetles (Halitschke et al., 2008).

Interestingly, larval stages of several Lepidoptera are attracted by volatiles emitted by plants that have been damaged by conspecific larvae. This was found for neonates of several Lepidoptera species, including *Ostrinia nubialis* (Hübner) and *Ostrinia furnacalis* (Guenée) (Lepidoptera: Pyralidae) on maize (Huang et al., 2009; Piesik et al., 2009), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) on maize and cowpea (Carroll et al., 2006, 2008), and *Estigmene acrea* (Drury) (Lepidoptera: Arctiidae) on soybean, tomato, and maize (Castrejon et al., 2009). Furthermore, caterpillars adapt their behavior depending on plant VOC emission (Shiojiri et al., 2006). This attraction to VOCs emitted by already infested host plants is puzzling, as it will lead to competition and may increase the risk of cannibalism and attack by natural enemies that are attracted to the same volatiles. Cannibalism is common among noctuid larvae, such as *Spodoptera littoralis* (Boisduval) (Abdel Salam and Fokhar. cited in Fox, 1975), *S. frugiperda* (Chapman et al., 1999), and *Helicoverpa armigera* (Hübner) (Kakimoto et al., 2009). The attraction of natural enemies to herbivore-induced volatiles has been shown for numerous tritrophic systems (Dicke

et al., 1990; Turlings et al., 1990; Heil, 2008; Dicke and Baldwin, 2010; Hare, 2011), which makes one wonder why lepidopteran larvae are attracted to the same volatiles. This apparent maladaptive behavior may be explained by a trade-off between risks: in the field harsh weather conditions and attempts to escape parasitoids and predators cause larvae to frequently fall off plants (personal observ.). In order to find back the same plant or new suitable plants the larvae will have to rely on dependable and available VOC signals. Induced VOCs may provide the best cues, as undamaged plants are often virtually odorless (Turlings et al., 1990). However, once on a plant, caterpillars may prefer sites with minimal VOC emissions, where it is less likely to encounter competitors and natural enemies.

We therefore hypothesized that caterpillars may initially be attracted to induced VOCs, but once they are on the plant they will feed preferentially in places with low GLV emissions. We tested this for larvae of the noctuid moth *Spodoptera littoralis* (Boisduval). First we confirmed attraction to induced plant volatiles in a four-arm olfactometer and then tested their growth rate as a measure of feeding behavior when they were exposed to GLVs. Previous feeding experiences were also taken into consideration, as larval attraction may be higher for volatiles that are emitted by plant species on which the larvae previously fed (Carlsson et al., 1999).

MATERIALS AND METHODS

PLANTS AND INSECTS

Maize plants (*Zea mays*, cv. Delprim) were grown individually in plastic pots (10 cm high, 4 cm diameter) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) and placed in a climate chamber (23°C, 60% r.h., 16:8 h L:D, 50000 lm/m^2). Maize plants used for the experiments were 10–12 days old and had three fully developed leaves. The evening before the experiments, plants were transferred into glass vessels, as described in Turlings et al. (2004) and kept under laboratory conditions (25 ± 2°C, 40 ± 10% r.h., 16:8 h L:D, and 8000 lm/m^2). *S. littoralis* larvae were reared from eggs provided by Syngenta (Stein, Switzerland). The eggs were kept in an incubator at 30.0 ± 0.5°C until emergence of the larvae. Subsequently, they were transferred on artificial diet at room temperature (24 ± 4°C).

OLFACTOMETER EXPERIMENTS

Two olfactometer experiments were performed with fourth-instar *S. littoralis* larvae. In the first experiment, the attraction of larvae to an *S. littoralis*-infested maize plant vs. healthy maize plant was compared. In the second experiment, the attraction of larvae to a maize plant with fresh (mechanically inflicted) damage was tested against a plant with old (mechanically inflicted) damage. In both experiments, the effect of previous feeding experience (either artificial diet or maize) was compared. All the larvae were initially reared on artificial diet as previously described (Turlings et al., 2004). Twenty-four hours before each experiment, 90 larvae were transferred on fresh maize leaves (maize feeding experience), and 90 on artificial diet (artificial diet feeding experience).

ATTRACTION OF FOURTH-INSTAR *S. littoralis* LARVAE TO INFESTED MAIZE PLANTS

A four-arm olfactometer (as described in D'Alessandro and Turlings, 2005) was modified to measure the attraction of *S. littoralis* larvae. The olfactometer consisted of a central glass choice arena (**Figure 1**) [6 cm internal diameter (ID), 5 cm length] with four arms (15 mm ID, 5 cm length), each with a glass elbow (5 cm length) and an upward connection for a glass bulb (50 ml). To avoid visual distraction of the larvae, a white cardboard cylinder was placed around the central choice arena.

The choice arena was connected to four glass bottles. One bottle contained a maize plant (cv Delprim) infested with 15 second-instar *S. littoralis* larvae that had been placed on the plant 16 h before the bioassay. The opposite bottle contained a healthy maize plant. The two remaining bottles remained empty. The position of the odor sources was changed between each experimental day, with the two odor sources always opposite to each other.

Thirty fourth-instar larvae were placed in a small plastic box (2 × 2 × 0.8 cm) with an open top, which was introduced in the center of the choice arena. The larvae would crawl out of the box into the central choice arena and a number of them entered one of the four arms. After 60 min, the number of larvae in each arm was counted. The larvae that did not leave the choice arena after 60 min were considered as having made "no choice" and all the larvae were removed from the olfactometer. Six such releases were done on a given day and this was repeated on 6 different days ($n = 6$).

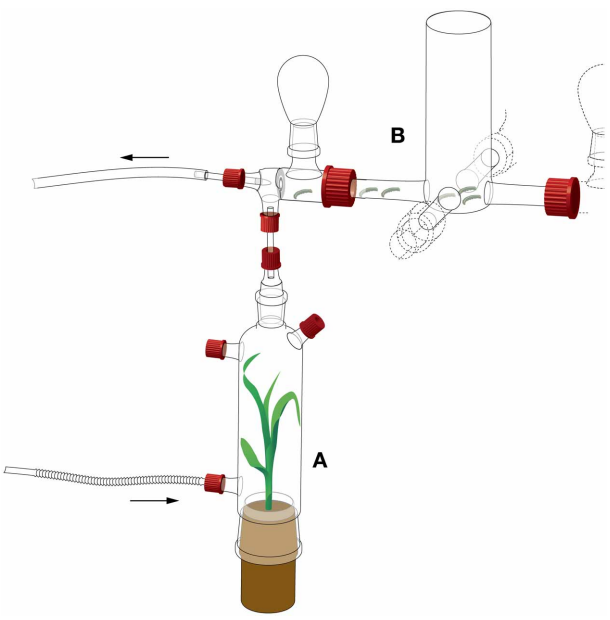


FIGURE 1 | Detail of the four-arm olfactometer setup for *S. littoralis* larval behavior. (A) Odor source. **(B)** Choice arena. Arrows indicate airflows. Four odor sources were compared, attached to each of the four arms of the choice arena. Drawing by Thomas Degen (www.thomas-degen.ch).

ATTRACTION OF FOURTH-INSTAR *S. littoralis* LARVAE TO PLANTS WITH OLD vs. PLANTS WITH FRESH DAMAGE

The same setup as described above was used, with the same experimental procedure, except for the odor sources. Two maize plants were brought to the laboratory 16 h prior to the bioassay. One plant was scratched on the underside of the two oldest leaves, damaging approximately 2 cm^2, on both sides of the central vein (Hoballah and Turlings, 2005). Caterpillar regurgitant, collected as described in Turlings et al. (1998), was applied to the two wounds. Both plants were then placed in a glass bottle and exposed to a carbon-filtered, humidified airflow of 300 ml/min for 15 h. The second plant was then scratched and regurgitant was applied. The two plants were then placed opposite to each other in the olfactometer, leaving two empty bottles between them. The airflow was then increased to 1200 ml/min through each bottle, of which 500 ml/min entered the olfactometer choice chamber. The position of the treatments was changed for each experimental day.

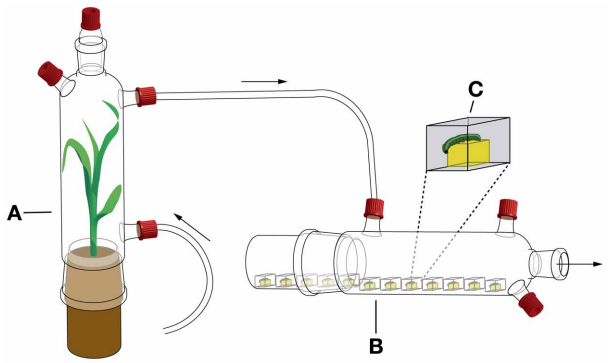


FIGURE 2 | Design of growth performance experiment. (A) Odor source bottle, which contained either a healthy maize plant or a caterpillar-damaged maize plant. **(B)** Bottles containing 12 larvae inside small plastic boxes. **(C)** Plastic box enlarged showing a *S. littoralis* larva feeding on a cube of artificial diet. Arrows indicate the direction of the airflow.

GLV DISPENSERS

To expose larvae to green leafy volatiles we made dispensers as described by von Mérey et al. (2011). The GLVs were first mixed together in an Erlenmeyer flask (100 mL) placed in ice. The composition of the mixture was 80% cis-3-hexen-1-al [92.5% purity, (NEAT), Bedoukian Research Inc., USA]; 10% cis-3-hexen-1-ol (>98%, GC, Sigma-Aldrich, CH-9471 Buchs, Switzerland); 8% cis-3-hexenyl acetate (>98%, SAFC Supply Solutions, 3050 Spruce street, St. Louis, MO 63103, USA); and 2% trans-2-hexenol (99%, ACROS Organics, New Jersey, USA). The mix was stored at -70°C until it was used. For the assays, 0.2 mL of the GLV mix was transferred into a 2 mL amber glass vial (11.6 × 32 mm) (Sigma-Aldrich, CH-9471 Buchs, Switzerland) containing clean fiberglass wool. Each vial was sealed with a PTFE/rubber septum pierced by a Drummond 2 µL micro-pipette in black polypropylene cap. This device allowed the constant release of GLVs, and their release rate was calibrated to the amount of GLVs that was found to be released by infested maize plants (*Zea mays* cv Delprim) (von Mérey et al., 2011). Control dispensers consisted of glass vials only containing fiberglass wool.

VOC-EXPOSURE EXPERIMENTS

Three experiments were conducted to measure the effect of VOCs on the growth of *S. littoralis* larvae. In the first experiment, the larvae were exposed to the volatiles of caterpillar-damaged maize plants. In the second experiment, they were exposed to amounts of a blend of synthetic GLVs that fall within the range of what is commonly emitted by a single, caterpillar-infested maize plant (see von Mérey et al., 2011 for details). In the third experiment, they were exposed to high concentrations of synthetic GLVs. In all three experiments we recorded, besides weight gain, mortality, and pupation of the larvae.

EFFECT OF EXPOSURE TO VOCs EMITTED BY CATERPILLAR-DAMAGED MAIZE PLANTS ON FEEDING RATE OF *S. littoralis* LARVAE

Second-instar *S. littoralis* were placed individually inside small plastic boxes (2 × 2 × 1.5 cm) that were covered with fine-meshed nylon tissue, fixed with an elastic band. The larvae were provided a 1 cm^3 cube of wheatgerm-based artificial diet (Turlings et al., 2004), which was changed every second day. Twelve such boxes were placed inside a glass bottle lying on its side, connected at its base with a Teflon tube to the top of an odor source bottle (**Figure 2**; see Turlings et al., 2004 for details on glass bottles and tubing). Odor source bottles contained either a maize plant infested with fifteen second-instar *S. littoralis* larvae (induced plant, VOCi, replaced with a new infested plant every third day) or an uninfested maize plant (control plant, VOCu, also replaced every third day). The odor source bottle was connected to a four-port air-delivery system (Model VCS-HADS-6AF6C6B; ARS Analytical Research Systems, Gainesville, FL, USA), providing a purified and humidified airflow of 300 ml/min. Two such four-port air-delivery systems were used simultaneously to introduce odors into eight exposure chambers, resulting in 48 larvae for each treatment.

Before placing the larvae inside the plastic boxes, they were weighed on a microbalance (Model MX5, Mettler Toledo, Greifensee, Switzerland). Weighing was repeated at the following time-points after placing the boxes inside the glass bottle: 5, 24, 48, 96, 144, 192, 240, 288, 312, 336, 360, 408, and 432 h. After this time-point, all larvae had pupated or had died and the experiment was terminated.

EFFECT OF EXPOSURE TO SYNTHETIC GLVs ON WEIGHT GAIN OF *S. littoralis* LARVAE

The same setup as described above was used for this experiment. In this case, the odor source bottles containing a dispenser built up as follows: a 2 ml amber glass vial (11.6 × 32 mm; Sigma-Aldrich, Buchs, Switzerland) containing 100 mg clean fiberglass wool. The vial was sealed with a PTFE/rubber septum (Sigma-Aldrich, Buchs, Switzerland) pierced with a 2 µl micro-pipette (Drummond, Millan SA, Plan-Les-Ouates, Switzerland). The length of the pipette was calibrated to release a controlled amount of GLVs, similar to the amount emitted by maize plants (cv Delprim). The GLV mixture consisted of 80%

(*Z*)-3-hexen-1-al [92.5% purity, (NEAT), Bedoukian Research, Danbury, CT, USA], 10% (*Z*)-3-hexenyl-acetate (<98%, SAFC Supply Solutions, St. Louis, MO, USA), 8% (*Z*)-3-hexenyl-Acetate (≥98%, SAFC Supply Solutions, 3050 Spruce Street, St. Louis, MO 63103, USA), and 2% (*E*)-2-hexenol (99%, ACROS Organics, Geel, Belgium). The same GLV dispenser was kept for the duration of the assay. Control bottles contained no dispenser.

In this experiment, the weighing of the larvae was repeated at 5, 12, 24, 48, 96, 120, and 144 h after placement in the bottles. The experiment was terminated at 144 h because the tests showed that larval weight was not affected by the volatiles at these concentrations.

EFFECT OF EXPOSURE TO HIGH CONCENTRATIONS OF GLVs ON WEIGHT GAIN OF *S. littoralis* LARVAE

In this experiment, larvae were placed individually in a plastic box (7.5 × 6.5 × 5 cm) containing a GLV dispenser (described above), and a piece of diet (2 × 1.5 × 1 cm). The box was closed, in order to increase the concentration of GLVs. As a control, an empty dispenser was placed inside the cage without GLVs inside. There were twelve larvae in each treatment and they were weighed before placing them inside the boxes. They were weighed again after 3, 6, 9, 12, 15, 24, 40, 48, 51, 54, 58, 72, 96, 120, and 168 h.

The larger plastic boxes allowed for more mobility, compared to the cages used in the previous experiments. In order to observe whether the high concentrations of GLV affected larval mobility, we recorded whether the larvae were on the diet or off the diet during the first 8 h of exposure.

STATISTICAL ANALYSIS

VOC-exposure data were compared using Student's *t*-test, provided they met the assumptions of normality (Shapiro-Wilk test) and equal variance (Levene's test). Else, a Mann-Whitney test was applied. Both treatments (VOCu and VOCi exposure) were compared at each time-point individually. Data on mortality and pupation of the larvae compared using a one-way analysis of variance (ANOVA). Data was tested with SigmaStat (version 3.5, STATCON, Witzenhausen, Germany). Data on mobility were analyzed in a general linear model (GLM) with binomial distribution (the larvae were observed either on the diet or off the diet) family in R (R Development Core Team, 2009). Olfactometer data was analyzed using the software package R (R Development Core Team, 2009), in a GLM, allowing to compensate for over-dispersed data, as previously described (D'Alessandro and Turlings, 2005; Tamò et al., 2006; Ricard and Davison, 2007). This means that any positional biases or effects of the individuals on each other's behavior are considered in the model and that calculated statistical differences are solely the result of differential attractiveness of the odor sources.

RESULTS

ATTRACTION OF FOURTH-INSTAR *S. littoralis* LARVAE TO INDUCED MAIZE PLANTS

The larvae that had fed on maize and the larvae fed on artificial diet were both more attracted toward caterpillar-damaged maize plants than to intact plants (GLM $P < 0.001$ and $P < 0.002$, respectively; **Figure 3**). However, the maize-fed larvae were attracted more strongly by the induced plants than the diet-fed

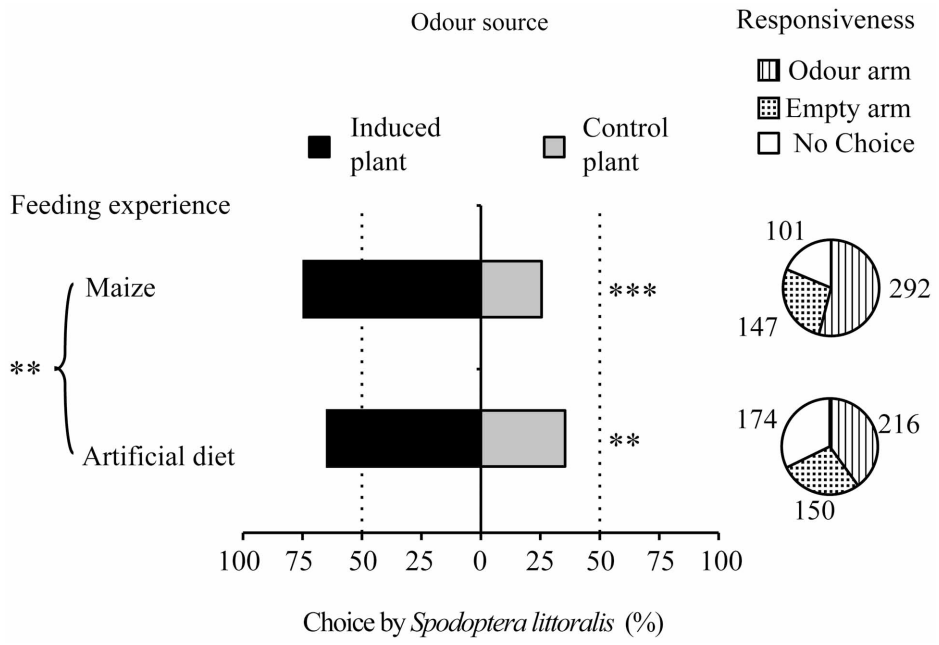


FIGURE 3 | Effect of feeding experience on the attraction of *S. littoralis* larvae to induced maize plants. Pie charts indicate overall responsiveness (number of larvae entering the different types of arms). GLMs were performed to test for differences between arms within each group of feeding experience, as well as to compare feeding experiences. ** $P < 0.01$, *** $P < 0.001$.

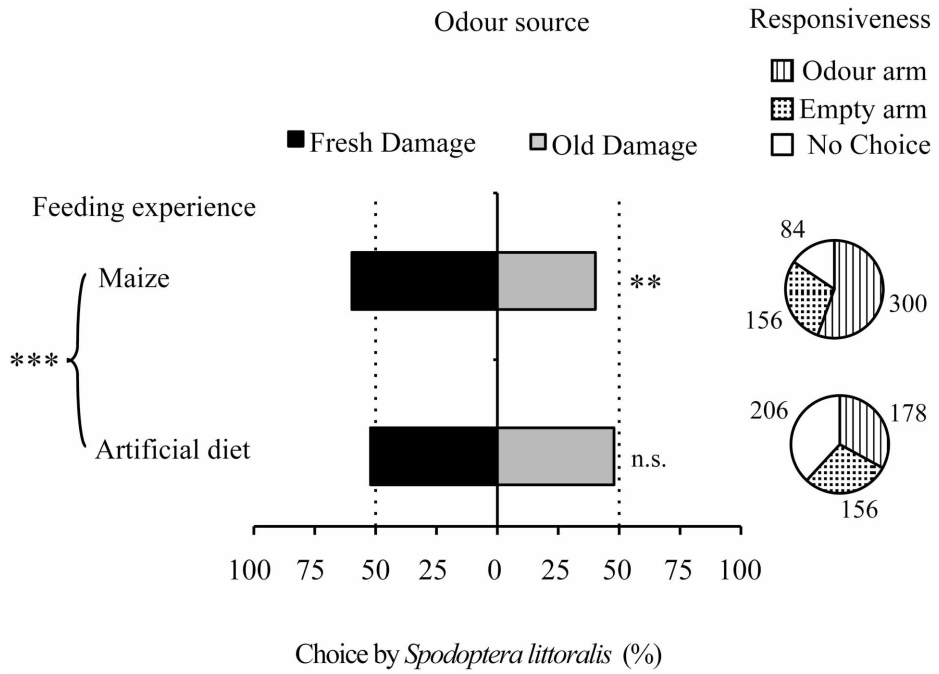


FIGURE 4 | Effect of feeding experience on the attraction of *S. littoralis* larvae to old and fresh damaged maize plants. Pie charts indicate overall responsiveness (number of larvae entering the different types of arms). GLMs were performed to test for differences between arms within each group of feeding experience, as well as to compare feeding experiences. n.s., no significant difference ($P > 0.05$); ** $P < 0.01$, *** $P < 0.001$.

larvae (GLM $P < 0.005$). Maize-fed larvae also displayed an increased responsiveness (80% entering an arm) compared to diet-fed larvae (66%).

ATTRACTION TO OLD vs. FRESH DAMAGE

Freshly damaged plants were more attractive to maize-fed larvae (GLM $P < 0.003$) than plants with older damage (**Figure 4**). Artificial diet-fed larvae did not show a preference between old and fresh damage. This difference in preference between maize-fed and diet-fed larvae was significant (GLM $P < 0.001$). Also in this case, overall responsiveness of maize-fed larvae (84%) was higher than the responsiveness of artificial diet-fed larvae (62%).

EXPOSURE TO VOCs FROM CATERPILLAR-DAMAGED MAIZE PLANTS

The larvae that were exposed to the VOCs emitted by caterpillar-damaged maize plants grew more slowly in the early stages of development (**Figure 5**). Initial weight of the larvae was equal across treatments. After 5 h, there was still no difference between the two treatment groups ($P < 0.356$). However, after 24 h, the larvae exposed to VOCs from damaged plants (VOCi) had gained significantly less weight than the larvae exposed to VOCs emitted by healthy plants (VOCu) ($P < 0.030$). This difference in growth rate persisted throughout the early weighing time points: 48 h ($P < 0.030$), 96 h ($P < 0.012$), 144 h ($P < 0.033$). After this, both treatment groups displayed similar weight gains until pupation. The weight of the pupae did not differ significantly ($P < 0.916$). There was also no difference in mortality between the larvae of the two treatment groups ($P < 0.839$).

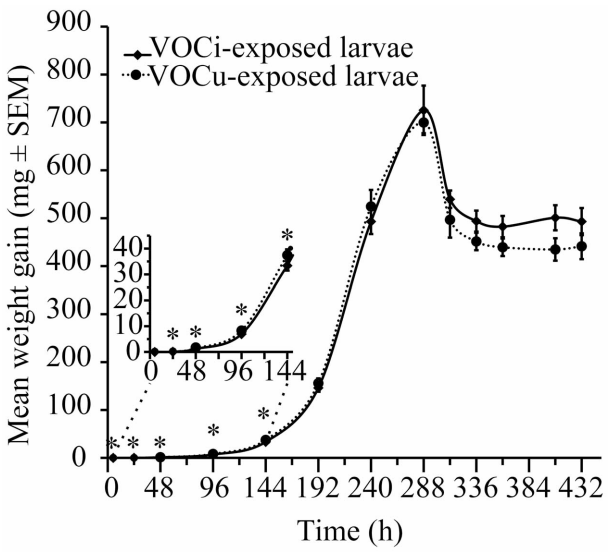


FIGURE 5 | Mean weight gain (mg ± SEM) of *S. littoralis* larvae exposed to VOCs emitted by *S. littoralis*-induced (VOCi) or healthy (VOCu) maize plants. *indicates a significant difference at $P < 0.05$ (Student's *t*-test).

EFFECT OF EXPOSURE TO LOW CONCENTRATIONS OF SYNTHETIC GLVs ON WEIGHT GAIN OF *S. littoralis* LARVAE

When larvae were exposed to the synthetic volatile blend we measured no difference either in larval weight gain (5 h: $P < 0.759$,

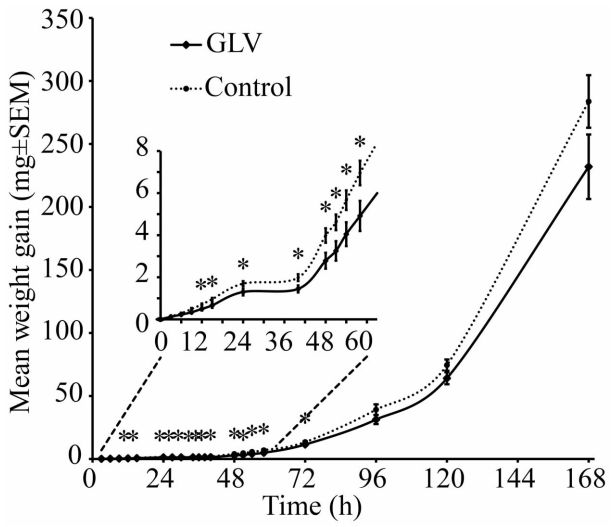


FIGURE 6 | Weight gain (mg ± SEM) of *S. littoralis* larvae exposed to GLV dispensers or control dispensers. An asterisk above the value point indicates significant difference between treatments ($P < 0.05$ Student's *t*-test).

12 h: $P < 0.286$, 24 h: $P < 0.267$, 48 h: $P < 0.502$, 72 h: $P < 0.506$, 96 h: $P < 0.833$, 120 h: $P < 0.833$, 144 h: $P < 0.646$), or mortality (0% in both treatments).

EFFECT OF EXPOSURE TO HIGH CONCENTRATIONS OF GLVs ON WEIGHT GAIN OF *S. littoralis* LARVAE

When larvae were exposed to high concentrations of GLVs, such as can be expected to be present in the immediacy of the feeding sites on the maize plants, the larvae were found to gain less weight at the early stages of their development (**Figure 6**). After 3 h ($P < 0.514$) and 6 h ($P < 0.173$), there was still no difference between the treatments. After exposure to GLVs for 9 h a strong trend of lower weight gain in GLV-exposed larvae was observed ($P < 0.051$) and at 12 h the difference between the two treatments was significant ($P < 0.025$). This difference persisted throughout the early part of the experimental time (15 h: $P < 0.036$; 24 h: $P < 0.027$; 40 h: $P < 0.031$; 48 h: $P < 0.030$; 51 h: $P < 0.033$; 54 h: $P < 0.039$; 58 h: $P < 0.038$; 72 h: $P < 0.047$). From 96 h, however, there was no longer a difference in weight gain between the treatments. Interestingly, the mobility of GLV-exposed larvae was slightly increased ($P < 0.060$), with a significant difference in number of larvae moving in the box after 6 h ($P < 0.048$). However, at 30 min ($P < 0.410$), 2 h ($P < 0.716$), 4 h ($P < 0.572$), and 8 h ($P < 0.423$), GLV-exposed and control larvae were equally on the diet and off the diet.

DISCUSSION

We show here that *Spodoptera littoralis* caterpillars are attracted to volatiles from maize plants that are under attack by conspecifics. This confirms the findings by Carroll et al. (2006, 2008), who obtained similar results for a related species, *S. frugiperda*, which was found to be attracted to inducible volatiles emitted from maize and cowpea seedlings. Similarly, neonate larvae of the codling moth, *Cydia pomonella*, are more attracted to apple fruits with other codling moth larvae than to uninfested fruits (Landolt et al., 2000). This is somewhat surprising, as these Lepidoptera are not known to aggregate, unlike many Coleoptera, for which both adults and larvae are often attracted to the volatiles of already infested plants (Crowe, 1995; Bolter et al., 1997; Müller and Hilker, 2000; Kalberer et al., 2001; Heil, 2004; Yoneya et al., 2010). It should be noted that in the case of *S. frugiperda*, Carroll et al. (2008) found linalool to be particularly attractive. This terpene alcohol is in fact also released, be it in lesser amounts, by undamaged maize plants, at least in some varieties (Degen et al., 2004), and therefore can be a reliable cue for the presence of maize in general. In adult Lepidoptera, however, increased linalool levels decreased oviposition (De Moraes et al., 2001; Kessler and Baldwin, 2001).

The larval response to herbivore-induced volatiles is in contrast to what is known for adult Lepidoptera, which avoid to oviposit on plants that are already under caterpillar attack (Landolt, 1993; De Moraes et al., 2001; Kessler and Baldwin, 2001; Huang et al., 2009). Such avoidance of already infested plants, which is also the case for aphids (Bernasconi et al., 1998), is expected, as it reduces the chances of competition and cannibalism, as well as predation and parasitism by natural enemies that are attracted to the same volatiles. Then why are the larvae attracted to volatiles that are indicative of these risks? To answer this it may help to list the potential disadvantages and discuss counter arguments why these may not be as important as potential advantages. The apparent disadvantages are: (1) VOCs emitting plants have mobilized their defenses and should be less suitable for caterpillar development, (2) The VOCs indicate the plants carry other larvae that will compete for the same resource and may even pose a cannibalism risk, (3) The VOCs are attractive to natural enemies of the caterpillars and therefore indicate a higher risk of predation and parasitism.

As for the counter argument, the most obvious reason to use herbivore-induced VOCs is the same as has been argued for the natural enemies (Vet and Dicke, 1992), the induced VOCs are emitted in large amounts and are therefore easily detectible and reliable cues for the presence of a host plant. Moreover, the alternative, the avoidance of inducible defenses by opting for healthy plants gives only an advantage for a very short period of time, as maize plants respond very rapidly, within hours, to an attack (Turlings et al., 1998). This is particularly true for plants that are neighboring already attacked plants and have their defenses primed in response to the volatiles emitted by the neighbor (Ton et al., 2007). This then only leaves the risk of competition and possibly cannibalism. This risk may be minor in light of the possibility of not finding a plant at all and unlike *S. frugiperda*, *S. littoralis* is not cannibalistic, at least not the colony that we used in our experiments. We therefore hypothesized that *Spodoptera* and other larvae of herbivorous insects have adapted to use the readily available and reliable herbivore-induced volatile signals to find host plants despite the risks they will face on these plants, because the likely alternative would be starvation. A similar argument formulated by Carroll et al. (2006) emphasizes the limited range at which caterpillars can forage, as compared to the highly mobile

adults. The far less mobile caterpillars, when fallen to the ground, have a high risk of predation and are fully exposed to unfavorable environmental conditions. Getting back on a plant should be high priority and in most cases the same plant will be the closest to crawl on. This may also explain why we found that a previous feeding experience has a significant impact on the attractiveness of the induced maize volatiles. Similar preferences for familiar odors in *S. littoralis* larvae were found by Anderson et al. (1995) and Carlsson et al. (1999) when they studied the caterpillar's responses to cotton volatiles. This effect of experience even extends to the adult moth, which prefers to oviposit on the same plant species on which it fed as a larva (Anderson et al., 1995). It is also known that caterpillars adapt their feeding physiology to plant diet on which they feed as neonates and will perform worse on an alternative diet (del Campo et al., 2001; Zalucki et al., 2002), the more reason for the larvae to forage for the same plant species.

Once on an already infested plant, however, caterpillars could lessen the risks of competition/cannibalism, which can be very severe in certain *Spodoptera* species (Chapman et al., 1999, 2000; Richardson et al., 2009), but this is not the case for *S. littoralis*. They will also reduce the risk of predation and parasitism by avoiding the most odorous plant parts (Turlings and Wäckers, 2004). This notion is tentatively supported by the effects of maize VOCs on caterpillar feeding behavior. *S. littoralis* larvae that were exposed to the VOCs induced by their conspecifics on maize plants were found to feed and grow less than larvae that were not exposed to the VOCs (**Figure 3**). This is indicative of an avoidance of the VOCs, which was only evident at high concentrations. Hence, the results of the current study support our hypothesis that on a plant the caterpillars prefer to commence feeding away from freshly damaged areas, i.e., sites from which large amounts of GLVs are emitted. Yet, alternative explanations should be considered. For instance, the larvae that were exposed to GLVs volatiles might have been attracted and searched for the source of the volatiles and therefore ate less on the diet that they were offered. We can also not exclude a direct (toxic) effect of the volatiles on the larvae.

In summary, we show here that *Spodoptera littoralis* larvae are attracted to the volatiles emitted by plants that are already damaged by conspecific larvae. Although such plants are less suitable for the larvae than undamaged plants, the larvae may simply opt to go for readily detectable signals. The notion that the larvae are attracted to reliable, familiar volatile signals even if it leads them to sub-optimal resources is further supported by the fact that previous experience with the odors enhances their attractiveness. But once they are on the plants they seem to avoid the volatiles and eat less when they detect high concentrations of them. We speculate that by doing so the larvae avoid the parts of the plant with up-regulated defenses, competition/cannibalism, and natural enemies that are attracted to the same volatiles.

An understanding of signals that are of importance for host plant foraging by caterpillars can be of use in the development of pest control strategies. In this context, current focus is on foraging of adults and this has found good use in "push-pull" strategies (Khan et al., 2000, 2008; Cook et al., 2007). Similarly, with the right combination of repellent and attractive volatiles, it may be possible to manipulate the foraging of caterpillar such that they are guided away from the crop and toward their demise on trap plants.

ACKNOWLEDGMENTS

We thank Matthias Erb for useful comments on the manuscript. Yves Borcard and several students of the University of Neuchâtel reared parasitoids, and Syngenta (Stein, Switzerland) provided *S. littoralis* eggs. This work received support by the Swiss National Science Foundation via the National Center of Competence in Research *Plant Survival*.

REFERENCES

Anderson, P., Hilker, M., and Löfqvist, J. (1995). Larval diet influence on oviposition behavior in *Spodoptera littoralis*. *Entomol. Exp. Appl.* 74, 71–82. doi: 10.1111/j.1570-7458.1995.tb01876.x

Arimura, G., Matsui, K., and Takabayashi, J. (2009). Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. *Plant Cell Physiol.* 50, 911–923. doi: 10.1093/pcp/pcp030

Bernasconi, M. L., Turlings, T. C. J., Ambrosetti, L., Bassetti, P., and Dorn, S. (1998). Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, shape *Rhopalosiphum maidis*. *Entomol. Exp. Appl.* 87, 133–142. doi: 10.1046/j.1570-7458.1998.00315.x

Bolter, C. J., Dicke, M., Van Loon, J. J. A., Visser, J. H., and Posthumus, M. A. (1997). Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *J. Chem. Ecol.* 23, 1003–1023. doi: 10.1023/B:JOEC.0000006385.70652.5e

Carlsson, M. A., Anderson, P., Hartlieb, E., and Hansson, B. S. (1999). Experience-dependent modification of orientational response to olfactory cues in larvae of *Spodoptera littoralis*. *J. Chem. Ecol.* 25, 2445–2454. doi: 10.1023/A:1020865922827

Carroll, M., Schmelz, E., and Teal, P. (2008). The attraction of *Spodoptera frugiperda* neonates to cowpea seedlings is mediated by volatiles induced by conspecific herbivory and the elicitor inceptin. *J. Chem. Ecol.* 34, 291–300. doi: 10.1007/s10886-007-9414-y

Carroll, M. J., Schmelz, E. A., Meagher, R. L., and Teal, P. E. A. (2006). Attraction of *Spodoptera frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *J. Chem. Ecol.* 32, 1911–1924. doi: 10.1007/s10886-006-9117-9

Castrejon, F., Virgen, A., and Rojas, J. C. (2009). Influence of chemical cues from host plants on the behavior of neonate *Estigmene acrea* larvae (Lepidoptera: Arctiidae). *Environ. Entomol.* 35, 700–707. doi: 10.1603/0046-225X-35.3.700

Chapman, J. W., Williams, T., Escribano, A., Caballero, P., Cave, R. D., and Goulson, D. (1999). Fitness consequences of cannibalism in the fall armyworm, *Spodoptera frugiperda*. *Behav. Ecol.* 10, 298–303. doi: 10.1093/beheco/10.3.298

Chapman, J. W., Williams, T., Martínez, A. M., Cisneros, J., Caballero, P., Cave, R. D., et al. (2000). Does cannibalism in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) reduce the risk of predation? *Behav. Ecol. Sociobiol.* 48, 321–327. doi: 10.1007/s002650000237

Cook, S. M., Kahn, Z. R., and Pickett, J. A. (2007). The use of push-pull strategies in integrated pest management. *Annu. Rev. Entomol.* 52, 375–400. doi: 10.1146/annurev.ento.52.110405.091407

Crowe, M. L. (1995). The effect of season and group-size on survivorship and larval growth in *Plagiodera versicolora*. *Ecol. Entomol.* 20, 27–32. doi: 10.1111/j.1365-2311.1995.tb00425.x

D'Alessandro, M., and Turlings, T. C. J. (2005). *In-situ* modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem. Senses* 30, 739–753. doi: 10.1093/chemse/bji066

D'Alessandro, M., and Turlings, T. C. J. (2006). Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods.

Analyst 131, 24–32. doi: 10.1039/b507589k

Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135, 1928–1938. doi: 10.1104/pp.104.039891

de Groot, P., Grant, G., Poland, T., Scharbach, R., Buchan, L., Nott, R., et al. (2008). Electrophysiological response and attraction of emerald ash borer to green leaf volatiles (GLVs) emitted by host foliage. *J. Chem. Ecol.* 34, 1170–1179. doi: 10.1007/s10886-008-9514-3

del Campo, M. L., Miles, C. I., Schroeder, F. C., Mueller, C., Booker, R., and Renwick, J. A. (2001). Host recognition by the tobacco hornworm is mediated by a host plant compound. *Nature* 411, 186–189. doi: 10.1038/35075559

De Moraes, C. M., Mescher, M. C., and Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410, 577–580. doi: 10.1038/35069058

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help". *Trends Plant Sci.* 15, 167–175. doi: 10.1016/j.tplants.2009.12.002

Dicke, M., Sabelis, M. W., Takabayashi, J., Bruin, J., and Posthumus, M. A. (1990). Plant strategies of manipulating predatorprey interactions through allelochemicals: prospects for application in pest control. *J. Chem. Ecol.* 16, 3091–3118. doi: 10.1007/BF00979614

Fox, L. R. (1975). Cannibalism in natural populations. *Annu. Rev. Ecol. Syst.* 6, 87–106. doi: 10.1146/annurev.es.06.110175.000511

Halitschke, R., Stenberg, J. A., Kessler, D., Kessler, A., and Baldwin, I. T. (2008). Shared signals—"alarm calls" from plants increase apparency to herbivores and their enemies in nature. *Ecol. Lett.* 11, 24–34. doi: 10.1111/j.1461-0248.2007.01123.x

Hansson, B. S., Larsson, M. C., and Leal, W. S. (1999). Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol.* 24, 121–126. doi: 10.1046/j.1365-3032.1999.00121.x

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annu. Rev. Entomol.* 56, 161–180. doi: 10.1146/annurev-ento-120709-144753

Heil, M. (2004). Direct defense or ecological costs: responses of herbivorous beetles to volatiles released by wild lima bean (*Phaseolus lunatus*). *J. Chem. Ecol.* 30, 1289–1295. doi: 10.1023/B:JOEC.0000030299.59863.69

Heil, M. (2008). Indirect defence via tritrophic interactions. *New Phytol.* 178, 41–61. doi: 10.1111/j.1469-8137.2007.02330.x

Hoballah, M. E., and Turlings, T. C. J. (2005). The role of fresh versus old leaf damage in the attraction of parasitic wasps to herbivore-induced maize volatiles. *J. Chem. Ecol.* 31, 2003–2018. doi: 10.1007/s10886-005-6074-7

Huang, C.-H., Yan, F.-M., Byers, J. A., Wang, R.-J., and Xu, C.-R. (2009). Volatiles induced by the larvae of the Asian corn borer (*Ostrinia furnacalis*) in maize plants affect behavior of conspecific larvae and female adults. *Insect Sci.* 16, 311–320. doi: 10.1111/j.1744-7917.2009.01257.x

Kakimoto, T., Fujisaki, K., and Miyatake, T. (2009). Egg laying preference, larval dispersion, and cannibalism in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 96, 793–798. doi: 10.1603/0013-8746(2003)096[0793:ELPLDA]2.0.CO;2

Kalberer, N. M., Turlings, T. C. J., and Rahier, M. (2001). Attraction of a leaf beetle (*Oreina cacaliae*) to damaged host plants. *J. Chem. Ecol.* 27, 647–661. doi: 10.1023/A:1010389500009

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144. doi: 10.1126/science.291.5511.2141

Khan, Z. R., James, D. G., Midega, C. A. O., and Pickett, J. A. (2008). Chemical ecology and conservation biological control. *Biol. Control* 45, 210–224. doi: 10.1016/j.biocontrol.2007.11.009

Khan, Z. R., Pickett, J. A., van den Berg, J., Wadhams, L. J., and Woodcock, C. M. (2000). Exploiting chemical ecology and species diversity: stem borer and striga control for maize and sorghum in Africa. *Pest Manage. Sci.* 56, 957–962. doi: 10.1002/1526-4998(200011)56:11<957::AID-PS236>3.0.CO;2-T

Landolt, P. J. (1993). Effects of host plant leaf damage on cabbage-looper moth attraction and oviposition. *Entomol. Exp. Appl.* 67, 79–85. doi: 10.1111/j.1570-7458.1993.tb01654.x

Landolt, P. J., Brumley, J. A., Smithhisler, C. L., Biddick, L. L., and Hofstetter, R. W. (2000). Apple fruit infested with codling moth are more attractive to neonate codling moth larvae and possess increased amounts of (E, E)-alpha-farnesene. *J. Chem. Ecol.* 26, 1685–1699. doi: 10.1023/A:1005595014589

Landolt, P. J., Tumlinson, J. H., and Alborn, D. H. (1999). Attraction of Colorado potato beetle (Coleoptera: Chrysomelidae) to damaged and chemically induced potato plants. *Environ. Entomol.* 28, 973–978.

Müller, C., and Hilker, M. (2000). The effect of a green leaf volatile on host plant finding by larvae of a herbivorous insect. *Naturwissenschaften* 87, 216–219. doi: 10.1007/s001140050706

Paré, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–332. doi: 10.1104/pp.121.2.325

Piesik, D., Rochat, D., van der Pers, J., and Marion-Poll, F. (2009). Pulsed odors from maize or spinach elicit orientation in European corn borer neonate larvae. *J. Chem. Ecol.* 35, 1032–1042. doi: 10.1007/s10886-009-9676-7

R Development Core Team. (2009). *R: A Language and Environment for Statistical Computing, 2.9.1 Edn.* Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org

Ricard, I., and Davison, A. C. (2007). Statistical inference for olfactometer data. *J. Roy. Stat. Soc. C-App* 56, 479–492. doi: 10.1111/j.1467-9876.2007.00588.x

Richardson, M. L., Mitchell, R. F., Reagel, P. F., and Hanks, L. M. (2009). Causes and consequences of cannibalism in noncarnivorous insects. *Annu. Rev. Entomol.* 55, 39–53. doi: 10.1146/annurev-ento-112408-085314

Rostas, M., and Hilker, M. (2002). Feeding damage by larvae of the mustard leaf beetle deters conspecific females from oviposition and feeding. *Entomol. Exp. Appl.* 103, 267–277. doi: 10.1046/j.1570-7458.2002.00984.x

Shiojiri, K., Ozawa, R., and Takabayashi, J. (2006). Plant volatiles, rather than light, determine the nocturnal behavior of a caterpillar. *PLoS Biol.* 4:e164. doi: 10.1371/journal.pbio.0040164

Tamò, C., Ricard, I., Held, M., Davison, A. C., and Turlings, T. C. J. (2006). A comparison of naïve and conditioned responses of three generalist endoparasitoids of lepidopteran larvae to host-induced plant odours. *Anim. Biol.* 56, 205–220. doi: 10.1163/157075606777304177

Ton, J., D'Alessandro, M., Jourdie, V., Jakab, G., Karlen, D., Held, M., et al. (2007). Priming by airborne signals boosts direct and indirect resistance in maize. *Plant J.* 49, 16–26. doi: 10.1111/j.1365-313X.2006.02935.x

Turlings, T. C. J., Davison, A. C., and Tamò, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. *Physiol. Entomol.* 29, 45–55. doi: 10.1111/j.1365-3032.2004.0362.x

Turlings, T. C. J., Lengwiler, U. B., Bernasconi, M. L., and Wechsler, D. (1998). Timing of induced volatile emissions in maize seedlings. *Planta* 207, 146–152. doi: 10.1007/s004250050466

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253. doi: 10.1126/science.250.4985.1251

Turlings, T. C. J., and Wäckers, F. (2004). Recruitment of predator and parasitoid by herbivore-injured plants. *Adv. Insect Chem. Ecol.* 2, 21–75. doi: 10.1017/CBO9780511542664.003

Vet, L. E. M., and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37, 141–172. doi: 10.1146/annurev.en.37.010192.001041

von Mérey, G., Veyrat, N., Mahuku, G., Valdez, R. L., Turlings, T. C. J., and D'Alessandro, M. (2011). Dispensing synthetic green leaf volatiles in maize fields increases the release of sesquiterpenes by the plants, but has little effect on the attraction of pest and beneficial insects. *Phytochemistry* 72, 1838–1847. doi: 10.1016/j.phytochem.2011.04.022

Whitman, D. W., and Eller, F. J. (1990). Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1, 69–76. doi: 10.1007/BF01325231

Yoneya, K., Ozawa, R., and Takabayashi, J. (2010). Specialist leaf beetle larvae use volatiles from willow leaves infested by conspecifics for reaggregation in a tree. *J. Chem. Ecol.* 36, 671–679. doi: 10.1007/s10886-010-9808-0

Zalucki, M. P., Clarke, A. R., and Malcolm, S. B. (2002). Ecology

and behavior of first instar larval Lepidoptera. *Annu. Rev. Entomol.* 47, 361–393. doi: 10.1146/annurev.ento.47.091201.145220

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2013; accepted: 02 June 2013; published online: 28 June 2013.
*Citation: von Mérey GE, Veyrat N, D'Alessandro M and Turlings TCJ (2013) Herbivore-induced maize leaf volatiles affect attraction and feeding behavior of Spodoptera littoralis caterpillars. Front. Plant Sci. **4**:209. doi: 10.3389/fpls.2013.00209*
This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.
Copyright © 2013 von Mérey, Veyrat, D'Alessandro and Turlings. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 12 April 2013
doi: 10.3389/fpls.2013.00074

E-2-hexenal promotes susceptibility to *Pseudomonas syringae* by activating jasmonic acid pathways in *Arabidopsis*

Alessandra Scala[1], Rossana Mirabella[1], Cynthia Mugo[2], Kenji Matsui[2], Michel A. Haring[1] and Robert C. Schuurink[1]*

[1] Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands
[2] Department of Biological Chemistry, Faculty of Agriculture, Graduate School of Medicine, Yamaguchi University, Yamaguchi, Japan

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Choong-Min Ryu, Korea Research Institute of Bioscience and Biotechnology, South Korea
Eric Kemen, Max Planck Institute for Plant Breeding Research, Germany
Marcel Dicke, Wageningen University, Netherlands

***Correspondence:**
Robert C. Schuurink, Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, Netherlands.
e-mail: r.c.schuurink@uva.nl

Green leaf volatiles (GLVs) are C6-molecules – alcohols, aldehydes, and esters – produced by plants upon herbivory or during pathogen infection. Exposure to this blend of volatiles induces defense-related responses in neighboring undamaged plants, thus assigning a role to GLVs in regulating plant defenses. Here we compared *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*) with a *hydroperoxide lyase* line, *hpl1*, unable to synthesize GLVs, for susceptibility to *Pseudomonas syringae* pv. *tomato* (DC3000). We found that the growth of DC3000 was significantly reduced in the *hpl1* mutant. This phenomenon correlated with lower jasmonic acid (JA) levels and higher salicylic acid levels in the *hpl1* mutant. Furthermore, upon infection, the JA-responsive genes *VSP2* and *LEC* were only slightly or not induced, respectively, in *hpl1*. This suggests that the reduced growth of DC3000 in *hpl1* plants is due to the constraint of JA-dependent responses. Treatment of *hpl1* plants with *E*-2-hexenal, one of the more reactive GLVs, prior to infection with DC3000, resulted in increased growth of DC3000 in *hpl1*, thus complementing this mutant. Interestingly, the growth of DC3000 also increased in *Ler* plants treated with *E*-2-hexenal. This stronger growth was not dependent on the JA-signaling component MYC2, but on ORA59, an integrator of JA and ethylene signaling pathways, and on the production of coronatine by DC3000. GLVs may have multiple effects on plant–pathogen interactions, in this case reducing resistance to *Pseudomonas syringae* via JA and ORA59.

Keywords: green leaf volatiles, *Pseudomonas syringae*, jasmonate, coronatine, hormone crosstalk

INTRODUCTION

Plants produce green leaf volatiles (GLVs), C6-aldehydes, C6-alcohols, and their acetates, through the lipoxygenase (LOX) and hydroperoxide lyase (HPL) pathways. Linoleic and linolenic acid are the substrates for dioxygenation and subsequent cleavage to obtain C6-volatile aldehydes that can be further modified by alcohol dehydrogenases (ADH), an isomerization factor and an acetyltransferase leading to the formation of a bouquet of these volatiles. Intact plants produce only trace amounts of GLVs, whereas these compounds are rapidly emitted in large amounts after wounding, herbivory or pathogen attack (Croft et al., 1993; Turlings et al., 1995; Fall et al., 1999; Shiojiri et al., 2000, 2006a; Heiden et al., 2003).

Green leaf volatiles have been reported to play important roles in different biological processes (Bate and Rothstein, 1998; Arimura et al., 2000; Farag and Paré, 2002; Engelberth et al., 2004; Farag et al., 2005; Ruther and Fürstenau, 2005; Ruther and Kleier, 2005). Herbivory induces very specific sets of GLVs that are perceived by natural predators of the herbivores (Kessler and Baldwin, 2001; Birkett et al., 2003; Gouinguené et al., 2005; Shiojiri et al., 2006a,b). Beside a role in indirect defenses, GLVs also act as airborne signaling molecules regulating plant defense responses. Several studies show that plants themselves upon exposure to GLVs respond by activating wound- and herbivore-induced defenses. Examples of this are found in *Zea mays* (maize), *Citrus jambhiri*, *Nicotiana attenuata* (tobacco), *Gossypium hirsutum*, *Lycopersicon esculentum* (tomato), and *Arabidopsis thaliana* plants where GLV perception induces the transcription of genes known to be involved in defense responses, or in biosynthesis of defense-related secondary metabolites (Bate and Rothstein, 1998; Arimura et al., 2001; Gomi et al., 2003; Weber et al., 2004; Farag et al., 2005; Kishimoto et al., 2005, 2006; Paschold et al., 2006), resulting in the production of defensive compounds (Zeringue, 1992; Bate and Rothstein, 1998; Farag and Paré, 2002; Engelberth et al., 2004; Farag et al., 2005; Ruther and Fürstenau, 2005; Kishimoto et al., 2006; Yan and Wang, 2006). Besides direct defense elicitation, exposure to GLVs, emitted from wounded leaves, has also been shown to prime systemic leaves for augmented defense responses upon future attacks (Engelberth et al., 2004; Kessler et al., 2006; Frost et al., 2007, 2008; Heil and Silva Bueno, 2007). Similarly, the *E*-2-hexenal released by rice upon planthopper infestation, induces expression of defense-related genes, increasing resistance to bacterial blight (Gomi et al., 2010). In some of these examples the effect of GLVs and jasmonic acid (JA) signaling have been linked (Engelberth et al., 2004; Halitschke et al., 2004; Kishimoto et al., 2006; Allmann et al., 2010; Tong et al., 2012).

Finally, GLVs possess fungicidal and bactericidal activity (Prost et al., 2005; Shiojiri et al., 2006b). Since GLVs are released after infection with pathogenic fungi and bacteria (Croft et al., 1993; Heiden et al., 2003; Shiojiri et al., 2006b), this suggests that a possible physiological role of these volatiles is to limit pathogen growth. Several observations support this hypothesis. For instance, upon infection with the pathogenic bacteria *Pseudomonas syringae*, *Phaseolus vulgaris* (lima bean) leaves release relatively high amounts of the C6-aldehyde *E*-2-hexenal and the C6-alcohol Z-3-hexenol (Croft et al., 1993). Moreover, pre-treatment with the C6-aldehyde *E*-2-hexenal as well as genetic manipulation to enhance C6-volatile production, resulted in increased resistance against the necrotrophic fungus *Botrytis cinerea* in *Arabidopsis*, most likely as a result of both activation of defense responses and direct inhibition of fungal growth (Kishimoto et al., 2005; Shiojiri et al., 2006b).

Since all this evidence indicates a role for GLVs in regulating plant responses to bacterial pathogens and GLV levels have been shown to increase in plants upon infection with *Pseudomonas syringae* (Croft et al., 1993; Heiden et al., 2003), we decided to further dissect the role of GLVs in the interaction of plants with this pathogen. Increased GLV levels could directly inhibit the pathogen and/or promote infection through downstream signaling favorable for the pathogen. *Pseudomonas syringae* pv. *tomato* DC3000 is a plant pathogen that enters leaves through stomata, multiplies in the apoplast, and produces necrotic lesions with chlorotic halos (Hirano and Upper, 2000). *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) causes bacterial speck on tomato (Cuppels, 1986), but also on *A. thaliana* (Whalen et al., 1991). DC3000 produces coronatine (COR), a toxin, responsible for chlorotic halos, which mimics the action of JA-isoleucine (JA-Ile), the active form of JA. With this phytotoxin DC3000 exploits the antagonistic interaction between JA and salicylic acid (SA) in order to shut down SA-dependent defenses that plant triggers to fight against *Pseudomonas* infections (Block et al., 2005; Glazebrook, 2005).

We especially focused on the role of *E*-2-hexenal during the *Arabidopsis–Pseudomonas* interaction. Although it is not the most abundant C6-volatile produced by HPL activity, *E*-2-hexenal is emitted during *Pseudomonas* ssp. infections in lima bean (Croft et al., 1993) and in tobacco (Heiden et al., 2003), and it has the highest bactericidal activity *in vitro* among oxylipins (Prost et al., 2005), likely because its α,β-unsaturated carbonyl moiety that can react with nucleophilic groups (Farmer and Davoine, 2007). Additionally, *E*-2-hexenal has been shown to induce several responses in *Arabidopsis*, including induction of defenses, inhibition of root growth and enhancement of resistance against the necrotrophic fungus *B. cinerea* (Bate and Rothstein, 1998; Kishimoto et al., 2005; Mirabella et al., 2008). In order to determine the role of GLVs in the responses against *Pseudomonas*, we set out to study *Arabidopsis* plants with and without a functional HPL (Shiojiri et al., 2012) and did complementation studies with *E*-2-hexenal. Remarkably we found that the presence of a working copy of HPL increased susceptibility of *Arabidopsis* to DC3000. Treatment with *E*-2-hexenal also enhanced the susceptibility to this bacterial pathogen. We found evidence that this is mediated by the transcription factor ORA59, one of the main players in the JA-signaling pathways, and required the production of the bacterial toxin COR.

MATERIALS AND METHODS

PLANT LINES

Arabidopsis thaliana ecotype Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) were used. The *hpl*1 mutant is an introgression line between Col-0 and L*er* (Shiojiri et al., 2012). The mutant *myc2* (*jin1-7*; Verhage et al., 2011), the transgenic lines RNAi-ORA59 and the 35S:GUS plants (Pré et al., 2008) were all in the Col-0 background. Plants were grown in soil in a growth chamber at 21°C, 70% relative humidity under an 11-h photoperiod with 100 $\mu E\ s^{-1}\ m^{-2}$.

BACTERIAL POPULATION COUNTS

Bacteria were grown overnight at 28°C in liquid King's broth (KB) medium (King et al., 1954) containing rifampicin (50 μg/ml) for the *Pseudomonas syringae* pv. *tomato* DC3000 strain, and kanamycin (100 μg/ml) for the cor$^-$ DC3682 mutant strain, unable to produce COR (Ma et al., 1991). Plants were inoculated with either a low dose (OD_{600} of 0.0007), for bacterial growth assays, or a high dose (OD_{600} of 0.007), for qRT-PCR and hormone quantification, of the bacterial suspension, and bacteria (colony forming units, cfu) were counted as reported in Park et al. (2010).

PLANT HORMONES EXTRACTION AND QUANTIFICATION

For JA and SA quantification, 12 leaves were harvested, in pools of 4, from 12 different mock-infiltrated (10 mM $MgSO_4$) or bacteria-infiltrated plants in two independent experiments. To extract JA and SA, frozen leaf material (50–150 mg) was ground and homogenized in 0.5 ml 70% methanol, spiked with 200 ng of D6-JA and D6-SA (internal standards for extraction efficiency; CDN Isotopes, Canada[1]), with a Precellys24 automated lyser (Bertin Technologies[2]). Samples were homogenized twice by shaking at 6,000 rpm for 40 s and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants of two extraction steps were pooled. Hormones were quantified by liquid chromatography–mass spectrometry (LC–MS) analysis on Varian 320 Triple Quad LC/MS/MS. Ten microliters of each sample were injected onto a C18 Pursuit 5 (50 mm × 2.0 mm) column (Varian) coupled to a double mass spectrometer in tandem (Varian 320 MS-MS[3]). The mobile phase comprised solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) as follows: 85% solvent A for 1 min 30 s (flow rate 0.4 ml/min), followed by 3 min in which solvent B increased till 98% (0.2 ml/min) which continued for 5 min 30 s with the same flow rate, followed by 2 min 30 s with increased flow rate (0.4 ml/min), subsequently returning to 85% solvent A in 1 min, conditions that were kept till the end of the run, in total 15 min. Compounds were detected in the electrospray ionization negative mode. Molecular ions $[M\text{-}H]^-$ at *m*/*z* 137 and 209 and 141 and 213 generated from endogenous SA and JA and their internal standards, respectively, were fragmented under 12 V collision energy. The ratios of ion intensities of their respective daughter ions, *m*/*z* 93 and 97 and *m*/*z* 59 and 63, were used to quantify endogenous SA and JA, respectively.

[1]www.cdnisotopes.com

[2]http://www.bertin.fr

[3]www.home.agilent.com

QUANTITATIVE RT-PCR

For analysis of transcript levels, total RNA was isolated using Trizol from 10 infiltrated leaves, harvested from 10 different plants, in three independent experiments and treated with TurBo DNA-free (Ambion[4]) to remove DNA. cDNA was synthesized from 1 μg of total RNA using M-MuLV reverse transcriptase (Fermentas[5]), as described by the manufacturer, in a 20-μl reaction that was diluted to 50 μl prior to using it for the real-time PCR. This was performed in a 20-μl volume containing 2 μl of cDNA, 0.4 pmol of specific primer sets for each gene and 10 μl of iTaq™ SYBR Green Supermix with ROX (Bio-Rad[6]). PCR conditions were as follows: 95°C for 2 min 30 s (first cycle), 95°C for 15 s and 60°C for 30 s (40 cycles). To ensure amplification of a single product during the qRT-PCR reactions, a dissociation protocol was performed in which samples were slowly heated from 55 to 95°C. qRT-PCR was performed using the ABI Prism 7000 real-time PCR detection system (Applied Biosystems) and the data were collected using software (ABI 7000 SDS version 1) provided by the supplier. Transcript levels were normalized to the levels of the *SAND* gene (At2g28390; Hong et al., 2010) and quantification was performed as described in previous work (Pfaffl, 2001). Primer sequences were as reported in (Anderson and Badruzsaufari, 2004; Czechowski et al., 2005; Park et al., 2010) for *PR1*, *VSP2*, *LEC*, and *SAND*, respectively.

TRYPAN BLUE AND ANILINE BLUE STAINING

Trypan blue staining solution was prepared by adding trypan blue to lactophenol (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, and 10 ml distilled water) to a concentration of 2.5 mg/ml. Two volumes of ethanol were added to the trypan blue–lactophenol solution. To visualize plant cell death, mock and DC3000 infected leaf tissues were placed in plates containing staining solution and heated in a microwave at intervals for 1 min. The plates were incubated for 2 h at room temperature, followed by destaining (three times) in chloral hydrate (2.5 g/ml). The leaf tissues were mounted in 70% glycerol for observations with a microscope. For detection of callose deposition, leaves were incubated for at least 24 h in 96% ethanol until all tissues were transparent and stained in 0.01% aniline blue in 0.15 M K_2HPO_4 (pH 8.5). Leaf tissues were incubated for 1.5–3 h, mounted on slides, and observed under an epifluorescence microscope (AF6000) with UV filter (excitation filter: BP 470/40 nm; emission filter: BP 525/50 nm).

CALLOSE QUANTIFICATION

Callose was quantified from digital photographs as the number of white pixels, covering the whole leaf material, using Photoshop CS7 software. Contrast settings of photographs were adjusted to obtain an optimal separation of the callose signal from the background signal. Callose was selected automatically, using the "Color Range" tool. In cases in which the contrast settings resulted in significant loss of callose signal, due to high autofluorescence of vasculature tissue, callose was selected manually, using the "Magic Wand" tool of Photoshop CS7. Relative callose intensities were quantified as the number of fluorescent callose-corresponding pixels relative to the total number of pixels covering plant material (Luna et al., 2011).

E-2-HEXENAL TREATMENT

Plants were grown for 3 weeks under the conditions mentioned above before being exposed to volatiles. For the volatile treatment, 10 plants in single pots were placed into airtight glass desiccators (22 l). *E*-2-hexenal was diluted in methanol, and applied to a sterile cotton swab, placed in an Erlenmeyer flask, between the plants in the desiccators to give a final concentration of 3 μM. For the control treatment, only methanol was applied. Plants were incubated in the desiccators for 24 h and subsequently taken out to be placed under the growth conditions described above for 1 h, prior to infiltration with bacteria or mock solution as mentioned above. *E*-2-hexenal was purchased from Sigma-Aldrich.

RESULTS

hpl1 INFLUENCES SUSCEPTIBILITY TO *Pseudomonas syringae* pv. *tomato* (DC3000)

In order to determine whether the ability to synthesize GLVs had an effect on *Arabidopsis* susceptibility to pathogenic bacteria, we compared Landsberg *erecta* (*HPL*, L*er*) and an introgression line between Col-0 and L*er* that can synthesize only trace amounts of GLVs, *hpl1* (Shiojiri et al., 2012), for the susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000. To ensure infection throughout the entire leaf, we used the syringe infiltration method since it overcomes stomatal defenses and maximizes the number of responding cells (de Torres Zabala et al., 2009), and bacterial populations were determined 72 hpi (hours post-infection). **Figure 1** shows that DC3000 populations were lower in the *hpl1* line. The difference measured in bacterial population between L*er* and *hpl1* (∼4.6-fold) was statistically significant (*t*-test $P < 0.05$). This indicates that the *hpl1* line is less susceptible to DC3000 than L*er*.

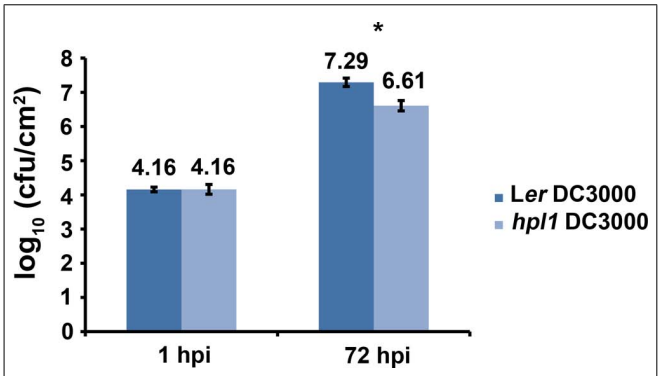


FIGURE 1 | *HPL* influences bacterial growth. Bacterial populations of DC3000 in infected L*er* and *hpl1* leaves 1 hours post infection (hpi) and 72 hpi. Values are the mean of 27 sets of two leaf disks from 20 plants. Error bars represent standard error. Bars annotated with an asterisk indicate significant differences among 72 hpi samples ($P < 0.05$, according to Student's *t*-test analysis). The data presented are from a representative experiment that was repeated four times with similar results.

[4]http://www.ambion.com

[5]http://www.fermentas.com

[6]www.bio-rad.com

hpl1 INFLUENCES JA AND SA LEVELS DURING THE INFECTION WITH DC3000

It is well known that the balance between JA and SA is crucial for the interaction that will be established between a pathogen and its host (Spoel and Dong, 2008; Grant and Jones, 2009; Pieterse et al., 2009). We therefore monitored the changes in JA and SA in L*er* and the *hpl1* plants, prior to the bacterial population measurement, at 2, 24, and 48 hpi. As shown in **Figure 2A**, the levels of JA were up at 2 hpi in all treatments, most likely because of the mechanical damage caused by the inoculation with the syringe. At 24 hpi, this wound response was reset, as JA levels were very low, comparable to the mock inoculation. The situation changed at 48 hpi when JA levels increased in DC3000 infested leaves, in L*er* approximately threefold higher than in *hpl1*. SA levels (**Figure 2B**) changed already at 24 hpi, with levels being approximately 1.7-fold higher in *hpl1* than in L*er*, suggesting that SA-related defenses are activated earlier in *hpl1*. In L*er*, the SA levels were higher than in *hpl1* at 48 hpi suggesting that these defenses are mounted later in L*er*.

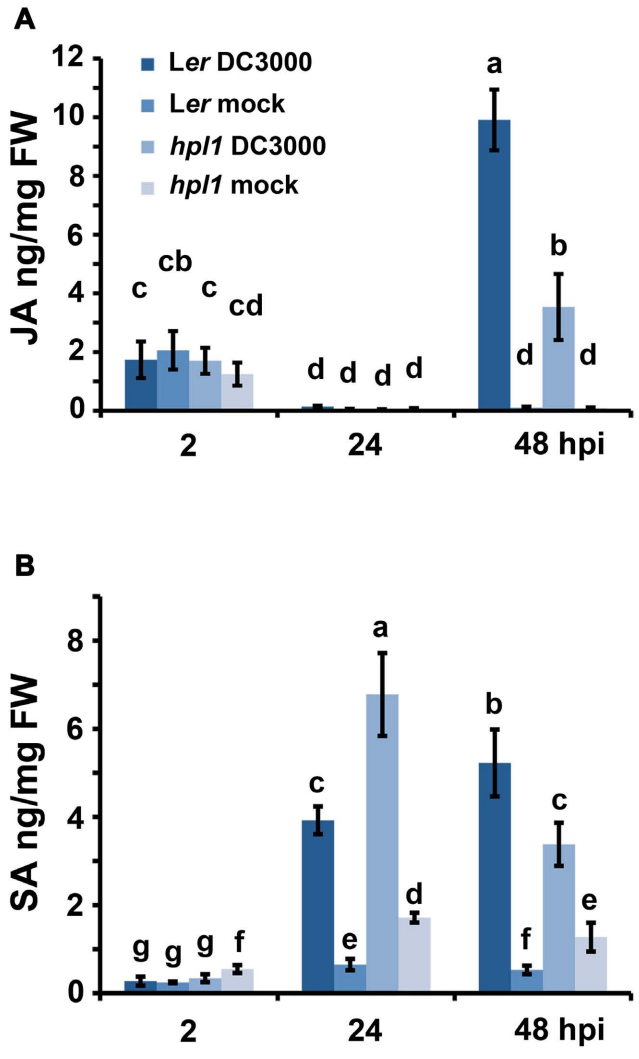


FIGURE 2 | DC3000 infection results in higher JA levels in L*er* plants and higher SA levels in *hpl1* plants. (A) JA levels in L*er* and *hpl1* infected with DC3000 at 2, 24, and 48 hpi; **(B)** SA levels in L*er* and *hpl1* plants infected with DC3000 at 2, 24, and 48 hpi. In both cases, the hormone levels in the 10 mM $MgSO_4$ (mock) infiltrated plants are also shown. For each timepoint and genotype, nine leaves were harvested, in pools of three from mock-infiltrated or bacteria-infiltrated plants and used for plant hormone quantification. Bars represent the mean of two independent experiments. Error bars represent standard error. Bars annotated with different letters indicate statistically different hormone levels [$P < 0.05$, according to analysis of variance (ANOVA), followed by a least significant difference (LSD) *post hoc* test].

JA MARKER GENES ARE LESS INDUCED IN *hpl1* THAN L*er* WHEN INFECTED WITH DC3000

In order to determine whether the differences in hormone levels had an effect on the expression of relevant marker genes in our system, we performed qRT-PCR for genes downstream of JA and SA. We chose *VSP2* and *LEC* for JA (Potter et al., 1993; Penninckx et al., 1998; Thomma et al., 1998; Liu et al., 2005; Pré et al., 2008) and *PR-1* for SA (Bowling et al., 1997; Clarke et al., 2001). *PR1* expression was clearly induced by DC3000 at 48 hpi, however, to similar levels in L*er* and *hpl1* plants (**Figure A1** in Appendix). In contrast, transcript levels of both *VSP2* and *LEC* at 48 hpi (and 24 hpi) were much lower in *hpl1* than in L*er* (**Figures 3A,B**). This result is consistent with the observed lower JA levels in *hpl1* at 48 hpi (**Figure 2A**).

Ler (*HPL*) AND *hpl1* DIFFER IN THE NUMBER OF DEAD CELLS AND IN CALLOSE DEPOSITION

To investigate further the differences between L*er* and *hpl1* in mounting plant defense responses, we decided to look at the appearance of dead cells and callose deposition. Dead cells are indicative of programed cell death (or the hypersensitive response, HR) and enhanced resistance, usually occurring when an pathogenic effector is recognized by the host (Alfano and Collmer, 1996), whereas callose is typically triggered by conserved pathogen-associated molecular patterns (PAMPs), such as flagellin, at the sites of infection during the relatively early stages of pathogen invasion (Brown et al., 1998; Gómez-Gómez et al., 1999; Jones and Dangl, 2006). Dead cells appeared earlier and more frequently in the more resistant *hpl1* while callose deposition occurred earlier and more abundantly in the more susceptible L*er* (**Figures 4A–C**). Dead cells appeared at day 2 in *hpl1*, whereas in L*er* they were not present at all, even at day 3. L*er* started to deposit callose massively at day 1, while much less papillae at this time could be observed in *hpl1*. Moreover, even at later stages of infection, at days 2 and 3, L*er* showed more callose deposition than *hpl1*.

E-2-HEXENAL TREATMENT INCREASES SUSCEPTIBILITY TO DC3000

Since *hpl1* is unable to produce GLVs, we addressed the question whether application of GLVs would restore its susceptibility to DC3000 comparable to L*er*. We chose to use the C6-aldehyde *E*-2-hexenal, one of the most active GLVs, and treated *hpl1* and L*er* plants with 3 μM aerial *E*-2-hexenal or with the carrier methanol (MeOH) for the control treatment. **Figure 5A** shows that the treatment with the C_6-aldehyde turned both *hpl1* and L*er* more susceptible to DC3000, as bacterial populations increased about five- and ninefold, respectively, in the *E*-2-hexenal pre-treated

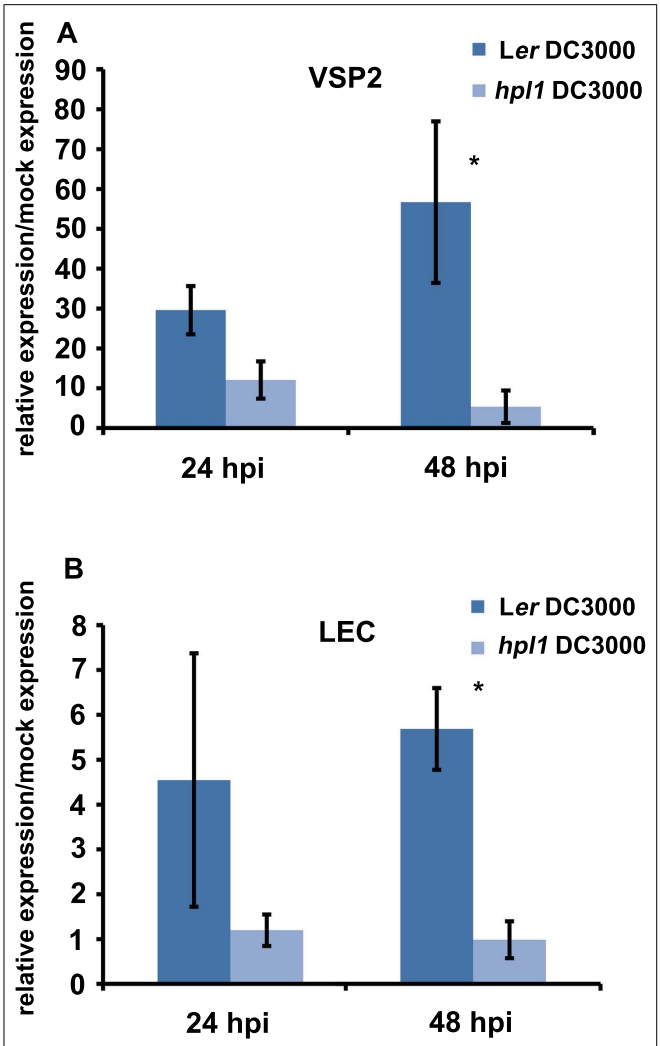


FIGURE 3 | JA-dependent gene expression is higher in infected L*er* plants. (A) VSP2 transcript levels and **(B)** LEC transcript levels were measured by qRT-PCR in L*er* and *hpl1* infected with DC3000 at 24 and 48 hpi and normalized for SAND transcript levels. Bars represent the ratio between the transcript levels in infected and mock samples. Three infected or mock infiltrated leaves were harvested from three different plants and pooled for RNA isolation. Bars represent the mean of three independent experiments. Error bars represent standard error. Bars annotated with asterisk indicate significant differences among samples ($P < 0.05$, according to *t*-test analysis).

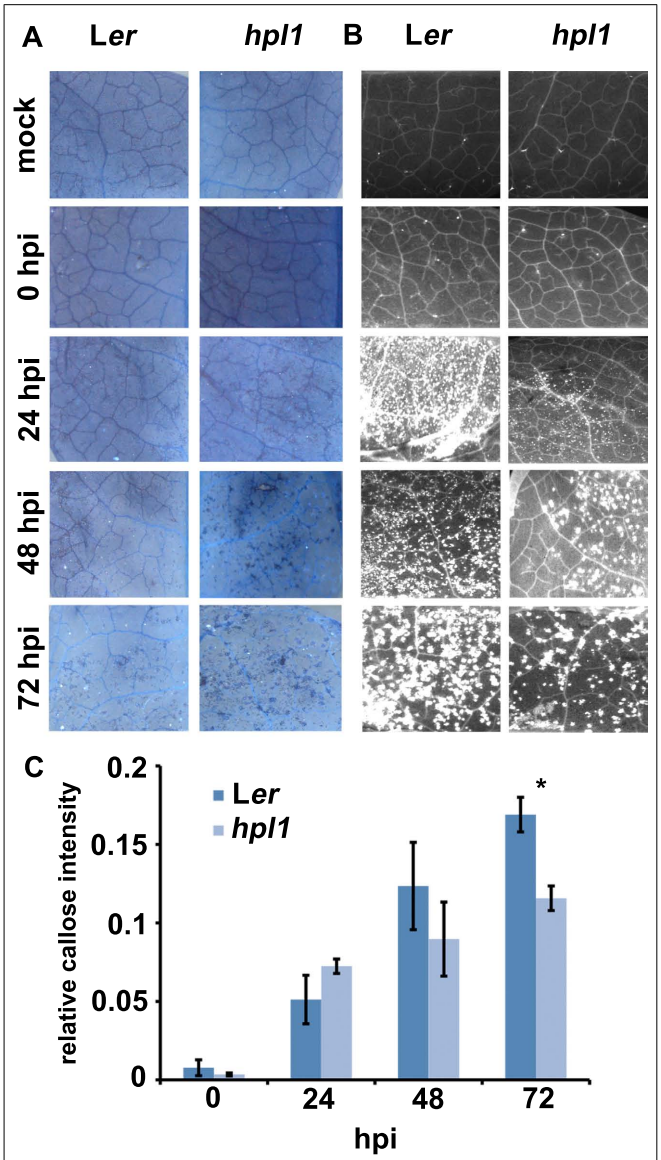


FIGURE 4 | Dead cells and callose deposition are different in L*er* and *hpl1*. (A) Trypan blue staining showing small clusters of dead cells in *hpl1* but not in L*er* leaves infected with DC3000. **(B)** Aniline blue stained leaf tissues observed under UV illumination showing earlier and higher callose deposition in L*er* than in *hpl1* leaves infected with DC3000. **(C)** Relative callose intensity. Bars represent the mean of three different experiments. Error bars represent standard error. Bars annotated with an asterisk indicate a significant difference among samples ($P < 0.05$, according to *t*-test analysis).

leaves compared to the control pre-treatment (**Figure 5B**). Additionally, we measured JA and SA levels in L*er* and *hpl1* plants infected with DC3000 after pre-treatment with *E*-2-hexenal or MeOH. Although JA and SA levels increased 48 hpi after DC3000 infection, no significant differences in hormone levels were detected between the *E*-2-hexenal and the control treatment or between L*er* and *hpl1* (**Figure A2** in Appendix).

THE EFFECT OF *E*-2-HEXENAL ON BACTERIAL GROWTH ACTS VIA ORA59.

Since a functional HPL leads to higher susceptibility and higher JA levels upon DC3000 infection and *E*-2-hexenal pre-treatment increased susceptibility of *Arabidopsis* to DC3000 we sought to elucidate part of the signaling pathways involved, by testing if *Arabidopsis* mutants in the JA-signaling pathway were still more susceptible to DC3000 after treatment with *E*-2-hexenal. We chose to analyze MYC2 and ORA59 impaired lines since these are the main players in regulating JA-dependent responses and are located in two different branches of the JA-signaling pathway (Lorenzo et al., 2003, 2004; Anderson and Badruzsaufari, 2004; Dombrecht et al., 2007; Oñate-Sánchez et al., 2007; Kazan and Manners, 2008;

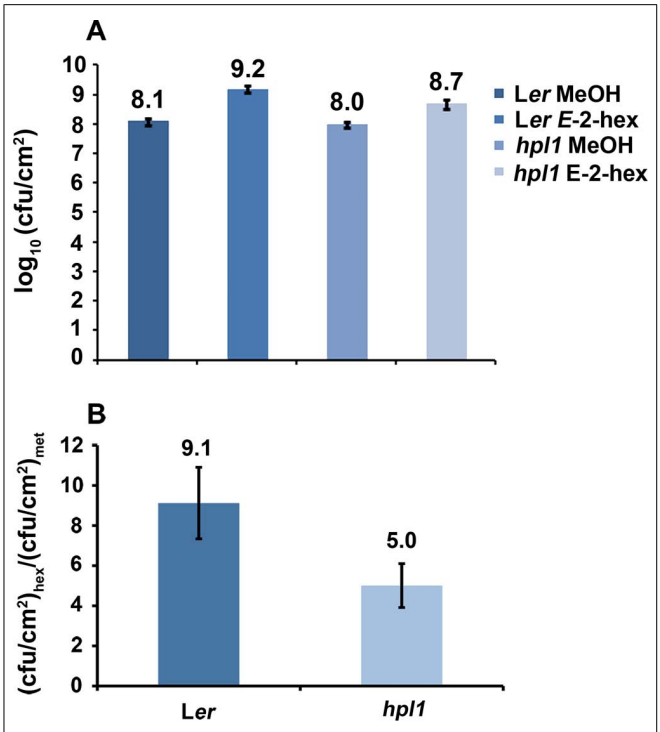


FIGURE 5 | *E*-2-hexenal pre-treatment increases susceptibility to DC3000. (A) DC3000 populations in L*er* and *hpl1* pre-treated with 3 μM *E*-2-hexenal or methanol were measured 72 hpi. Values are the mean of 16 sets of two leaf disks from 12 plants. Error bars represent standard error. The data presented are from a representative experiment that was repeated four times with similar results. All pre-treatments with *E*-2-hexenal were significantly different from the control treatment ($P < 0.05$, according to Student's *t*-test analysis). **(B)** Bars represent the ratio between cfu/cm^2 with *E*-2-hexenal pre-treatment and cfu/cm^2 with methanol pre-treatment (control). Values are the mean of three independent experiments. Error bars represent standard error.

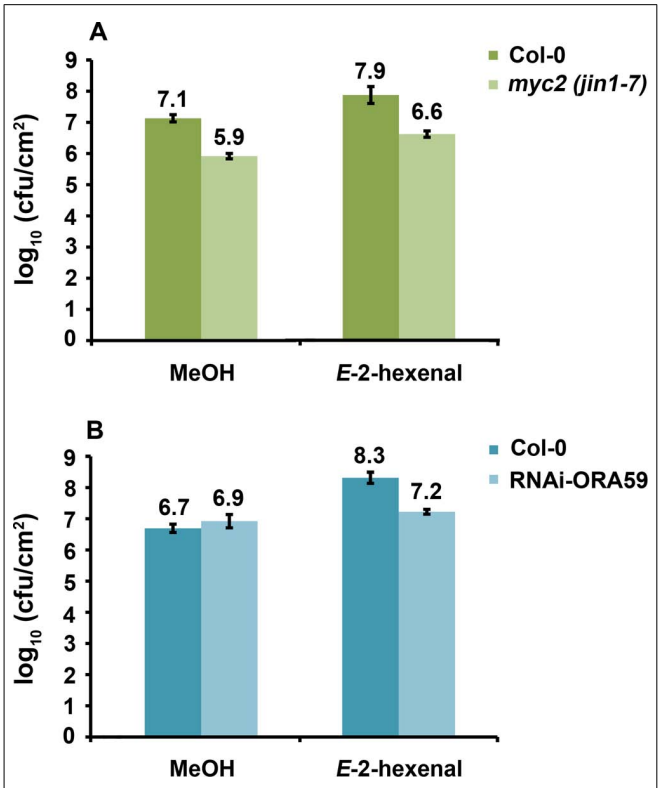


FIGURE 6 | Reduction of *ORA59* expression influences *E*-2-hexenal effect on bacterial growth. (A) Bacterial populations of DC3000 in inoculated *myc2* (*jin1-7*) and Col-0 leaves 72 hpi. Plants were pre-treated 24 h with 3 μM *E*-2-hexenal or methanol. **(B)** Bacterial populations of DC3000 in inoculated RNAi-ORA59 and 35S:GUS plants at 72 hpi. Plants were pre-treated with 3 μM *E*-2-hexenal or methanol for 24 h. Values are the mean of 24 sets of two leaf disks from 20 plants. Error bars represent standard error. All pre-treatments with *E*-2-hexenal were significantly different from the control treatment ($P < 0.05$, according to Student's *t*-test analysis), except for RNAi-ORA59. The data presented are from a representative experiment that was repeated three times with similar results.

Pré et al., 2008). As shown in **Figure 6A**, *myc2* (*jin1-7*) plants were more resistant to DC3000 as has been reported (Fernández-Calvo et al., 2011). Moreover, *myc2* as well as wild-type plants showed increased susceptibility to DC3000 when pre-treated with *E*-2-hexenal, seemingly excluding a role for MYC2 in mediating this phenomenon. In contrast, the same assay performed on RNAi-ORA59 plants (Pré et al., 2008) showed that the bacterial populations increased significantly less in the ORA59 silenced plants compared to the corresponding control line after *E*-2-hexenal treatment (**Figure 6B**). This indicates an involvement of ORA59 in this response to *E*-2-hexenal.

THE *E*-2-HEXENAL EFFECT IS CORONATINE DEPENDENT

Pseudomonas syringae pv. *tomato* strain DC3000 synthesizes COR (Mitchell, 1982), a phytotoxin that mimics JA-Ile (Thines et al., 2007; Yan et al., 2009), in order to antagonize the SA-dependent defenses (Brooks et al., 2005; Glazebrook, 2005). Therefore, we also determined whether the production of COR was necessary for DC3000 to proliferate more in *E*-2-hexenal treated plants. For this, L*er* and *hpl1* plants were infected with the *Pseudomonas syringae* mutant strain DC3682 (Ma et al., 1991), that is unable to produce COR, after pre-treatment with *E*-2-hexenal or methanol. **Figure 7** shows that the bacterial populations of the *cor* mutant were only slightly, but significantly, higher in L*er* or *hpl1* plants treated with *E*-2-hexenal compared to the control plants, but that this increase was much lower than for DC3000 (**Figure 1**). Thus COR seems to be necessary for DC3000 to benefit from the *E*-2-hexenal treatment.

DISCUSSION

Green leaf volatiles have received considerable attention for their ability to induce direct and indirect defense responses in plants and can be considered important players in the already complex network regulated during biotic stress. However the mechanisms by which GLVs influence pathogenesis, and the signaling pathways involved in these responses, are not well known. To address this, we used L*er* and its *Arabidopsis* introgression line, *hpl1*, lacking GLV synthesis, and analyzed their response during infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (DC3000). DC3000 was chosen because in some plant species such as lima bean and tobacco, infection triggers *E*-2-hexenal emission (Croft

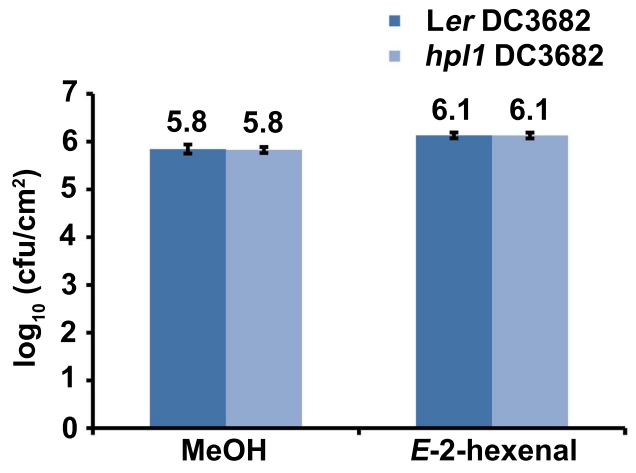


FIGURE 7 | The effect of *E*-2-hexenal is partially dependent on coronatine. Bacterial populations of the *cor* mutant (DC3682) in inoculated L*er* and *hpl1* leaves at 72 hpi. Plants were pre-treated 24 h with 3 μM *E*-2-hexenal or methanol. Values are the mean of 24 sets of two leaf disks from 20 plants. Error bars represent standard error. All pre-treatments with *E*-2-hexenal were significantly different from the control treatment ($P < 0.05$, according to Student's *t*-test analysis). The data presented are from a representative experiment that was repeated three times with similar results.

et al., 1993; Heiden et al., 2003). We hypothesized that *hpl1* plants would be more susceptible to DC3000 since there is evidence that GLVs and *E*-2-hexenal have antimicrobial properties (Prost et al., 2005), induce defense-related genes or biosynthesis of defense-related secondary metabolites (Bate and Rothstein, 1998; Arimura et al., 2001; Gomi et al., 2003; Weber et al., 2004; Farag et al., 2005; Kishimoto et al., 2005, 2006; Paschold et al., 2006), and increase resistance against *B. cinerea* (Kishimoto et al., 2005). However, we found the opposite result: plants impaired in GLV production were more resistant to DC3000 (**Figure 1**). A similar result was very recently shown in rice where the mutant Os*hpl3*, not able to synthesize GLVs, was more resistant to *Xanthomonas oryzae* pv. *oryzae* (Tong et al., 2012).

Subsequently, we investigated some of the mechanisms underlying this result by analyzing the levels of SA and JA since it is well known that these phytohormones and their antagonism are crucial for the development of pathogenesis in *Arabidopsis* (Spoel and Dong, 2008; Grant and Jones, 2009; Pieterse et al., 2009). Hormone measurements clearly showed that JA levels were much lower in *hpl1* than in L*er* (**Figure 2A**). Conversely, *hpl1* showed an earlier induction of SA than L*er* (**Figure 2B**). These data suggest that a non-functional *HPL* gene influences the JA-branch of the oxylipin pathway, leading to lower production of JA when *Arabidopsis* is challenged with *Pseudomonas*. Thus, this is not related to substrate competition as previously shown in *Arabidopsis* where ectopic expression of *HPL* led to lower JA levels upon wounding (Chehab et al., 2006). Reduction of *HPL* expression in rice and *N. attenuata* also influenced JA levels but differently: Os*hpl3* and *asHPL1* had increased JA levels (Halitschke et al., 2004; Tong et al., 2012), in *N. attenuata* probably due to crosstalk between the GLV and JA pathway (Allmann et al., 2010).

Since JA-signaling downstream of COI1 occurs via two different branches, regulated by MYC2 or ORA59, we used markers for both branches to study their activation after DC3000 infection. *LEC*, a lectin-like gene, was used for the ORA59 pathway since it is induced by methyl-jasmonate and upon ORA59 overexpression (Schenk et al., 2000; Pré et al., 2008), while *VSP2* was used for the MYC2 pathway (Abe et al., 2003; Dombrecht et al., 2007). Both *VSP2* and *LEC* transcript levels were much lower in *hpl1* than in L*er* (**Figures 3A,B**) concurrent with the lower JA levels. Thus DC3000 activates in L*er*, with an active HPL unlike Col-0 (Duan et al., 2005), with which most DC3000 experiments are carried out, both branches of the JA-signaling pathway and antagonistic control of these distinct branches of the JA pathway (Verhage et al., 2011) is apparently minor. Transcript levels of the SA-marker *PR-1* were higher upon DC3000 infection, similarly in *hpl1* and L*er* (**Figure A1** in Appendix), probably because the differences in SA levels between the two genotypes were not big enough to cause a difference. Thus it seems that the lower JA levels in *hpl1* plants leads to less activation of the JA-signaling pathways and renders them less susceptible to DC3000.

A hallmark of basal plant defenses to pathogen infection is the deposition of callose. PAMP-induced callose deposition has recently been defined with essential roles for the DC3000 type III effector HopM1 and COR suppressing callose deposition, the latter being, interestingly, partly COI1-independent (Geng et al., 2012). Our results showed that in *hpl1*, although with smaller bacterial populations than in L*er*, clearly less callose was deposited (**Figures 4B,C**). Ethylene (ET) signaling it is crucial for callose deposition in response to flagellin (Clay et al., 2009). It is possible that this ET signaling is less activated in *hpl1*, leading to less callose deposition. Support for this comes from our complementation studies with the *hpl1* mutant, a response that is largely dependent on ORA59, a TF that integrates JA and ET signaling (**Figure 6B**). Perhaps related to this is the fact that DC3000 is apparently less effective in preventing cell death in *hpl1* than in L*er* (**Figure 4A**), with fewer living cells producing less callose. DC3000 apparently triggers in *hpl1* a higher rate of cell death, which is related to higher resistance (Jones and Dangl, 2006).

With the aim to overcome the *hpl1* phenotype in response to DC3000 infection, we decided to treat these, and L*er*, plants with *E*-2-hexenal. The pre-treatment with 3 μM *E*-2-hexenal for 24 h prior to DC3000 infection made *hpl1* plants considerably more susceptible to DC3000 (**Figures 5A,B**). The increase in bacterial populations was about ninefold in L*er* and fivefold in *hpl1* plants. Thus L*er* plants remained more susceptible to DC3000 than *hpl1* plants, most likely due to the functional HPL. Due to its high reactivity for being a reactive electrophile species (RES), *E*-2-hexenal, either induced during the HR or exogenously applied, can undergo conjugation to glutathione (GSH), leading to the formation of *E*-2-hexenal-GSH adducts in the form of 1-hexanol-3-GSH (Davoine et al., 2006; Mirabella et al., 2008). Conjugation to GSH is a well-known mechanism to inactivate reactive molecules (Coleman et al., 1997). Additionally, conjugation to cellular proteins has been reported to occur for several RES, including *E*-2-hexenal (Davoine et al., 2006; Myung et al., 2007; Dueckershoff et al., 2008; Mueller et al., 2008; Yamauchi et al., 2008). Therefore, we cannot exclude the possibility that, through conjugation, *E*-2-hexenal affects the

function of proteins involved in the plant defense responses to DC3000, making *Arabidopsis* more susceptible to this pathogen. A similar effect has been reported for syringolin, a toxin with an unsaturated α,β carbonyl moiety, that makes it a RES, produced by, e.g., *Pseudomonas syringae* pv. *syringae*. This toxin specifically inhibits the proteasome in order to suppress host defenses (Groll et al., 2008; Schellenberg et al., 2010).

Analyses of phytohormone levels after treatment of *E*-2-hexenal and DC3000 infection showed that there were no statistically significant differences in SA and JA levels between control and treatment (**Figure A2** in Appendix). So far only in monocots (maize) an increase in JA has been measured after a GLV treatment (Engelberth et al., 2004; Engelberth, 2011). In the JA-signaling pathway COI1 plays a central role and mutants in this gene are blocked in almost all JA responses (Feng et al., 2003; Devoto et al., 2005; Wang et al., 2008). Downstream of COI1, different TFs regulate specific JA-dependent responses: MYC2 and ORA59 are the main players involved. The MYC2-dependent branch is associated with wound response, responses against herbivores and is also regulated by abscisic acid (ABA; Lorenzo et al., 2003). This basic helix-loop-helix (bHLH) transcription factor regulates a large number of JA-responsive genes (Dombrecht et al., 2007), among which VEGETATIVE STORAGE PROTEIN2 (VSP2; Liu et al., 2005). In the other branch, ORA59 integrates JA and ET signaling (Pré et al., 2008). Interestingly, in spite of the absence of difference in JA and SA levels, the higher susceptibility of *Arabidopsis* plants to DC3000 after *E*-2-hexenal treatment was dependent on ORA59. The DC3000 bacterial populations increased only slightly in ir-ORA59 plants after *E*-2-hexenal treatment as compared to control (35S-GUS) plants (**Figure 6B**), indicating the relevance of JA signaling, and perhaps ET signaling. A role for MYC2 in this process was excluded based on the fact that *myc2* mutants still responded to exogenous *E*-2-hexenal treatment (**Figure 6A**).

From the bacterial side we investigated whether the production of COR was necessary to benefit from the *E*-2-hexenal treatment. For this we employed *cor*, a COR-deficient strain, to infect plants, after the *E*-2-hexenal or control treatment. The result showed that there was a small but significant increase in bacterial populations of the *cor* strain after the *E*-2-hexenal treatment (**Figure 7**). Nevertheless this difference was much smaller than for DC3000, suggesting that COR is necessary for DC3000 to fully benefit from GLVs.

Our data show that a functional *HPL* in *Arabidopsis* promotes susceptibility to DC3000. This effect is partially mediated by ORA59 in the plant and by COR in the bacteria.

The question remains how DC3000 precisely exploits HPL or its products, GLVs or the C_{12} compounds that are also formed in the HPL pathway (Kallenbach et al., 2011), for its benefit. Since it is clear that some herbivores can lower *HPL* transcript levels (Halitschke et al., 2004; Savchenko et al., 2012), we propose that HPL may be a target for DC3000 to employ in *Arabidopsis*, albeit to its own advantage.

ACKNOWLEDGMENTS

We kindly acknowledge Johan Memelink (Leiden University, Netherlands) for providing the ir-ORA59 seeds and Saskia van Wees (University of Utrecht, Netherlands) for *myc2* seeds. Alessandra Scala was supported by NWO-ALW grant 818.02.017.

REFERENCES

Abe, H., Urao, T., Ito, T., and Seki, M. (2003). *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 15, 63–78.

Alfano, J. R., and Collmer, A. (1996). Bacterial pathogens in plants: life up against the wall. *Plant Cell* 8, 1683–1698.

Allmann, S., Halitschke, R., Schuurink, R. C., and Baldwin, I. T. (2010). Oxylipin channelling in *Nicotiana attenuata*: lipoxygenase 2 supplies substrates for green leaf volatile production. *Plant Cell Environ.* 33, 2028–2040.

Anderson, J., and Badruzsaufari, E. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in. *Plant Cell* 16, 3460–3479.

Arimura, G., Ozawa, R., Horiuchi, J., Nishioka, T., and Takabayashi, J. (2001). Plant–plant interactions mediated by volatiles emitted from plants infested by spider mites. *Biochem. Syst. Ecol.* 29, 1049–1061.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., and Takabayashi, J. (2000). Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* 406, 512–515.

Bate, N. J., and Rothstein, S. J. (1998). C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. *Plant J.* 16, 561–569.

Birkett, M. A., Chamberlain, K., Guerrieri, E., Pickett, J. A., Wadhams, L. J., and Yasuda, T. (2003). Volatiles from whitefly-infested plants elicit a host-locating response in the parasitoid, *Encarsia formosa*. *J. Chem. Ecol.* 29, 1589–1600.

Block, A., Schmelz, E., Jones, J. B., and Klee, H. J. (2005). Coronatine and salicylic acid: the battle between *Arabidopsis* and *Pseudomonas* for phytohormone control. *Mol. Plant Pathol.* 6, 79–83.

Bowling, S. A., Clarke, J. D., Liu, Y., Klessig, D. F., and Dong, X. (1997). The cpr5 mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9, 1573–1584.

Brooks, D. M. D., Bender, C. L. C., and Kunkel, B. N. B. (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 6, 629–639.

Brown, I., Trethowan, J., and Kerry, M. (1998). Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J.* 15, 333–343.

Chehab, E., Raman, G., and Walley, J. (2006). Rice HYDROPEROXIDE LYASES with unique expression patterns generate distinct aldehyde signatures in *Arabidopsis*. *Plant Physiol.* 141, 121–134.

Clarke, J. D., Aarts, N., Feys, B. J., Dong, X., and Parker, J. E. (2001). Constitutive disease resistance requires EDS1 in the *Arabidopsis* mutants cpr1 and cpr6 and is partially EDS1-dependent in cpr5. *Plant J.* 26, 409–420.

Clay, N., Adio, A., and Denoux, C. (2009). Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323, 95–101.

Coleman, J. O. D., Randall, R., and Blake-Kalff, M. M. A. (1997). Detoxification of xenobiotics in plant cells by glutathione conjugation and vacuolar compartmentalization: a fluorescent assay using monochlorobimane. *Plant Cell Environ.* 20, 449–460.

Croft, K., Juttner, F., and Slusarenko, A. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. *Plant Physiol.* 101, 13–24.

Cuppels, D. A. (1986). Generation and characterization of Tn5 insertion mutations in. *Appl. Environ. Microbiol.* 51, 323–327.

Czechowski, T., Stitt, M., and Altmann, T. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* 139, 5–17.

Davoine, C., Falletti, O., Douki, T., Iacazio, G., Ennar, N., Montillet, J. L., et al. (2006). Adducts of oxylipin electrophiles to glutathione reflect a 13 specificity of the downstream lipoxygenase pathway in the tobacco hypersensitive response. *Plant Physiol.* 140, 1484–1493.

de Torres Zabala, M., Bennett, M. H., Truman, W. H., and Grant, M.

R. (2009). Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. *Plant J.* 59, 375–386.

Devoto, A., Ellis, C., Magusin, A., Chang, H.-S., Chilcott, C., Zhu, T., et al. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Mol. Biol.* 58, 497–513.

Dombrecht, B., Xue, G. P., Sprague, S. J., Kirkegaard, J. A., Ross, J. J., Reid, J. B., et al. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. *Plant Cell* 19, 2225–2245.

Duan, H., Huang, M., Palacio, K., and Schuler, M. (2005). Variations in CYP74B2 (hydroperoxide lyase) gene expression differentially affect hexenal signaling in the Columbia and Landsberg *erecta* ecotypes of *Arabidopsis*. *Plant Physiol.* 139, 1529–1544.

Dueckershoff, K., Mueller, S., Mueller, M. J., and Reinders, J. (2008). Impact of cyclopentenone-oxylipins on the proteome of *Arabidopsis thaliana*. *Biochim. Biophys. Acta* 1784, 1975–1985.

Engelberth, J. (2011). Selective inhibition of jasmonic acid accumulation by a small α, β-unsaturated carbonyl and phenidone reveals different modes of octadecanoid signalling activation in response to insect elicitors and green leaf volatiles in *Zea mays*. *BMC Res. Notes* 4:377. doi: 10.1186/1756-0500-4-377

Engelberth, J., Alborn, H. T., Schmelz, E. A., and Tumlinson, J. H. (2004). Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1781–1785.

Fall, R., Karl, T., Hansel, A., Jordan, A., and Lindiger, W. (1999). Volatile organic compounds emitted after leaf wounding: on-line analysis by proton-transfer-reaction mass spectrometry. *J. Geophys. Res. Atmos.* 104, 15963–15974.

Farag, M. A., Fokar, M., Abd, H., Zhang, H., Allen, R. D., and Paré, P. W. (2005). (Z)-3-hexenol induces defense genes and downstream metabolites in maize. *Planta* 220, 900–909.

Farag, M. A., and Paré, P. W. (2002). C6-green leaf volatiles trigger local and systemic VOC emissions in tomato. *Phytochemistry* 61, 545–554.

Farmer, E. E., and Davoine, C. (2007). Reactive electrophile species. *Curr. Opin. Plant Biol.* 10, 380–386.

Feng, S., Ma, L., and Wang, X. (2003). The COP_9 signalosome interacts physically with SCF^{COI1} and modulates jasmonate responses. *Plant Cell* 15, 1083–1094.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.-M., Gimenez-Ibanez, S., Geerinck, J., et al. (2011). The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* 23, 701–715.

Frost, C. J., Appel, H. M., Carlson, J. E., De Moraes, C. M., Mescher, M. C., and Schultz, J. C. (2007). Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores. *Ecol. Lett.* 10, 490–498.

Frost, C. J., Mescher, M. C., Carlson, J. E., and De Moraes, C. M. (2008). Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiol.* 146, 818–824.

Geng, X., Cheng, J., Gangadharan, A., and Mackey, D. (2012). The coronatine toxin of *Pseudomonas syringae* is a multifunctional suppressor of *Arabidopsis* defense. *Plant Cell* 24, 4763–4774.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227.

Gómez-Gómez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* 18, 277–284.

Gomi, K., Satoh, M., Ozawa, R., Shinonaga, Y., Sanada, S., Sasaki, K., et al. (2010). Role of hydroperoxide lyase in white-backed planthopper (*Sogatella furcifera* Horváth)-induced resistance to bacterial blight in rice, *Oryza sativa* L. *Plant J.* 61, 46–57.

Gomi, K., Yamasaki, Y., Yamamoto, H., and Akimitsu, K. (2003). Characterization of a hydroperoxide lyase gene and effect of C6-volatiles on expression of genes of the oxylipin metabolism in *Citrus*. *J. Plant Physiol.* 160, 1219–1231.

Gouinguené, S., Pickett, J. A., Wadhams, L. J., Birkett, M. A., and Turlings, T. C. J. (2005). Antennal electrophysiological responses of three parasitic wasps to caterpillar-induced volatiles from maize (*Zea mays mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*). *J. Chem. Ecol.* 31, 1023–1038.

Grant, M. R., and Jones, J. D. G. (2009). Hormone (dis)harmony moulds plant health and disease. *Science* 324, 750–752.

Groll, M., Schellenberg, B., Bachmann, A. S., Archer, C. R., Huber, R., Powell, T. K., et al. (2008). A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. *Nature* 452, 755–758.

Halitschke, R., Ziegler, J., Keinänen, M., and Baldwin, I. T. (2004). Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata*. *Plant J.* 40, 35–46.

Heiden, A. C., Kobel, K., Langebartels, C., Schuh-Thomas, G., and Wildt, J. (2003). Emissions of oxygenated volatile organic compounds from plants part I: emissions from lipoxygenase activity. *J. Atmos. Chem.* 45, 143–172.

Heil, M., and Silva Bueno, J. C. (2007). Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5467–5472.

Hirano, S. S., and Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* 64, 624–653.

Hong, S. M., Bahn, S. C., Lyu, A., Jung, H. S., and Ahn, J. H. (2010). Identification and testing of superior reference genes for a starting pool of transcript normalization in *Arabidopsis*. *Plant Cell Physiol.* 51, 1694–1706.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Kallenbach, M., Gilardoni, P. A., Allmann, S., Baldwin, I. T., and Bonaventure, G. (2011). C12 derivatives of the hydroperoxide lyase pathway are produced by product recycling through lipoxygenase-2 in *Nicotiana attenuata* leaves. *New Phytol.* 191, 1054–1068.

Kazan, K., and Manners, J. M. (2008). Jasmonate signaling: toward an integrated view. *Plant Physiol.* 146, 1459–1468.

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144.

Kessler, A., Halitschke, R., Diezel, C., and Baldwin, I. T. (2006). Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*. *Oecologia* 148, 280–292.

King, E. O., Ward, M. K., and Raney, D. E. (1954). Two simple media for the demonstration of phycocyanin and fluorescin. *J. Lab. Clin. Med.* 44, 301–307.

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2005). Volatile C6-aldehydes and Allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. *Plant Cell Physiol.* 46, 1093–1102.

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2006). ETR1-, JAR1- and PAD2-dependent signaling pathways are involved in C6-aldehyde-induced defense responses of *Arabidopsis*. *Plant Sci.* 171, 415–423.

Liu, Y., Ahn, J., Datta, S., and Salzman, R. (2005). *Arabidopsis* vegetative storage protein is an anti-insect acid phosphatase. *Plant Physiol.* 139, 1545–1556.

Lorenzo, O., Chico, J., Sánchez-Serrano, J. J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. *Plant Cell* 16, 1938–1950.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15, 165–178.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193.

Ma, S.-W., Morris, V. L., and Cuppels, D. A. (1991). Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Mol. Plant Microbe Interact.* 4, 69–74.

Mirabella, R., Rauwerda, H., Struys, E. A., Jakobs, C., Triantaphylidès, C., Haring, M. A., et al. (2008). The *Arabidopsis* her1 mutant implicates GABA in E-2-hexenal responsiveness. *Plant J.* 53, 197–213.

Mitchell, R. (1982). Coronatine production by some phytopathogenic pseudomonads. *Physiol. Plant Pathol.* 20, 83–89.

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Mueller, M. J., et al. (2008). General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. *Plant Cell* 20, 768–785.

Myung, K., Hamilton-Kemp, T. R., and Archbold, D. D. (2007). Interaction

with and effects on the profile of proteins of *Botrytis cinerea* by C6 aldehydes. *J. Agric. Food Chem.* 55, 2182–2188.

Oñate-Sánchez, L., Anderson, J. P., Young, J., and Singh, K. B. (2007). AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant Physiol.* 143, 400–409.

Park, D. H., Mirabella, R., Bronstein, P. A., Preston, G. M., Haring, M. A., Lim, C. K., et al. (2010). Mutations in gamma-aminobutyric acid (GABA) transaminase genes in plants or *Pseudomonas syringae* reduce bacterial virulence. *Plant J.* 64, 318–330.

Paschold, A., Halitschke, R., and Baldwin, I. T. (2006). Using 'mute' plants to translate volatile signals. *Plant J.* 45, 275–291.

Penninckx, I. A., Thomma, B. P., Buchala, A., Métraux, J. P., and Broekaert, W. F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10, 2103–2113.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45.

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316.

Potter, S., Uknes, S., and Lawton, K. (1993). Regulation of a hevein-like gene in *Arabidopsis*. *Mol. Plant Microbe Interact.* 6, 680–685.

Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C. M. J., and Memelink, J. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiol.* 147, 1347–1357.

Prost, I., Dhondt, S., and Rothe, G. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiol.* 139, 1902–1913.

Ruther, J., and Fürstenau, B. (2005). Emission of herbivore-induced volatiles in absence of a herbivore – response of *Zea mays* to green leaf volatiles and terpenoids. *Z. Naturforsch. C* 60, 743–756.

Ruther, J., and Kleier, S. (2005). Plant–plant signaling: ethylene synergizes volatile emission in *Zea mays* induced by exposure to (Z)-3-hexen-1-ol. *J. Chem. Ecol.* 31, 2217–2222.

Savchenko, T., Pearse, I., and Ignatia, L. (2012). Insect herbivores selectively suppress the HPL branch of the oxylipin pathway in host plants. *Plant J.* 73, 653–662.

Schellenberg, B., Ramel, C., and Dudler, R. (2010). *Pseudomonas syringae* virulence factor syringolin A counteracts stomatal immunity by proteasome inhibition. *Mol. Plant Microbe Interact.* 23, 1287–1293.

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C., et al. (2000). Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11655–11660.

Shiojiri, K., Kishimoto, K., Ozawa, R., Kugimiya, S., Urashimo, S., Arimura, G., et al. (2006a). Changing green leaf volatile biosynthesis in plants: an approach for improving plant resistance against both herbivores and pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16672–16676.

Shiojiri, K., Ozawa, R., Matsui, K., Kishimoto, K., Kugimiya, S., and Takabayashi, J. (2006b). Role of the lipoxygenase/lyase pathway of host-food plants in the host searching behavior of two parasitoid species, *Cotesia glomerata* and *Cotesia plutellae*. *J. Chem. Ecol.* 32, 969–979.

Shiojiri, K., Ozawa, R., Matsui, K., Sabelis, M. W., and Takabayashi, J. (2012). Intermittent exposure to traces of green leaf volatiles triggers a plant response. *Sci. Rep.* 2, 378.

Shiojiri, K., Takabayashi, J., Yano, S., and Takafuji, A. (2000). Flight response of parasitoids toward plant-herbivore complexes: a comparative study of two parasitoid-herbivore systems on cabbage plants. *Appl. Entomol. Zool.* 35, 87–92.

Spoel, S. H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. *Cell Host Microbe* 3, 348–351.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448, 661–665.

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P., et al. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. U.S.A.* 95, 15107–15111.

Tong, X., Qi, J., Zhu, X., Mao, B., Zeng, L., Wang, B., et al. (2012). The rice hydroperoxide lyase OsHPL3 functions in defense responses by modulating the oxylipin pathway. *Plant J.* 71, 763–775.

Turlings, T., Loughrin, J., McCall, P. J., Röse, U. S., Lewis, W. J., and Tumlinson, J. H. (1995). How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4169–4174.

Verhage, A., Vlaardingerbroek, I., Raaymakers, C., Van Dam, N. M., Dicke, M., Van Wees, S. C. M., et al. (2011). Rewiring of the jasmonate signaling pathway in *Arabidopsis* during insect herbivory. *Front. Plant Sci.* 2:47. doi: 10.3389/fpls.2011.00047

Wang, L., Mitra, R. M., Hasselmann, K. D., Sato, M., Lenarz-Wyatt, L., Cohen, J. D., et al. (2008). The genetic network controlling the *Arabidopsis* transcriptional response to *Pseudomonas syringae* pv. *maculicola*: roles of major regulators and the phytotoxin coronatine. *Mol. Plant Microbe Interact.* 21, 1408–1420.

Weber, H., Chételat, A., Reymond, P., and Farmer, E. E. (2004). Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant J.* 37, 877–888.

Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3, 49–59.

Yamauchi, Y., Furutera, A., Seki, K., Toyoda, Y., Tanaka, K., and Sugimoto, Y. (2008). Malondialdehyde generated from peroxidized linolenic acid causes protein modification in heat-stressed plants. *Plant Physiol. Biochem.* 46, 786–793.

Yan, J., Zhang, C., Gu, M., Bai, Z., Zhang, W., Qi, T., et al. (2009). The *Arabidopsis* CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell* 21, 2220–2236.

Yan, Z., and Wang, C. (2006). Wound-induced green leaf volatiles cause the release of acetylated derivatives and a terpenoid in maize. *Phytochemistry* 67, 34–42.

Zeringue, H. J. (1992). Effects of C6-C10 alkenals and alkanals on eliciting a defence response in the developing cotton boll. *Phytochemistry* 31, 6–9.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 December 2012; accepted: 15 March 2013; published online: 12 April 2013.

Citation: Scala A, Mirabella R, Mugo C, Matsui K, Haring MA and Schuurink RC (2013) E-2-hexenal promotes susceptibility to Pseudomonas syringae by activating jasmonic acid pathways in Arabidopsis. Front. Plant Sci. ***4****:74. doi: 10.3389/fpls.2013.00074*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Scala, Mirabella, Mugo, Matsui, Haring and Schuurink. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

APPENDIX

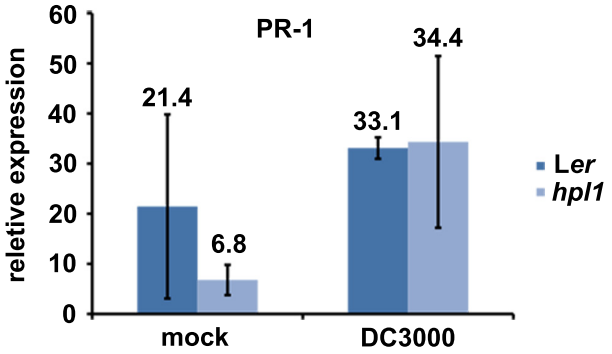


FIGURE A1 | *PR-1* expression is equally induced in L*er* and *hpl1*. *PR-1* transcript levels were measured by qRT-PCR in L*er* and *hpl1* infected with DC3000 48 hpi and normalized for SAND transcript levels. Error bars represent standard error.

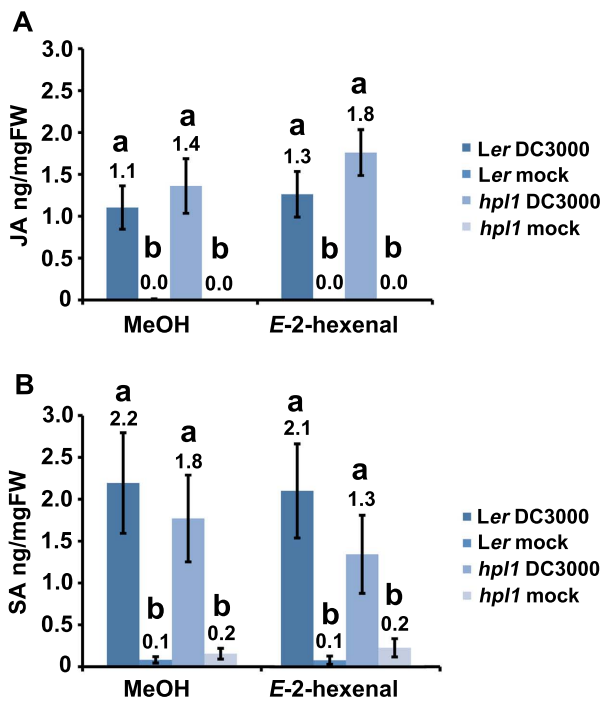


FIGURE A2 | *E*-2-hexenal does not induce changes in JA and SA levels in L*er* and *hpl1* plants infected with DC3000. (A) JA levels in L*er* and *hpl1* plants pre-treated with *E*-2-hexenal or MeOH and subsequently infected with DC3000 (48 hpi); **(B)** SA levels in L*er* and *hpl1* plants pre-treated with *E*-2-hexenal or MeOH and subsequently infected with DC3000 (24 hpi). In both cases the hormone levels in the 10 mM $MgSO_4$ (mock) infiltrated plants are also shown. Nine leaves were harvested, in pools of three from mock-infiltrated or bacteria-infiltrated plants at specified timepoints and used for plant hormone quantification. Bars represent the mean of three independent experiments. Error bars represent standard error. Bars annotated with different letters indicate statistically different hormone levels ($P < 0.05$, according to ANOVA, followed by a LSD *post hoc* test).

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 29 April 2013
doi: 10.3389/fpls.2013.00115

Jasmonate-mediated induced volatiles in the American cranberry, *Vaccinium macrocarpon*: from gene expression to organismal interactions

Cesar R. Rodriguez-Saona[1]*, James Polashock[2] and Edi A. Malo[3]

[1] *Department of Entomology, Philip E. Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, USA*
[2] *Genetic Improvement of Fruits and Vegetables Lab, Philip E. Marucci Center for Blueberry and Cranberry Research, United States Department of Agriculture-Agricultural Research Service, Chatsworth, NJ, USA*
[3] *Grupo de Ecología de Artrópodos y Manejo de plagas, El Colegio de la Frontera Sur, Tapachula, Chiapas, México*

Edited by:
Erik Poelman, Wageningen University, Netherlands

Reviewed by:
Nicole M. Van Dam, Radboud University Nijmegen, Netherlands
Colette Broekgaarden, Wageningen University, Netherlands

****Correspondence:***
Cesar R. Rodriguez-Saona, Philip E. Marucci Center for Blueberry and Cranberry Research, 125A Lake Oswego Road, Chatsworth, NJ 08019, USA.
e-mail: crodriguez@aesop.rutgers.edu

Jasmonates, i.e., jasmonic acid (JA) and methyl jasmonate (MeJA), are signaling hormones that regulate a large number of defense responses in plants which in turn affect the plants' interactions with herbivores and their natural enemies. Here, we investigated the effect of jasmonates on the emission of volatiles in the American cranberry, *Vaccinium macrocarpon*, at different levels of biological organization from gene expression to organismal interactions. At the molecular level, four genes (*BCS*, *LLS*, *NER1*, and *TPS21*) responded significantly to gypsy moth larval feeding, MeJA, and mechanical wounding, but to different degrees. The most dramatic changes in expression of *BCS* and *TPS21* (genes in the sesquiterpenoid pathway) were when treated with MeJA. Gypsy moth-damaged and MeJA-treated plants also had significantly elevated expression of *LLS* and *NER1* (genes in the monoterpene and homoterpene biosynthesis pathways, respectively). At the biochemical level, MeJA induced a complex blend of monoterpene and sesquiterpene compounds that differed from gypsy moth and mechanical damage, and followed a diurnal pattern of emission. At the organismal level, numbers of *Sparganothis sulfureana* moths were lower while numbers of parasitic wasps were higher on sticky traps near MeJA-treated cranberry plants than those near untreated plants. Out of 11 leaf volatiles tested, (*Z*)-3-hexenyl acetate, linalool, and linalool oxide elicited strong antennal (EAG) responses from *S. sulfureana*, whereas sesquiterpenes elicited weak EAG responses. In addition, mortality of *S. sulfureana* larvae increased by about 43% in JA treated cranberry plants as compared with untreated plants, indicating a relationship among adult preference, antennal sensitivity to plant odors, and offspring performance. This study highlights the role of the jasmonate-dependent defensive pathway in the emissions of herbivore-induced volatiles in cranberries and its importance in multi-trophic level interactions.

Keywords: methyl jasmonate, jasmonic acid, herbivore-induced plant volatiles, *Sparganothis sulfureana*, electroantennograms, multi-trophic interactions

INTRODUCTION

Plants can sometimes change their phenotype after herbivore feeding damage by becoming more protected from future enemy attacks (Karban and Baldwin, 1997; Walling, 2000; Heil, 2010). These induced phenotypic changes can reduce the performance of herbivores or alter their behavior, i.e., reduce the herbivore's feeding or oviposition on plants (Agrawal, 1998; Denno et al., 2000; De Moraes et al., 2001; Kessler and Baldwin, 2001; Van Zandt and Agrawal, 2004; Viswanathan et al., 2005), and can thus be classified as direct defenses if they provide a fitness benefit to plants (Agrawal, 1998, 2000). They can also indirectly defend plants by changing the behavior of the herbivores' natural enemies (Price et al., 1980). One such indirect defense is the emission of volatiles – so-called herbivore-induced plant volatiles (HIPVs) – that attract predators and parasitoids of insect herbivores to the attacked plant (Turlings et al., 1990; Vet and Dicke, 1992; Dicke and Vet, 1999). Manipulation of these defensive traits in plants can serve as a natural pest control tactic in agriculture (Thaler, 1999a; Rodriguez-Saona et al., 2012).

An important signaling pathway involved in the induction of plant defenses, including HIPV emissions, is the octadecanoid pathway (Karban and Baldwin, 1997; Arimura et al., 2005). In many plants, activation of the octadecanoid pathway by herbivore feeding may lead to increased production of the plant growth regulator, jasmonic acid (JA) (Farmer et al., 1992; Staswick and Lehman, 1999), which in turn can elicit multiple direct and indirect defenses in plants (Karban and Baldwin, 1997). For example, increases in HIPV emissions similar to those induced by insect feeding have been reported in response to exogenous applications of jasmonates, i.e., JA or its volatile methyl ester methyl jasmonate (MeJA), in many plant species including gerbera, *Gerbera jamesonii* Bolus (Gols et al., 1999),

cotton, *Gossypium hirsutum* L. (Rodriguez-Saona et al., 2001), Manchurian ash, *Fraxinus mandshurica* Rupr. (Rodriguez-Saona et al., 2006), and sacred datura, *Datura wrightii* Regel (Hare, 2007).

Activation of the octadecanoid pathway by jasmonate treatment is known to affect the preference and performance of herbivores on plants. Thaler et al. (2001), for example, found fewer caterpillars, flea beetles, aphids, and thrips on tomato plants that were sprayed with JA. *Manduca quinquemaculata* Haworth oviposition was reduced when *Nicotiana attenuata* Torr ex. S. Watts plants were treated with MeJA (Kessler and Baldwin, 2001). Similarly, Bruinsma et al. (2007) showed that two species of cabbage white butterflies, *Pieris rapae* L. and *P. brassicae* L., laid fewer eggs on JA-treated Brussels sprouts, *Brassica oleracea* L., plants compared to untreated plants. *Pieris rapae* also preferred untreated leaves over JA-treated leaves of black mustard plants, *Brassica nigra* L., for oviposition (Bruinsma et al., 2008). Jasmonate-induced changes can also affect members of higher trophic levels. For example, JA treatment increased parasitism of caterpillars in tomato fields (Thaler, 1999b), but negatively affected predatory hoverflies due to a decrease in prey (aphid) abundance (Thaler, 2002). JA or MeJA also induced the emission of plant volatiles that attracted predatory mites (Dicke et al., 1999; Gols et al., 2003).

Studies that integrate multiple research approaches, such as gene expression, metabolite induction, and ecological interactions, are needed for a better understanding of plant interactions with arthropods across different levels of biological organization (Zheng and Dicke, 2008). Previously, we showed that gypsy moth, *Lymantria dispar* L. (Lep., Lymantriidae), larval feeding, and MeJA induce emissions of several monoterpenes and sesquiterpenes in a perennial ericaceous crop, the American cranberry (*Vaccinium macrocarpon* Ait.) (Rodriguez-Saona et al., 2011). In the present study, we linked volatile induction with transcriptional induction and organismal level interactions by investigating the effects of jasmonates on the up-regulation of several key terpene biosynthesis genes, induced volatile emissions, and the preference-performance of a polyphagous herbivore, *Sparganothis sulfureana* Clemens (Sparganothis fruitworm; Lep., Tortricidae), in cranberries. *Sparganothis sulfureana* is an important pest of cranberries in the United States (USA); in spring, the overwintered first instar larvae feed on foliage, adults emerge in late spring to mid-summer, and in June to early July second generation larvae feed on foliage and burrow into developing fruit (Beckwith, 1938; Averill and Sylvia, 1998). Specifically, we conducted studies to: (1) determine the effects of MeJA, gypsy moth larval feeding, and mechanical wounding on expression of eight genes involved in terpenoid biosynthesis; (2) compare the volatile emissions of cranberries in response to MeJA application, gypsy moth feeding, and mechanical wounding, and investigate the diurnal pattern of volatile emissions; (3) examine the response of key herbivores (*S. sulfureana* and leafhoppers) and natural enemies [hoverflies (Dip., Syrphidae), minute pirate bugs (Hem., Anthocoridae), spiders (Araneae), and parasitic wasps (Hymenoptera)] to MeJA application on cranberry plants; (4) test the effect of JA-treated cranberry foliage on *S. sulfureana* larval mortality; and, (5) investigate the electrophysiological response of *S. sulfureana* antennae (EAG) to various cranberry leaf volatiles.

MATERIALS AND METHODS

PLANTS AND INSECTS

Cranberries are propagated clonally and grow from single shoots by producing new uprights and runners. Cranberries, *V. macrocarpon* var. Stevens, were grown from rooted cuttings in 10 cm pots in a greenhouse ($22 \pm 2^{\circ}$C; $70 \pm 10\%$ RH; 15:9 L:D) at the Rutgers P. E. Marucci Center for Blueberry and Cranberry Research and Extension (Chatsworth, NJ, USA). Three cuttings were rooted per pot. Plants were allowed to grow in the greenhouse for at least a year before being used in experiments, and were fertilized biweekly with PRO-SOL 20-20-20 N-P-K All Purpose Plant Food (Pro Sol Inc., Ozark, AL, USA) at a rate of 165 ppm N and watered daily. At the time of bioassays, plants from each rooted cutting contained 3–5 uprights and runners. Runners were pruned as needed, and all plants were at the vegetative stage and insect-free when used in experiments.

Lymantria dispar caterpillars and *Sparganothis sulfureana* adults and caterpillars were obtained from a laboratory colony maintained at the Rutgers Marucci Center. Caterpillars were reared on a wheat germ diet (Bell et al., 1981) in 30 ml clear plastic cups at $24 \pm 1^{\circ}$C, 65% RH, and 14:10 L:D. Field-collected caterpillars were added yearly to the laboratory colony.

TREATMENT APPLICATIONS

Plants in each pot were bagged with a spun polyester sleeve (Rockingham Opportunities Corp., Reidsville, NC, USA) and then subjected to one of the following treatments:

(1) Jasmonate treatment: potted plants were sprayed with 1 ml of a 1 mM JA or MeJA solution (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.4% acetone or 0.1% Tween 20, respectively. Treated plants were used for bioassays (gene expression, volatile collections, and field experiments, see below) ∼16 h after jasmonate application (unless otherwise stated); this time period was sufficient to induce a volatile response from cranberry leaves in previous studies (Rodriguez-Saona et al., 2011).

(2) Caterpillar treatment: cranberry leaves were damaged by third–fourth instar gypsy moth caterpillars. Four or eight caterpillars were placed on plants and allowed to feed for 2 days before used in bioassays (gene expression and volatile collections); this amount of feeding time by gypsy moth was sufficient to induce a volatile response from cranberry leaves (Rodriguez-Saona et al., 2011).

(3) Mechanical treatment: mechanical damage was inflicted for 2 days by cutting the tips of 40 leaves (20 leaves were mechanically damaged on day 1 and 20 leaves on day 2) with scissors to simulate the amount of leaf area removed by gypsy moth caterpillars. Following, plants were used for gene expression and volatile collections.

(4) Control treatment: plants were either sprayed with 1 ml of distilled water with 0.4% acetone, 0.1% Tween 20, or received no treatment.

Bags were opened just prior to, and closed soon after, treatment.

GENE EXPRESSION ANALYSIS

Leaves were harvested from jasmonate (MeJA)-treated, caterpillar-damaged, mechanically wounded, and control (Tween) plants ($N = 3$ individual plants or biological replicates per treatment). We used eight rather than four gypsy moth caterpillars in these studies because this herbivore density induces a stronger volatile response in cranberries (see Results). We also harvested undamaged leaves from plants upon which gypsy moths fed and from mechanically wounded plants (systemic response). All samples were collected at 10:00. RNA was immediately extracted from the harvested leaves using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA) according to the manufacturer's directions. The total RNA was eluted in 100 μl sterile dH_2O and quantified using the ND-1000 Nanodrop Spectrophotometer (Nanodrop Products; Wilmington, DE, USA). The cDNA was synthesized using 100 ng of RNA per reaction and the Superscript VILO cDNA Synthesis kit (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol.

The genes targeted for real-time PCR and the primers used for each are listed in **Table 1**. The eight genes were selected for expression analysis based partly on published reports of terpenoid biosynthesis gene induction when jasmonate treated or in response to herbivores and partly on our own previous work on the volatiles that cranberry plants emit (Rodriguez-Saona et al., 2011). These include (−)-β-caryophyllene synthase (*BCS*), farnesyl diphosphate synthase (*FDS*), *R*-linalool synthase (*LLS*), (*E*)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (*MDS*), (3*S*,6*E*)-nerolidol synthase (*NER1*), phosphomevalonate kinase (*PMK*), terpene 1,8-cineole synthase (*TPS*), and α-humulene/β-caryophyllene synthase (*TPS21*). Actin (*ACT*) and RNA Helicase 8 (*RH8*) were used as endogenous controls. The gene sequences were selected from an assembled total genome sequence of cranberry (Georgi et al., 2013 and unpublished). Each target gene sequence was identified by homology to published sequences from other plant species. The primers were designed using the predicted coding sequence of each gene and the Primer Express 3.0 software (Applied Biosystems; Foster City, CA, USA). Real-time PCR reactions were set up using the Power SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's directions and run on an Applied Biosystems 7500 real-time PCR machine. Thermocycling conditions were 50°C–2 min, 95°C–10 min followed by 40 cycles at 95°C–15 s, 60°C–1 min with melt curve set at 95°C–15 s, 60°C–1 min, 95°C–30 s, 60°C–15 s. There were three biological replicates (individual plants) of each sample and three technical replicates were run for each biological replicate. The technical replicates for each biological replicate were averaged. Relative expression levels were calculated by the ΔΔCT method using the DataAssist 3.0 software package (Applied Biosystems) and using *ACT* and *RH8* to normalize the expression.

HEADSPACE COLLECTION

Volatiles emitted from cranberry plants that were either damaged by four or eight gypsy moth caterpillars, treated with MeJA, or left undamaged were collected using a push-pull volatile collection system (Tholl and Röse, 2006; Rodriguez-Saona, 2011) in a greenhouse (under conditions described above). The system consisted of four 4 L glass chambers (Analytical Research Systems, Inc., Gainesville, FL, USA). The chambers had a guillotine-like split plate with a hole in the center at the base that closed loosely around the stem of the plant. Incoming purified air entered each chamber at 2 L min^{-1} and was pulled through a filter trap containing 30 mg of Super-Q adsorbent (Alltech; Nicholasville, KY, USA) with a vacuum pump at 1 L min^{-1}. Volatile collections were initiated at 10:00. After 4 h of volatile collection, fresh weight of plants was measured to account for differences in plant size. Each treatment was replicated four times.

To determine the diurnal pattern of emissions, volatiles were collected from MeJA-treated at five different times of the day (06:00–10:00, 10:00–14:00, 14:00–18:00, 18:00–22:00, and 22:00–06:00). The experiment was replicated three times.

VOLATILE ANALYSIS

Collected volatiles were desorbed from the Super-Q traps with dichloromethane (150 μl) and added 400 ng of *n*-octane (Sigma-Aldrich; St. Louis, MO, USA) as internal standard (IS). Headspace samples (1 μl aliquots) were injected onto a Hewlett Packard 6890 Series Gas Chromatograph (GC) equipped with a flame ionization detector and a HP-1 column (10 m × 0.53 mm ID × 2.65 μm film thickness; Agilent Technologies, Palo Alto, CA, USA), with helium flow of 5 ml min^{-1}. The temperature program started at 40°C (1 min hold), and rose at 14°C min^{-1} to 180°C (2 min hold), then at 40°C min^{-1} to 200°C (2 min hold). Compounds (relative

Table 1 | Targets used for real-time qPCR, enzyme commission (EC) numbers and primer sequences.

Target (abbreviation)	EC number	Real-time F primer	Real-time R primer
Farnesyl diphosphate synthase (FDS)	2.5.1.10	CGAGGTCAGCCATGTTGGTA	CCATCATTTGCAGCAATCAAA
(*E*)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (MDS)	1.17.7.1	GCACAGGCGTTACTTCGATTT	CCCTCTTTTTGGATTGGCAAT
Phosphomevalonate kinase (PMK)	2.7.4.2	TTCCCTTCCACCGTTTACATCT	AGGCTTGCGAGTTTCTGAATTT
Terpene 1,8-cineole synthase (TPS)	4.2.3.108	GGTGGGATATAACTGCAATGGAA	TGAAAAGAGCAAGGAAGCAAATC
(−)-β-Caryophyllene synthase (BCS)	4.2.3.57	TCCGGTGGTTACAAAATGCTT	CACCAAATCCCCCATGACA
R-Linalool synthase (LLS)	4.2.3.26	GGGCAAGTTTGTGCAATGC	GGCAACTGCCCAGAAGCA
α-Humulene/β-caryophyllene synthase (TPS21)	4.2.3.	TCCGGTGGTTACAAAATGCTT	CACCAAATCCCCCATGACA
(3*S*,6*E*)-nerolidol synthase (NER1)	4.2.3.48	GGGCAAGTTTGTGCAATGC	GGCAACTGCCCAGAAGCA
Actin (ACT)	–	TTCACCACCACGGCTGAAC	AGCCACGTATGCAAGCTTTTC
RNA Helicase-Like 8 (RH8)	3.6.4.13	TGCCAAGATGCTTCAAGATCA	GCATGCACCATTCCGAAAAT

amounts) were quantified based on comparison of peak areas with that of the IS without FID response factor correction.

Identification of compounds was performed on a Varian 3400 GC coupled to a Finnigan MAT 8230 mass spectrometer (MS) equipped with a MDN-5S column (30 m × 0.32 mm ID × 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). The temperature program was initiated at 35°C (1 min hold), rose at 4°C min^{-1} to 170°C, then at 15°C min^{-1} to 280°C. The MS data were recorded and processed in a Finnigan MAT SS300 data system. The eluted compounds were identified by comparing the mass spectra with those from NIST library spectra and comparison of their retention times to those of commercially available compounds (Rodriguez-Saona et al., 2011).

ELECTROANTENNOGRAPHIC ANALYSIS

The relative antennal receptivity of adult males and females of *S. sulfureana* to 11 synthetic volatile compounds emitted from cranberry plants (this study; Rodriguez-Saona et al., 2011) was compared by EAG. The insect head was cut off carefully, and a reference electrode was inserted into its base with a glass capillary filled with physiological saline solution (Malo et al., 2004). The distal end of the antenna was inserted into the tip of the recording glass capillary electrode. EAGs were measured using 5–10 moths of each sex per volatile compound. The signals generated by the antennae were passed through a high-impedance amplifier (NL 1200; Syntech, Hilversum, Netherlands) and displayed on a monitor by Syntech software for processing EAG signals. A stimulus flow controller (CS-05; Syntech) was used to generate a stimulus at 1 min intervals. A current of humidified pure air (0.7 L min^{-1}) was constantly directed onto the antenna through a 10-mm-diameter glass tube. Dilutions of the synthetic compounds were prepared in HPLC-grade hexane to make 10 μg per μl solutions. A standard aliquot (1 μl) of each test dilution was pipetted onto a piece of filter paper (0.5 cm × 3.0 cm; Whatman, No. 1), exposed to air for 20 s to allow the solvent to evaporate, then inserted into a glass Pasteur pipette or sample cartridge, and left for 40 s before applying to antennae. A new cartridge was prepared for each insect. To present a stimulus, the pipette tip containing the test compound was inserted through a side hole located at the midpoint of a glass tube through which humidified pure air flowed at 0.5 L min^{-1}. The duration of stimulus was 1 s. The continuous flow of clean air through the airflow tube and over the preparation ensured that odors were removed immediately from the vicinity. The synthetic compounds (10 μg) were presented in random order. Control stimuli (air or filter paper with hexane) were presented at the beginning and end of each EAG analysis. Synthetic chemicals were purchased from Sigma-Aldrich and Bedoukian Research (Danbury, CT, USA), and the purities were >95% based on the results with GC.

ECOLOGICAL LEVEL ANALYSIS

The goal of this field study was to investigate the response of arthropods to MeJA-treated cranberry plants. The experiment was conducted in June–July of 2009 to coincide with *S. sulfureana* peak flight activity (Averill and Sylvia, 1998), on a commercial cranberry farm located in Chatsworth, New Jersey (39°72′ N, 74°50′ W), using potted cranberry plants (grown as described above). Potted plants were either treated with MeJA or left untreated (controls) ($N = 15$ pots per treatment). Pots were placed in a 5 × 6 grid pattern within an established cranberry bed (∼2 ha), and were separated by at least 10 m from each other. Treatments were assigned randomly to each location and applied at 17:00. The following morning (08:00), a sticky trap was placed in each pot to monitor abundances of all colonizing arthropods. The sticky traps were 5 cm × 5 cm pieces of green cardboard, covered on both sides (e.g., bi-directional trap) with a thin layer of Tangle-Trap (The Tanglefoot Co., Grand Rapids, MI, USA), and mounted on wooden stakes about 10 cm from the soil, such that the traps were next to but not touching the plants. Traps were left in the field for 48 h, after which they were removed, wrapped in plastic film, and stored in a refrigerator (4°C) for arthropod identification. The entire experiment was replicated three times with a new set of plants each time.

To determine the effects of jasmonate-mediated responses in cranberries on *S. sulfureana* larval survival, an experiment was conducted in a cranberry bed located at the Rutgers Marucci Center. Plots (60 cm × 60 cm) were treated with JA or left untreated (controls), replicated three times, and separated by 60 cm. Applications were made with an R&D (Bellspray Inc., Opelousas, LA, USA) CO_2 backpack sprayer, using a 1 L plastic bottle, calibrated to deliver 418 L per hectare at 241 kPa (∼15.4 ml per plot). Treatments were applied at 06:00 in early August, 2007. Four hours after treatment, five uprights were randomly collected from each plot and inserted in florists' water picks, enclosed in a ventilated 40-dram plastic vial, and secured on polystyrene foam trays. Each replicate consisted of a total of 10 vials per treatment ($N = 30$ vials per treatment). Four *S. sulfureana* neonates were added to each vial, and vials were placed in the laboratory at ∼25°C. The number of live larvae was recorded after 7 days.

To confirm that the effects of the JA treatment on *S. sulfureana* larval survival were the result of plant effects rather than any direct toxic effects of JA, an additional experiment was conducted to test the toxicity of JA to *S. sulfureana* neonates. *Sparganothis sulfureana* neonates were placed in 30 ml plastic cups (one neonate per cup), containing 10 ml of wheat germ diet. The diet in each cup was sprayed with either 1 mM JA solution with 0.4% acetone or distilled water with 0.4% acetone 4 h prior to placing the caterpillars ($N = 50$ cups per treatment). The number of live larvae was recorded after 7 days.

DATA ANALYSIS

The normalized gene expression levels were log-transformed, to satisfy the homogeneity of variance assumption for ANOVA. However, even with this transformation, responses of two genes (*BCS* and *TPS21*) to one treatment (systemic response in mechanically wounded plants) had excessively large variances, so were excluded from the analyses**.** Linear mixed models were fit with the lme4 package (Bates et al., 2011) in R (R Development Core Team, 2012) (with biological replicate as a random effect) to a subset of genes whose expression levels changed over treatments, means separations within a gene were done using the multcomp package (Hothorn et al., 2008).

Principal component analysis (PCA) was used to visualize overall differences among blends emitted from each treatment (gypsy moth feeding, MeJA, mechanical wounding, and control) using

Minitab v. 16 (Minitab Inc., State College, PA, USA). PCA was initially performed on the data because individual volatile compounds within blends are not independent (Hare, 2011). We also used multivariate analysis of variance (MANOVA; Minitab) to analyze the effects of treatment on volatile emissions. Volatile compounds were grouped into esters, monoterpenes, homoterpenes, sesquiterpenes, or others (only groups containing more than two compounds were considered for MANOVA). A significant MANOVA was followed by ANOVA (Minitab) to determine which compounds within a group were affected by treatment (Scheiner, 2001). Similarly, PCA and ANOVA were used to test the effect of time of day on volatiles emissions from MeJA-treated plants (06:00–10:00, 10:00–14:00, 14:00–18:00, 18:00–22:00, and 22:00–06:00). Volatile emissions data were either $\ln(x)$- or $\ln(x+0.5)$-transformed prior to analysis to satisfy assumptions of normality and homogeneity of variances. Differences in the emissions of individual compounds among treatments were analyzed by Tukey *post hoc* comparisons ($\alpha = 0.05$).

Sticky trap data were first analyzed by functional group, i.e., herbivores and natural enemies (predators and parasitoids), using MANOVA. MANOVA was initially performed on the data because densities of individual arthropod groups are not independent (Scheiner, 2001). The model included treatment (MeJA versus control), time of year (date), and treatment × date. A significant MANOVA was followed by ANOVA for individual arthropod groups. When needed, data were $\ln(x)$ or $\ln(x+0.5)$-transformed before analysis. Mortality data were arcsine square-root transformed before analysis with ANOVA. A chi-square test was used to determine differences in *S. sulfureana* mortality rate when fed diets sprayed with JA versus unsprayed diets.

The values of the EAG depolarization amplitude after exposure to the volatile compounds were transformed $[\ln(x+0.5)]$ prior to analysis with two-way ANOVA with sex of insect and type of chemical compound as the two factors. Significant ANOVA results were followed by Tukey test for means comparison.

RESULTS

GENE EXPRESSION

To determine if the various treatments induced expression of key enzymes in the terpenoid biosynthetic pathway, we tested the relative expression levels of eight selected genes – *BCS*, *FDS*, *LLS*, *MDS*, *NER1*, *PMK*, *TPS*, *TPS21* – using real-time PCR. Four of the genes tested responded significantly to gypsy moth larval feeding, MeJA, and mechanical wounding (*BCS*, *LLS*, *NER1*, and *TPS21*), but to different degrees (**Figure 1**). The most dramatic changes in expression of *BCS* and *TPS21* were when treated with MeJA. All

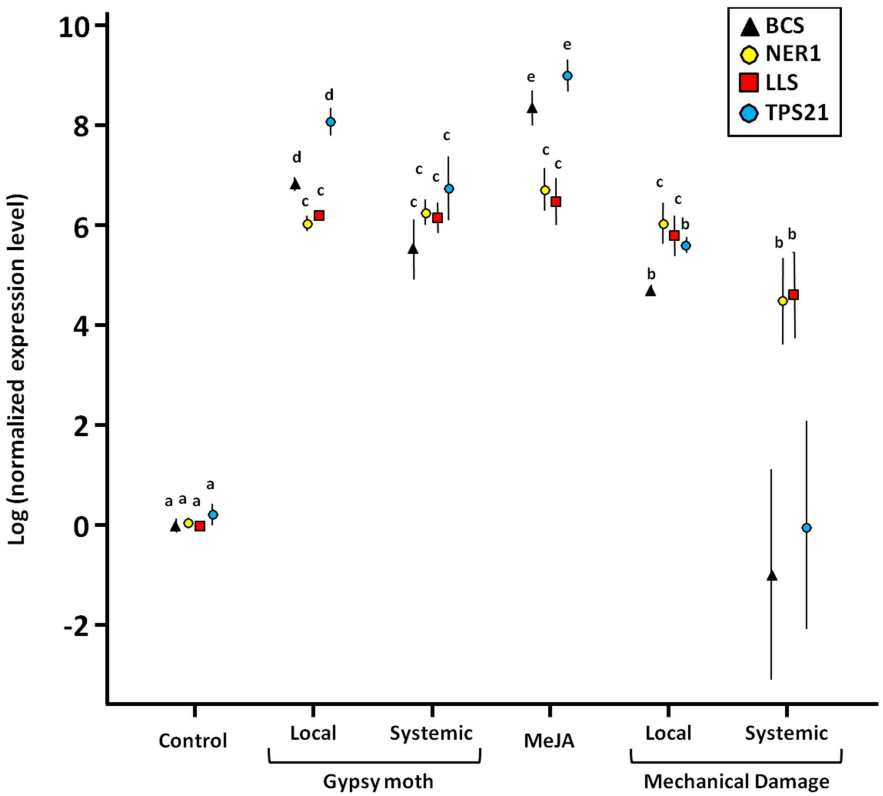


FIGURE 1 | Expression of terpene genes (*BCS, NER1, LLS,* and *TPS21*) in cranberries, *Vaccinium macrocarpon,* in response to gypsy moth larval feeding, MeJA, and mechanical damage as compared to control plants. Targets: *BCS* = (-)-β-caryophyllene synthase; *NER1* = (3*S*,6*E*)-nerolidol synthase; *LLS* = *R*-linalool synthase; *TPS21* = α-humulene/β-caryophyllene synthase. Local = expression at the site of feeding damage or mechanical damage; Systemic = expression of undamaged leaves on damaged plants. Values are the mean ±1 SE. Different letters indicate significant differences among treatments (adjusted for multiple comparisons); there are four sets of means separation letters, one for each gene. *BCS* and *TPS21* values for "Mechanical Damage-Systemic" were not included in the statistical analysis due to excessively high variance.

MeJA-treated plants also had significantly elevated expression of *LLS* and *NER1*. For those genes that responded to gypsy moth feeding (*BCS*, *LLS*, *NER*, and *TPS21*), the changes in gene expression were also evident in the undamaged tissue on the same plant. This was also true in mechanically wounded plants, but only for *NER1* and *LLS*. The undamaged leaves from the mechanically wounded plants had a wide variance in gene expression for *BCS* and *TPS21*. Expression of the other four genes tested (*FDS*, *MDS*, *PMK*, *TPS*) were unchanged for any of the treatments ($P > 0.05$; data not shown).

VOLATILE EMISSIONS

Cranberry plants responded to gypsy moth feeding in a density-dependent manner such that volatile emissions were ∼2.5 times greater when plants were damaged by eight caterpillars as compared with four caterpillars (four caterpillars [mean emissions in ng h^{-1} g^{-1} fresh tissue ± SE]: 81.9 ± 24.1; eight caterpillars: 199.9 ± 18.4; $F = 10.41$, $df = 1.6$, $P = 0.018$). Eleven out of 22 volatiles were significantly induced by herbivory from cranberry plants compared with undamaged plants (**Table 2**); with eucalyptol/limonene, linalool, DMNT, indole, β-caryophyllene, α-humulene, and germacrene-D emitted in highest quantities.

Total emissions of volatiles were 9 and 14-fold higher in gypsy moth-damaged (eight caterpillars) and MeJA-treated plants than in control plants, respectively; whereas emissions from mechanically wounded plants were variable and not significantly different from control plants (**Table 2**). The PCA resulted in a model with the first two PC components explaining 65.7% of the total

Table 2 | Volatiles identified in the headspace of cranberry, *Vaccinium macrocarpon*, plants damaged by eight gypsy moth larvae, sprayed with 1 mM MeJA solution, mechanically damaged with scissors, or left undamaged (control)[1].

Compound	Control[2]		Gypsy moth		MeJA		Mechanical wounding		*F*[3]	*P*
LIPOXYGENASE PATHWAY PRODUCTS										
(Z)-3-Hexenyl acetate[4]	3.8 ± 0.7	(18)b	8.7 ± 1.0	(4)ab	39.2 ± 31.0	(13)ab	313.5 ± 170.6	(92)a	4.88	0.019
ISOPRENOID PATHWAY PRODUCTS										
Monoterpenes										
α-Pinene	n.d.	b	n.d.	b	1.5 ± 0.6	(<1)a	n.d.	b	8.10	0.003
Camphene	1.5 ± 0.1	(7)bc	5.1 ± 0.7	(3)a	3.0 ± 0.5	(1)ab	0.7 ± 0.5	(<1)c	10.87	0.001
Sabinene	0.5 ± 0.5	(2)b	4.3 ± 0.8	(2)a	3.3 ± 0.5	(1)ab	9.9 ± 5.2	(3)a	5.56	0.013
β-Pinene	n.d.	b	n.d.	b	1.3 ± 0.4	(<1)a	n.d.	b	8.93	0.002
Myrcene	n.d.	b	2.3 ± 0.3	(1)ab	1.0 ± 0.6	(<1)a	0.3 ± 0.3	(<1) b	7.01	0.006
Eucalyptol/Limonene[5]	0.8 ± 0.4	(4)b	9.2 ± 3.6	(5)a	3.4 ± 0.8	(1)ab	7.3 ± 3.5	(2)ab	5.22	0.015
Linalool oxide	n.d.	b	1.4 ± 0.8	(1)ab	1.5 ± 0.3	(<1)a	n.d.	b	5.93	0.01
Linalool	3.9 ± 0.4	(18)b	23.3 ± 3.6	(12)a	42.6 ± 10.3	(14)a	3.4 ± 0.8	(1)b	35.93	<0.001
Borneol	n.d.	a	n.d.	a	0.4 ± 0.4	(<1)a	n.d.	a	1.00	0.426
Homoterpenes										
α-Copaene	n.d.	b	0.9 ± 0.5	(<1)ab	2.1 ± 0.4	(1)a	n.d.	b	11.66	0.001
β-Cubebene	n.d.	a	n.d.	a	1.6 ± 1.0	(1)a	n.d.	a	2.93	0.077
β-Caryophyllene	n.d.	b	28.0 ± 1.8	(14)a	37.3 ± 18.5	(12)a	n.d.	b	28.71	<0.001
α-Humulene	n.d.	b	15.9 ± 1.1	(8)a	20.6 ± 9.6	(7)a	n.d.	b	41.67	<0.001
Germacrene-D	n.d.	b	8.8 ± 1.0	(4)a	10.6 ± 5.1	(3)a	n.d.	b	32.26	<0.001
α-Farnesene	n.d.	a	n.d.	a	1.1 ± 0.7	(<1)a	n.d.	a	2.89	0.079
δ-Cadinene	n.d.	b	1.4 ± 0.5	(1)a	1.5 ± 0.6	(<1)a	n.d.	b	5.55	0.013
Sesquiterpenes										
4,8-Dimethyl-1,3,7-nonatriene[6]	6.9 ± 1.8	(32)b	57.5 ± 7.4	(29)a	100.0 ± 31.2	(32)a	4.1 ± 0.7	(1)b	45.46	<0.001
SHIKIMIC ACID /PHENYLPROPANOID PATHWAY PRODUCTS										
Indole	n.d.	b	15.4 ± 3.0	(8)a	17.2 ± 2.9	(6)a	0.3 ± 0.3	(<1)b	87.98	<0.001
Methyl salicylate	2.8 ± 0.2	(13)ab	3.9 ± 0.2	(2)a	2.7 ± 0.7	(1)ab	1.8 ± 0.4	(1)b	3.88	0.038
Phenylethyl ester	n.d.	b	7.4 ± 0.9	(4)a	6.7 ± 1.5	(2)a	n.d.	b	160.23	<0.001
Benzoic acid, ethyl ester	1.4 ± 0.8	(6)a	6.3 ± 5.2	(3)a	9.4 ± 2.7	(3)a	0.8 ± 0.5	(<1)a	2.66	0.095
Total	21.5 ± 1.3	b	199.9 ± 18.4	a	307.9 ± 71.7	a	342.3 ± 180.6	ab	6.29	0.008

[1] *N = 4.*

[2] *Mean ng n-octane units h^{-1} g^{-1} of fresh tissue (±SE). In parenthesis are percent values based on total amounts. n.d. = not detected (zero values were assigned to non-detectable values for statistical analysis).*

[3] *df = 3.12*

[4] *For each compound, different letters indicate significant differences between the samples.*

[5] *Peaks of these volatile compounds co-eluted in the GC.*

[6] *This compound was misidentified as myrcenone (based on spectral library match) in Rodriguez-Saona et al. (2011).*

variation in volatile blends. The score plot of PC1 versus PC2 shows no overlap among volatile blends of gypsy moth, MeJA, and control treatments, while the mechanically wounded and control treatments overlapped (**Figure 2A**). The first PC component explained 52.3% of the variation in volatile blend and separated the gypsy moth and MeJA treatments from the mechanically wounded and control treatments; while the second PC component explained 13.4% of the variation in the data and separated the gypsy moth from MeJA treatments (**Figure 2A**).

Two major biosynthetic pathways in the regulation of plant volatiles were influenced by herbivory and MeJA treatments in cranberries; these included the isoprenoid pathway, that resulted in increased emissions of monoterpenes (MANOVA: Wilks' $\lambda < 0.001$; $F = 4.98$; $P = 0.003$), sesquiterpenes (Wilks' $\lambda < 0.001$; $F = 19.77$; $P < 0.001$), and homoterpenes, and the shikimic acid/phenylpropanoid pathway (Wilks' $\lambda = 0.006$; $F = 11.94$; $P < 0.001$). In particular, emission of linalool, DMNT, phenylethyl ester, indole, β-caryophyllene, α-humulene, germacrene-D, and γ-cadinene were higher in the gypsy moth and MeJA treatments compared with the other treatments (**Table 2**). Only (*Z*)-3-hexenyl acetate emissions were greater in the mechanically wounded plants compared with the control plants (**Table 2**).

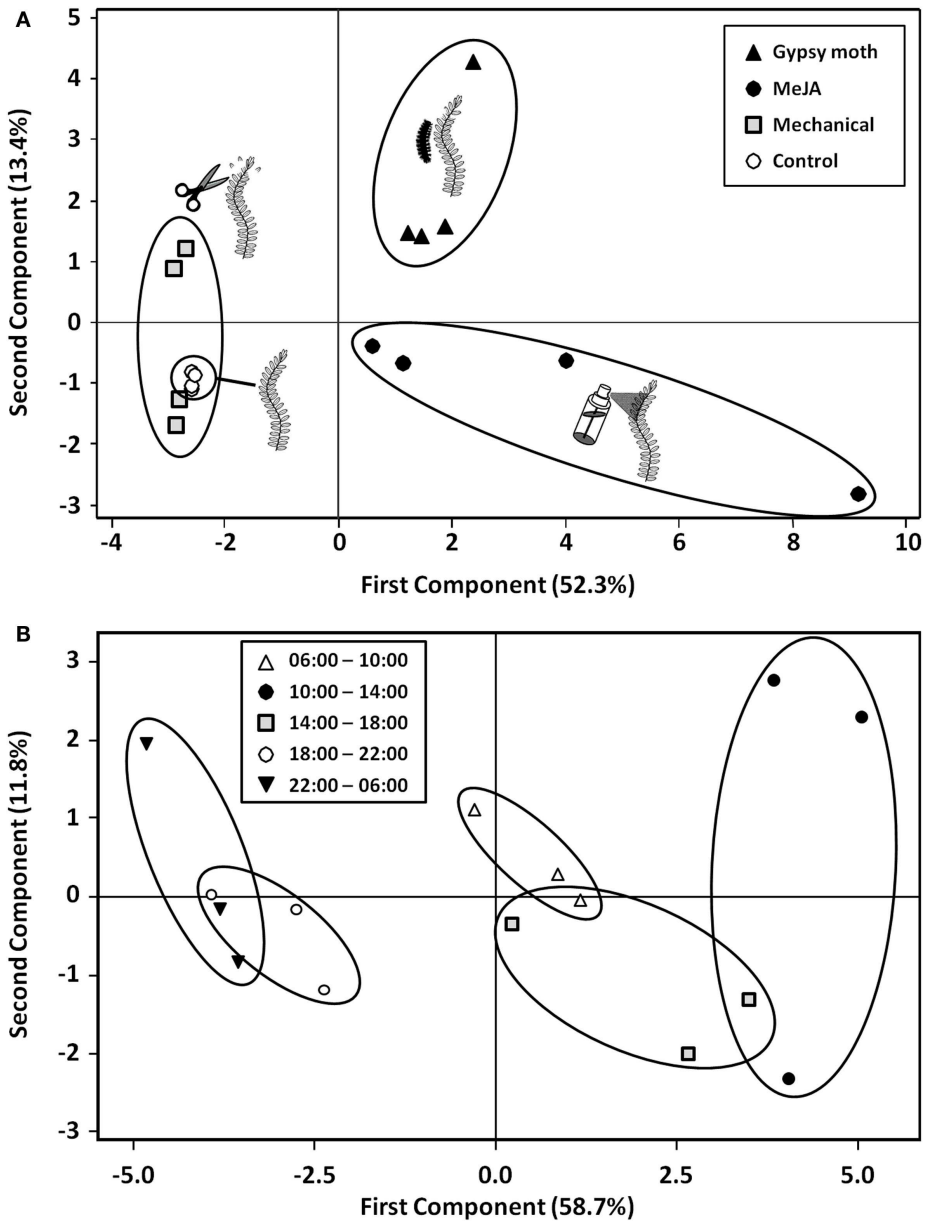


FIGURE 2 | Score plot of principal component analysis (PCA) for (A) the effects of gypsy moth larval feeding, MeJA, and mechanical damage as compared to control plants on the volatile profiles of cranberries (*Vaccinium macrocarpon*) leaves, and (B) the effects of time of day on volatile emissions from MeJA-treated plants. Percent variation explained by each principal component is indicated in parenthesis.

The score plot shows no overlap between volatiles emitted from MeJA-treated cranberry plants at 10:00–14:00 versus those emitted at 06:00–10:00, 18:00–22:00, and 22:00–06:00 (**Figure 2B**). Total volatile emissions from MeJA-treated cranberries peaked between 10:00 and 14:00 and were lowest between 18:00 and 06:00 (**Table 3**). Emissions of linalool, DMNT, methyl salicylate, phenylethyl ester, indole, α-copaene, β-cubebene, β-caryophyllene, α-humulene, germacrene-D, and γ-cadinene peaked between 10:00 and 14:00. Only a few compounds (e.g., linalool, DMNT, indole, β-caryophyllene, α-humulene, and germacrene-D) were emitted in detectable amounts at dusk (18:00–22:00) and during the scotophase (in the dark) (**Table 3**).

ELECTROANTENNOGRAPHY

We used EAG to determine the antennal activity of a polyphagous herbivore, *S. sulfureana*, to various cranberry volatiles. The magnitude of the EAG response was significantly different among the tested chemical compounds ($F = 2.29$, $df = 10,129$, $P = 0.017$). The EAG responses between males and females were, however, not different ($F = 1.28$, $df = 1,129$, $P = 0.261$), nor the interaction between chemical compound and sex of insect ($F = 0.99$, $df = 10,129$, $P = 0.458$). Antennal responses were greater to (*Z*)-3-hexenyl acetate, β-pinene, linalool, and linalool oxide compared with β-caryophyllene, α-cubebene, α-humulene, and β-farnesene (**Figure 3**).

ECOLOGICAL LEVEL

Three herbivores were most abundant on sticky traps; these were: the *Sparganothis* fruitworm, *S. sulfureana*, the sharp-nosed leafhopper, *Scaphytopius magdalensis* Provancher (Hem., Cicadellidae), and the blunt-nosed leafhopper, *Limotettix vaccinii* (Van Duzee) (Hem., Cicadellidae). The most abundant groups of natural enemies on traps were hoverflies [mainly *Toxomerus marginatus* (Say)], minute pirate bugs (*Orius* spp.), spiders, and parasitic wasps.

Table 3 | Time course of volatile emissions from cranberry, *Vaccinium macrocarpon*, plants sprayed with 1 mM MeJA solution[1].

Compound	06:00–10:00[2]		10:00–14:00		14:00–18:00		18:00–22:00		22:00–06:00		F[3]	P
LIPOXYGENASE PATHWAY PRODUCTS												
(Z)-3-Hexenyl acetate[4]	10.3 ± 0.9	ab	13.1 ± 0.7	a	33.9 ± 24.8	a	n.d.	b	n.d.	b	31.41	<0.001
ISOPRENOID PATHWAY PRODUCTS												
Monoterpenes												
α-Pinene	n.d.		n.d.		n.d.		n.d.		n.d.		–	–
Camphene	7.8 ± 1.1	a	12.8 ± 2.3	a	11.0 ± 1.3	a	n.d.	b	0.8 ± 0.8	b	28.81	<0.001
Sabinene	2.0 ± 2.2	a	5.2 ± 2.6	a	n.d.	a	n.d.	a	n.d.	a	2.14	0.151
β-Pinene	n.d.	b	4.8 ± 2.4	a	n.d.	b	n.d.	b	n.d.	b	3.98	0.035
Myrcene	n.d.	a	2.7 ± 2.7	a	2.6 ± 2.6	a	n.d.	a	n.d.	a	0.75	0.58
Eucalyptol/Limonene[5]	9.3 ± 2.9	ab	17.0 ± 2.6	a	15.0 ± 2.8	a	1.6 ± 1.6	bc	0.7 ± 0.7	c	9.69	0.002
Linalool oxide	n.d.	a	2.5 ± 2.5	a	n.d.	a	n.d.	a	n.d.	a	1.00	0.452
Linalool	85.7 ± 20.3	a	135.8 ± 23.3	a	52.8 ± 14.4	ab	9.4 ± 0.9	bc	2.5 ± 1.3	c	19.85	<0.001
Borneol	n.d.		n.d.		n.d.		n.d.		n.d.		–	–
Sesquiterpenes												
α-Copaene	5.2 ± 2.8	ab	13.2 ± 1.9	a	10.6 ± 0.7	ab	2.0 ± 2.0	b	0.9 ± 0.9	b	3.75	0.041
β-Cubebene	2.3 ± 2.3	ab	19.0 ± 3.9	a	9.8 ± 4.9	ab	n.d.	b	1.4 ± 1.4	b	3.84	0.038
β-Caryophyllene	123.8 ± 31.0	ab	365.5 ± 104.3	a	264.2 ± 34.3	a	75.1 ± 20.4	ab	43.7 ± 16.6	b	7.63	0.004
α-Humulene	66.4 ± 16.6.6	ab	193.7 ± 55.6	a	152.2 ± 21.5	a	42.5 ± 10.9	ab	25.9 ± 10.2	b	6.68	0.007
Germacrene-D	36.0 ± 10.5.5	ab	104.9 ± 48.1	a	74.1 ± 14.3	ab	18.5 ± 6.7	ab	9.3 ± 4.7	b	3.87	0.038
α-Farnesene	n.d.	a	14.1 ± 7.2	a	8.6 ± 4.3	a	n.d.	a	n.d.	a	3.00	0.072
δ-Cadinene	n.d.	b	17.7 ± 6.8	a	10.0 ± 5.1	a	n.d.	b	n.d.	b	9.30	0.002
Homoterpenes												
4,8-Dimethyl-1,3,7-nonatriene	223.1 ± 75.2	ab	635.8 ± 252.4	a	450.3 ± 128.2	ab	80.2 ± 34.4	bc	24.5 ± 10.5	c	11.90	0.001
SHIKIMIC ACID/PHENYLPROPANOID PATHWAY PRODUCTS												
Indole	39.8 ± 4.8	ab	56.8 ± 11.2	a	41.1 ± 12.2	ab	20.0 ± 3.2	b	12.9 ± 2.8	b	8.72	0.003
Methyl salicylate	9.8 ± 1.9	a	11.3 ± 2.8	a	4.5 ± 2.3	ab	n.d.	b	1.2 ± 1.2	ab	6.80	0.007
Phenylethyl ester	13.3 ± 6.8	ab	53.2 ± 18.3	a	15.4 ± 15.4	ab	n.d.	b	n.d.	b	4.49	0.025

[1] *N = 3. MeJA was applied 16 h before volatile collections.*

[2] *Mean ng n-octane units h^{-1} g^{-1} of fresh tissue (±SE). n.d. = not detected (zero values were assigned to non-detectable values for statistical analysis).*

[3] *df = 4, 10.*

[4] *For each compound, different letters indicate significant differences between the samples.*

[5] *Peaks of these volatile compounds co-eluted in the GC.*

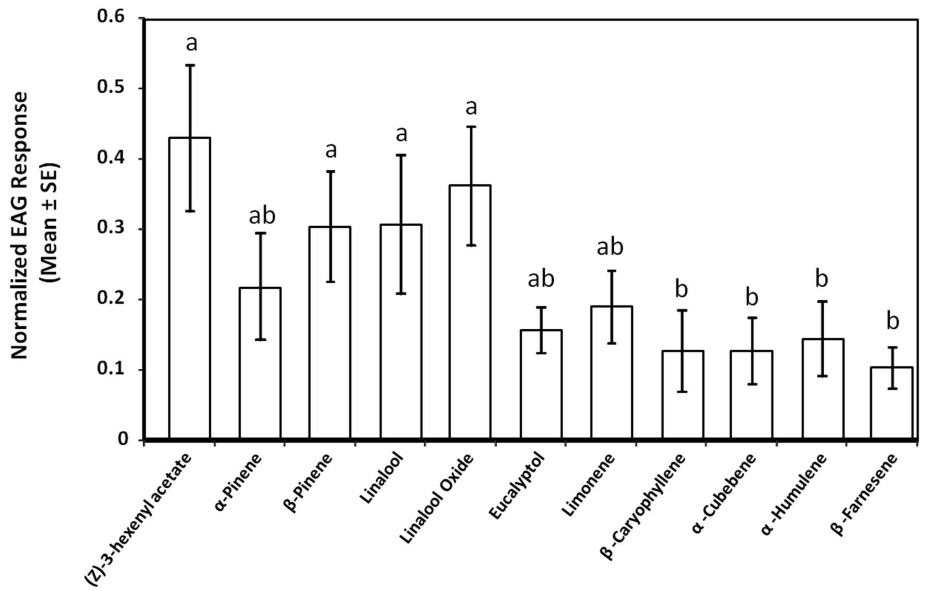


FIGURE 3 | Electroantennogram (EAG) responses of *Sparganothis* fruitworm, *Sparganothis sulfureana*, to various cranberry, *Vaccinium macrocarpon*, leaf volatiles. For analysis, control depolarizations (hexane) were subtracted from the test stimuli values. Different letters indicate significant differences among means ($P \leq 0.05$). Results of male and female *S. sulfureana* responses were combined in the graph ($N = 13$–17).

MeJA treatment (MANOVA: Wilks' $\lambda = 0.86$, $F = 3.36$, $df = 4.81$, $P = 0.014$), date (Wilks' $\lambda = 0.29$, $F = 16.85$, $df = 8.162$, $P < 0.001$), but not treatment × date interaction (Wilks' $\lambda = 0.87$, $F = 1.39$, $df = 8.162$, $P = 0.201$) had a significant negative effect on herbivore abundance on sticky traps. When analyzed in more detail, MeJA had an effect on *S. sulfureana* moths (treatment: $F = 6.10$, $df = 1.84$, $P = 0.016$; date: $F = 13.66$, $df = 2.84$, $P < 0.001$; treatment × date interaction: $F = 1.90$, $df = 2.84$; $P = 0.156$) (**Figure 4A**), but not on the leafhoppers *L. vaccinii* (treatment: $F = 2.10$, $df = 1.84$, $P = 0.151$; date: $F = 30.83$, $df = 2.84$, $P < 0.001$; treatment × date interaction: $F = 1.06$, $df = 2.84$; $P = 0.352$) (**Figure 4B**), or *S. magdalensis* (treatment: $F = 1.51$, $df = 1.84$, $P = 0.223$; date: $F = 29.43$, $df = 2.84$, $P < 0.001$; treatment × date interaction: $F = 0.45$, $df = 2.84$; $P = 0.638$) (**Figure 4C**).

Abundance of natural enemies on sticky traps were positively affected by MeJA treatment (MANOVA: Wilks' $\lambda = 0.68$, $F = 9.122$, $df = 4.81$, $P < 0.001$), date (Wilks' $\lambda = 0.28$, $F = 17.57$, $df = 8.162$, $P < 0.001$), and treatment × date interaction (Wilks' $\lambda = 0.47$, $F = 9.10$, $df = 8.162$, $P < 0.001$). When analyzed in more detail, MeJA had a positive effect on parasitic wasps (treatment: $F = 36.43$, $df = 1.84$, $P < 0.001$; date: $F = 91.56$, $df = 2.84$, $P < 0.001$; treatment × date interaction: $F = 36.48$, $df = 2.84$; $P < 0.001$) (**Figure 4F**), but not on the hoverfly *T. marginatus* (treatment: $F = 0.31$, $df = 1.84$, $P = 0.582$; date: $F = 4.35$, $df = 2.84$, $P = 0.016$; treatment × date interaction: $F = 1.91$, $df = 2.84$; $P = 0.155$) (**Figure 4D**), minute pirate bugs (treatment: $F = 1.19$, $df = 1.84$, $P = 0.278$; date: $F = 2.67$, $df = 2.84$, $P = 0.075$; treatment × date interaction: $F = 2.52$, $df = 2.84$; $P = 0.087$) (**Figure 4E**), or spiders (treatment: $F = 1.21$, $df = 1.84$, $P = 0.275$; date: $F = 0.08$, $df = 2.84$, $P = 0.919$; treatment × date interaction: $F = 0.01$, $df = 2.84$; $P = 0.988$) (**Figure 4G**).

Sparganothis sulfureana larval mortality was significantly higher when fed foliage from JA-treated cranberry plants as compared with those fed foliage from untreated plants [mean (± SE) percent mortality = JA: 42.5% (±13.5); control = 0% (±0); $F = 33.37$, $df = 1.6$, $P = 0.001$]. Moreover, the negative effects of JA on larval mortality were attributable to the activation of plant defenses by JA and not to direct toxicity because mortality rates were similar on JA-treated (4%) and untreated diets (8%) ($\chi^2 = 0.67$; $df = 1$; $P = 0.414$).

DISCUSSION

Jasmonates such as jasmonic acid and MeJA can serve as an important tool in insect pest management for plant protection against herbivorous pests (Thaler, 1999a; Rohwer and Erwin, 2008; Rodriguez-Saona et al., 2012). Before this can be achieved, however, studies are needed that link the activation of defensive genes by these hormones in plants, the plant's biochemical changes, and the response of both antagonistic and mutualistic organisms to jasmonate-induced plants. In this study, we showed that: (1) herbivory by gypsy moth caterpillars, jasmonate treatment, and mechanical wounding induce, albeit differently, the expression of genes from two different pathways in the biosynthesis of terpene compounds, i.e., the mevalonic acid (MVA) pathway that produces sesquiterpenes and the methylerythritol phosphate (MEP) pathway that leads to monoterpene production (Lichtenthaler et al., 1997; Paré and Tumlinson, 1999; Bartram et al., 2006; Dudareva et al., 2013); (2) herbivore feeding and jasmonate treatment, but not mechanical wounding, induced emissions of monoterpene, homoterpene, and sesquiterpene volatiles; however, blends

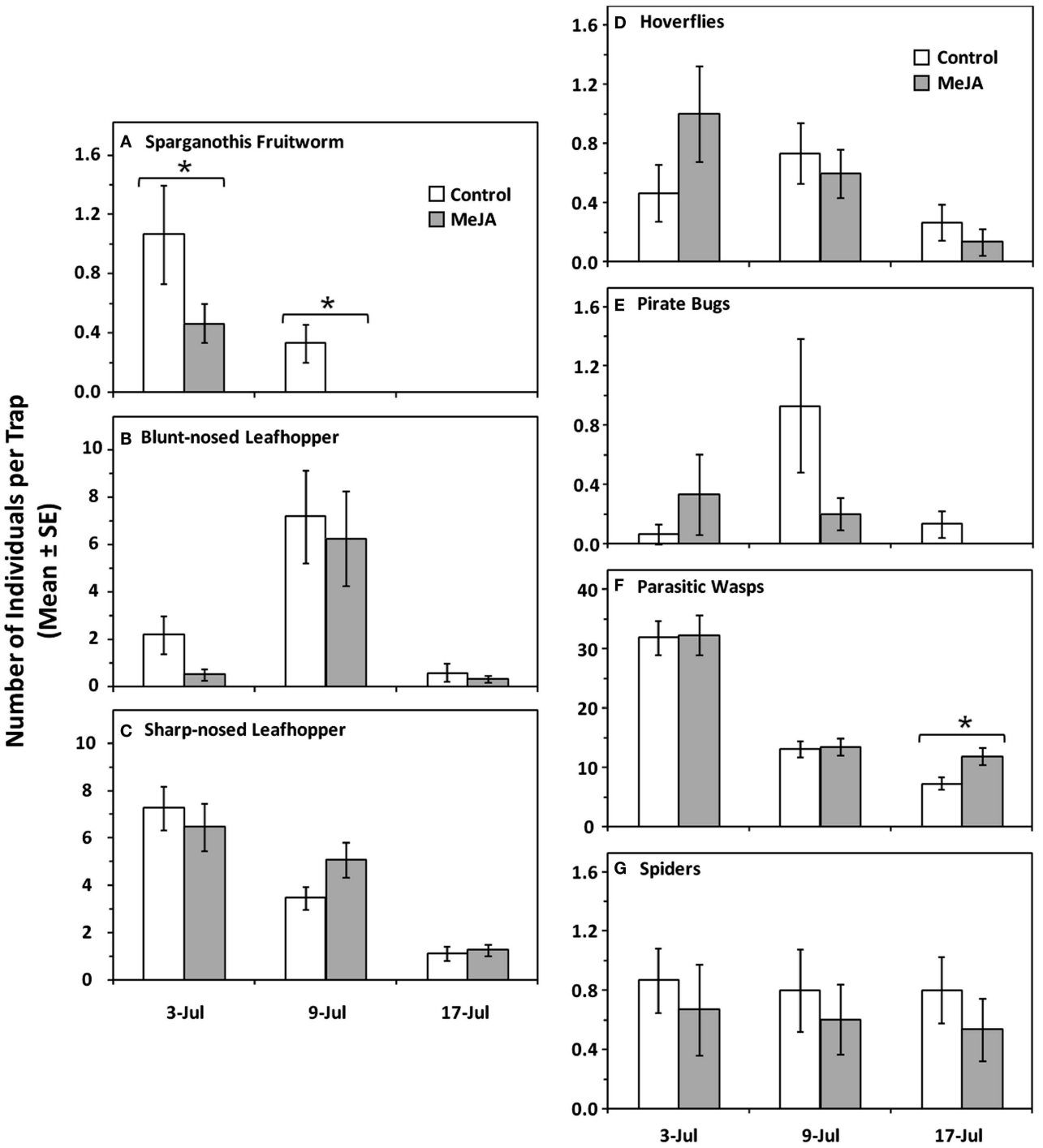


FIGURE 4 | Mean numbers of arthropods per sticky trap placed near cranberry plants treated with exogenous MeJA and untreated plants (control). Herbivores: **(A)** *Sparganothis* fruitworm, *Sparganothis sulfureana* (Lep., Tortricidae), **(B)** blunt-nosed leafhoppers, *Limotettix vaccinii* (Hem., Cicadellidae), **(C)** sharp-nosed leafhopper, *Scaphytopius magdalensis* (Hem., Cicadellidae). Natural enemies: **(D)** hoverflies (mainly *Toxomerus marginatus*) (Dip., Syrphidae), **(E)** minute pirate bugs (*Orius* spp.) (Hem., Anthocoridae), **(F)** parasitic wasps (Hymenoptera), and **(G)** spiders (Araneae). Asterisks (*) indicate statistically significant differences between MeJA and control treatments ($P \leq 0.05$); all other comparisons were non-significant ($P > 0.05$).

were distinct from one another; and, (3) jasmonate treatment reduced preference and performance of the herbivore *S. sulfureana* to cranberry plants, and increased colonization by parasitic wasps. Furthermore, *S. sulfureana* antennae were highly sensitive to inducible monoterpenes, in particular linalool, which might explain the repellence effects of jasmonate-induced plants.

At the molecular level, expression of genes from two isoprenoid biosynthetic pathways – the MVA and MEP pathways – were activated by gypsy moth feeding, MeJA, and mechanical wounding. Of the eight genes targeted in this study – *BCS, FDS, LLS, MDS, NER1*, *PMK*, *TPS*, *TPS21*, four responded significantly to gypsy moth larval feeding, MeJA, and mechanical wounding (*BCS*, *LLS*, *NER1*, and *TPS21*), but to different degrees. Expression of the other four genes (*FDS*, *MDS*, *PMK*, and *TPS*) did not change. *MDS*, *PMK*, and *FDS* are higher up in the terpenoid pathway (Terpenoid Backbone Biosynthesis pathway; KEGG map00900), so the whole pathway is not being induced by larval feeding, MeJA, or mechanical wounding but rather the genes involved in the synthesis of specific terpenes. These data thus indicate that cranberry leaves use pre-formed intermediates to rapidly make specific terpenes. The most dramatic changes in expression of *BCS* and *TPS21* were when treated with MeJA; these genes are in the MVA pathway and the enzymes encoded catalyze the production of β-caryophyllene and α-humulene, respectively. Gypsy moth-damaged and MeJA-treated plants also had elevated expression of *LLS* and *NER1* [*LLS* is in the MEP pathway and the encoded enzyme catalyzes the synthesis of linalool; *NER1* is in the homoterpene biosynthesis pathway and the encoded enzyme catalyzes the synthesis of nerolidol-derived DMNT (Boland et al., 1998)]. Interestingly, for those genes that responded to gypsy moth feeding (*BCS*, *LLS*, *NER1*, and *TPS21*), the changes in gene expression were also evident in the undamaged tissue on the same plant, indicating a systemic response in gene expression. This was also true in mechanically wounded plants, but only for *NER1* and *LLS*; thus, genes from the MVA pathway do not appear to be expressed systemically by mechanical wounding.

At the biochemical level, volatiles from the MVA pathway (e.g., β-caryophyllene, α-humulene, and germacrene-D) were strongly induced by gypsy moth feeding but not by mechanical wounding in cranberries. Similarly, volatiles from the MEP pathway (e.g., camphene, eucalyptol/limonene, and linalool) were strongly induced by gypsy moth feeding but not or only weakly induced by mechanical wounding. These data suggest that insect-derived elicitors (see Alborn et al., 1997) are required for the induction of terpene emissions in cranberries. In a previous study, Rodriguez-Saona et al. (2011) found differences in the volatile response to gypsy moth feeding among five cranberry varieties, indicating genotypic variation in the volatile response of cranberries to herbivore feeding. Contrary, they reported similar induction of volatiles by MeJA among these varieties (Rodriguez-Saona et al., 2011). In this study, we also showed that MeJA induces a blend of volatiles in cranberries that was different from herbivore feeding. Other studies have reported a high degree of resemblance, but also some qualitative and quantitative differences, between jasmonate and herbivore-induced volatile profiles (e.g., Dicke et al., 1999; Rodriguez-Saona et al., 2001, 2006; Gols et al., 2003). Altogether, previous data (Rodriguez-Saona et al., 2011) and the present data indicate that signals (i.e., hormones) other than jasmonates might be involved in the emission of HIPVs in cranberries. For example, ethylene was found to interact with JA and the insect-derived elicitor volicitin in the induction of volatile emissions from maize seedlings (Schmelz et al., 2003).

Herbivory and MeJA also induced indole and phenylethyl ester emissions, both products of the shikimic acid pathway (Paré and Tumlinson, 1999), indicating that induced volatiles in cranberries originate mainly from the isoprenoid and shikimic acid pathways. On the other hand, mechanical wounding increased emissions of (*Z*)-3-hexenyl acetate, a product of the lipoxygenase pathway and that is often emitted rapidly in plants as a result of wounding (Paré and Tumlinson, 1999; Chehab et al., 2008).

At the organismal level, jasmonates were involved in the activation of both direct (i.e., plant chemicals that deter or kill the herbivore) and indirect (i.e., chemicals such as HIPVs that attract the herbivores' natural enemies) resistance in cranberries. Host-plant preference and performance were correlated for the polyphagous herbivore *S. sulfureana*: adults were less attracted to, and larval survival was reduced on, jasmonate-induced plants. **Table 4** summarizes studies on the effects of jasmonates on herbivorous arthropod performance and preference – we limit this list to studies that used methodologies similar to ours, i.e., jasmonates (JA or MeJA) were sprayed exogenously on plants; thus, investigated only local responses. Out of 37 herbivore-plant interactions reported in these studies, 31 (84%) showed a negative effect of jasmonates on herbivores, while positive effects accounted for only 8%. Negative effects on the herbivores included lower abundance, performance, colonization, and oviposition on jasmonate-induced plants (**Table 4**) – likely resulting in increased foraging time and reduced overall fitness; while positive effects included greater abundance, attraction, and oviposition (**Table 4**). Interestingly, the western flower thrips, *Frankliniella occidentalis* Pergande, performs poorly on tomato (Thaler et al., 2001) and Chinese cabbage (Abe et al., 2009) induced by JA, whereas it has higher performance on JA-induced cotton (Omer et al., 2001). Likewise, the diamondback moth, *Plutella xylostella* L., was more attracted for oviposition to JA-treated compared with untreated common cabbage, while it was less attracted to JA-treated than untreated Chinese cabbage (Lu et al., 2004). Thus, the outcome of these interactions can be host-plant dependent.

The mechanism of the repellent effects of MeJA on adult *S. sulfureana* remains unknown – it is not known which compound(s) is responsible for these effects. Both male and female *S. sulfureana* antennae responded strongly to four cranberry volatiles [(*Z*)-3-hexenyl acetate, β-pinene, linalool, and linalool oxide]; however, only linalool was emitted in detectable amounts from MeJA-treated cranberry plants during dusk and nighttime (**Table 3**), when moths are expected to be most active. The more attractive cranberry plants to *S. sulfureana* (controls) emitted low quantities of linalool constitutively; whereas less attractive plants (MeJA-treated) emitted this compound in high amounts (**Table 2**). This compound has important behavioral effects on other moth species both as an attractant (e.g., Suckling et al., 1996; Raguso et al., 2003) and a repellent (e.g., Kessler and Baldwin, 2001; McCallum et al., 2011), and possibly also on *S. sulfureana*. Also, the herbivore *Spodoptera frugiperda* Smith showed an increment in the antennal response (EAG) to linalool at higher doses (Malo et al., 2004). In our study we could not discard the possibility that jasmonates themselves caused the effect. It was also difficult to determine the gender of the moths on sticky cards because of their poor

Table 4 | Effects of jasmonic acid (JA) and methyl jasmonate (MeJA) sprayed exogenously to plants on herbivorous arthropods[1].

Arthropod Species	Common Name	Feeding Guild	Elicitor	Concentration (mM)	Ecological Effects	Overall Effect	Plant	Conditions	Reference
Spodoptera exigua	Beet armyworm	Chewer	JA	0.5	Lower growth rate/feeding	Negative	Tomato	Laboratory	Thaler et al. (1996)
Manduca sexta	Tobacco hornworm	Chewer	JA	1	Lower growth rate	Negative	Tomato	Greenhouse	Cipollini and Redman (1999)
Unknown	Unknown	Chewer	JA	0.5, 1.5	Lower feeding damage	Negative	Tomato	Field	Thaler (1999c)
Daktulosphaira vitifoliae	Grape phylloxera	Phloem feeder (roots)	JA	1	Lower performance	Negative	Grape	Greenhouse	Omer et al. (2000)
Tetranychus pacificus	Pacific spider mite	Cell-content feeder	JA	1	Lower performance	Negative	Grape	Greenhouse	Omer et al. (2000)
Spodoptera exigua	Beet armyworm	Chewer	JA	0.5, 1.5	Lower performance	Negative	Tomato	Field	Thaler et al. (2001)
Macrosiphum euphorbiae	Potato aphid	Phloem feeder	JA	0.5, 1.5	Lower abundance	Negative	Tomato	Field	Thaler et al. (2001)
Myzus persicae	Green peach aphid	Phloem feeder	JA	0.5, 1.5	Lower abundance	Negative	Tomato	Field	Thaler et al. (2001)
Frankliniella occidentalis	Western flower thrips	Cell-content feeder	JA	0.5, 1.5	Lower abundance	Negative	Tomato	Field	Thaler et al. (2001)
Epitrix hirtipennis	Flea beetle	Chewer	JA	0.5, 1.5	Lower abundance	Negative	Tomato	Field	Thaler et al. (2001)
Aphis gossypii	Cotton aphid	Phloem feeder	JA	1	Lower abundance	Negative	Cotton	Greenhouse/ laboratory	Omer et al. (2001)
Tetranychus urticae	Two-spotted spider mite	Cell-content feeder	JA	1	Lower abundance	Negative	Cotton	Greenhouse/ laboratory	Omer et al. (2001)
Frankliniella occidentalis	Western flower thrips	Cell-content feeder	JA	1	Higher abundance	Positive	Cotton	Greenhouse/ laboratory	Omer et al. (2001)
Aphis gossypii	Aphid nymphs	Phloem feeder	JA	1	Lower performance	Negative	Cotton	Greenhouse/ laboratory	Omer et al. (2001)
Tetranychus urticae	Two-spotted spider mite	Cell-content feeder	JA	0.1–1	Lower abundance	Negative	Lima bean	Laboratory	Gols et al. (2003)
Unknown	Unknown	Chewer	JA	1	Lower feeding damage	Negative	Lima bean	Field	Heil (2004)
Plutella xylostella	Diamonback moth	Nectar feeder	JA	0.01–1	Lower oviposition	Negative	Chinese cabbage	Field/ laboratory	Lu et al. (2004)
Plutella xylostella	Diamonback moth	Nectar feeder	JA	0.01–1	Higher oviposition	Positive	Common cabbage	Field/ laboratory	Lu et al. (2004)
Macrosiphum euphorbiae	Potato aphid	Phloem feeder	JA	1.5	Lower performance	Negative	Tomato	Greenhouse	Cooper and Goggin (2005)
Ips typographus	Spruce bark beetle	Chewer	MeJA	100	Lower colonization/higher resistance	Negative	Norway spruce	Field	Erbilgin et al. (2006)

(Continued)

Table 4 | Continued

Arthropod Species	Common Name	Feeding Guild	Elicitor	Concentration (mM)	Ecological Effects	Overall Effect	Plant	Conditions	Reference
Agrilus planipennis	Emerald ash borer	Chewer	MeJA	1.4	Higher attraction	Positive	Manchurian ash	Laboratory	Rodriguez-Saona et al. (2006)
Myzus persicae	Green peach aphid	Phloem feeder	MeJA	1, 5, 10	Lower population growth	Negative	Tomato	Greenhouse	Boughton et al. (2006)
Pieris rapae	Small cabbage white	Nectar feeder	JA	0.1	Lower oviposition	Negative	Brussels sprouts	Greenhouse	Bruinsma et al. (2007)
Pieris brassicae	Cabbage white	Nectar feeder	JA	0.1	Lower oviposition	Negative	Brussels sprouts	Greenhouse	Bruinsma et al. (2007)
Pieris rapae	Small cabbage white	Nectar feeder	JA	0.5	Lower oviposition	Negative	Black mustard	Greenhouse	Bruinsma et al. (2008)
Nilaparvata lugens	Brown planthopper	Phloem feeder	JA	2.5, 5	Increase resistance	Negative	Rice	Greenhouse	Senthil-Nathan et al. (2009)
Frankliniella occidentalis	Western flower thrips	Cell-content feeder	JA	0.05	Increase resistance	Negative	Chinese cabbage	Greenhouse	Abe et al. (2009)
Tetranychus urticae	Two-spotted spider mite	Cell-content feeder	MeJA	0.1	Increase dispersal/resistance	Negative	Impatiens, Pansy, Tomato	Greenhouse	Rohwer and Erwin (2010)
Tetranychus urticae	Two-spotted spider mite	Cell-content feeder	MeJA	N/A[2]	Increase resistance	Negative	Apple, strawberry	Greenhouse	Warabieda and Olszak (2010)
Dendrolimus superans	Larch caterpillar moth	Chewer	JA	0.01–1	Lower oviposition	Negative	Larch	Laboratory	Meng et al. (2011)
Lymantria dispar	Gypsy moth	Chewer	JA	1	Increase resistance	Negative	Cranberry	Laboratory	Rodriguez-Saona et al. (2011)
Helicoverpa armigera	Cotton bollworm	Nectar feeder	MeJA	1.5	No effect on oviposition	None	Tomato	Greenhouse	Tan et al. (2012)
Gynandrobrotica guerreroensis	–	Chewer	JA	0.001–1	Repellency (females)	Negative	Lima bean	Laboratory	Ballhorn et al. (2013)
Cerotoma ruficornis	–	Chewer	JA	0.001–1	Repellency (females)	Negative	Lima bean	Laboratory	Ballhorn et al. (2013)
Sparganothis sulfureana	*Sparganothis* fruitworm	Nectar feeder	MeJA, JA	1	Lower attraction/performance	Negative	Cranberry	Field	This study
Scaphytopius magdalensis	Sharp-nosed leafhopper	Phloem feeder	MeJA	1	No effect on abundance	None	Cranberry	Field	This study
Limotettix vaccinii	Blunt-nosed leafhopper	Phloem feeder	MeJA	1	No effet on abundance	None	Cranberry	Field	This study

[1] *Listed in chronological order.*

[2] *Not indicated*

Table 5 | Effects of jasmonic acid (JA) and methyl jasmonate (MeJA) sprayed exogenously to plants on natural enemy behavior through induction of volatiles[1].

Arthropod species	Common name	Feeding guild	Elicitor	Concentration (mM)	Ecological effects	Overall effect	Plant	Conditions	Reference
Hyposoter exigua	Parasitic wasp	Parasitoid	JA	0.5	Higher parasitism	Positive	Tomato	Field	Thaler (1999b)
Phytoseiulus persimilis	Predatory mite	Predator	JA	1	Higher attraction	Positive	Lima bean	Laboratory	Dicke et al. (1999)
Cotesia rubecula	Parasitic wasp	Parasitoid	JA	1	Higher attraction	Positive	*Arabidopsis thaliana*	Laboratory	van Poecke and Dicke (2002)
Unknown	Hoverfly	Predator	JA	0.5	Lower abundance	Negative	Tomato	Field	Thaler (2002)
Hyposoter exigua	Parasitic wasp	Parasitoid	JA	0.5	No effect on adult abundance	None	Tomato	Field	Thaler (2002)
Hippodamia convergens	Convergent lady beetle	Predator	JA	0.5	No effect	None	Tomato	Field	Thaler (2002)
Aphelinid spp.	Aphid parasitoid	Parasitoid	JA	0.5	No effect	None	Tomato	Field	Thaler (2002)
Phytoseiulus persimilis	Predatory mite	Predator	JA	0.1–1	Higher attraction	Positive	Lima bean	Laboratory	Gols et al. (2003)
Cotesia kariyai	Parasitic wasp	Parasitoid	JA	1	Higher attraction	Positive	Corn	Laboratory	Ozawa et al. (2004)
Cotesia glomerata	Parasitic wasp	Parasitoid	JA	0.5	Higher attraction	Positive	Black mustard	Greenhouse	Bruinsma et al. (2008)
Eristalis tenax	Syrphid fly	Predator	JA	0.5	No effect on adults	None	Black mustard	Field	Bruinsma et al. (2008)
Cotesia glomerata	Parasitic wasp	Parasitoid	JA	1	Higher attraction	Positive	Brussels sprouts	Laboratory	Bruinsma et al. (2009)
Cotesia rubecula	Parasitic wasp	Parasitoid	JA	1	Higher attraction	positive	Brussels sprouts	Laboratory	Bruinsma et al. (2009)
Diadegma semiclausum	Parasitic wasp	Parasitoid	JA	1	Higher attraction	Positive	Brussels sprouts	Laboratory	Bruinsma et al. (2009)
Anastatus japonicas	Parasitic wasp	Parasitoid	JA	0.01–1	Higher attraction	Positive	Larch	Laboratory	Meng et al. (2011)
unknown (complex)	Parasitic wasp	Parasitoid	MeJA	1	Higher attraction	Positive	Cranberry	Field	This study
Toxomerus marginatus	Hoverfly	Predator	MeJA	1	No effect on adults	None	Cranberry	Field	This study
Orius spp.	Pirate bug	Predator	MeJA	1	No effect	None	Cranberry	Field	This study

[1] *Listed in chronological order.*

condition, i.e., moths were either too dry or covered with adhesive. Further studies are needed on the dose-dependent effects of linalool and other induced volatile compounds on *S. sulfureana* foraging behavior, and to determine if gender differences exist in *S. sulfureana*'s response to jasmonate-induced plants and, in particular, the effects of induced volatiles on oviposition.

In contrast to herbivores, the majority (11 out of 18 or 61%) of natural enemy-plant interactions involving jasmonate-induce plants were positive (**Table 5**). All these interactions involved greater attraction to induced plants likely through increases in volatile emissions. Only 1 out of 18 case studies (6%) reported a negative effect of jasmonates on natural enemies (**Table 5**). Thaler (2002) reported reduced number of hoverfly eggs laid on induced plants due to a decrease in aphid (prey) abundance. Interestingly, Thaler (2002) found no effect on adult *Hyposoter exiguae* Viereck caught on sticky traps placed beneath the canopy of control and JA-induce tomato plants. However, a previous study (Thaler, 1999b) showed higher parasitism of *Spodoptera exigua* Hübner larvae by *H. exiguae* on JA-induced induced tomato plants, indicating a possible discrepancy between adult attraction and parasitism rate. In the present study, higher numbers of parasitic wasps were caught on sticky traps near MeJA-treated plants than on untreated plants. This attraction coincided with the time of *S. sulfureana* egg laying and larval development; whether this effect translates to greater parasitism of *S. sulfureana* eggs or larvae (or other herbivore pest) requires further investigation. Additional studies are also needed to address the identity and function of these parasitic wasps.

In summary, this study demonstrates the potential of jasmonates as natural plant protectants against herbivorous pests in cranberries. It also summarizes the overall effects of these phytohormones on herbivorous arthropods and their natural enemies (**Tables 4** and **5**). These studies show that jasmonates provide protection against herbivores from multiple feeding guilds (chewers, phloem feeders, and cell-content feeders) and increase natural enemy attraction in various agro-ecosystems. However, jasmonate-induced responses can be costly in the absence of herbivores (Baldwin, 1998; Cipollini et al., 2003; but see Thaler, 1999c), or could lead to ecological costs due to trade-offs between resistance to herbivores and pathogens (Felton and Korth, 2000). Further studies are needed to measure these costs in cranberries. Other remaining concerns include: the cost of spraying cranberry fields with MeJA, the length of the effect, and the possibility of inducing all plants and producing a no-choice situation.

In addition, this study integrates multiple levels of biological organization from gene expression to biochemical activation to ecological consequences. Such studies are needed for a better understanding of plant-arthropod interactions (Zheng and Dicke, 2008); yet, we are not aware of any similar studies that investigated the effects of jasmonates (e.g., JA or MeJA) on plants and arthropods at all of these three levels of biological organization (molecular, biochemical, and organismal) in a single study (but see Birkett et al., 2000). Our data show general agreement across all three biological levels of organization: key genes from the terpene pathway were highly expressed in MeJA-treated cranberry plants and terpene volatiles were induced by MeJA, which in turn led to repellency of an herbivore and attraction of certain natural enemies. There were, however, some discrepancies: mechanical wounding induced the local expression of four terpene genes – *BCS*, *LLS*, *NER1*, and *TPS21* – that encode the enzymes that catalyze the synthesis of β-caryophyllene, linalool, DMNT, and α-humulene, respectively; however, none of these volatiles were emitted in quantities different from unwounded plants. Therefore, we highlight the need of multiple approaches for a more complete assessment on the effects of various environmental stresses that activate the jasmonate signaling – such as herbivory – on plant-insect interactions.

ACKNOWLEDGMENTS

The authors thank the topic editors for their invitation to submit this contribution and two anonymous reviewers for helpful comments on an earlier draft. Thanks also to Robert Holdcraft, Vera Kyryczenko-Roth, Kristia Adams, Jennifer Frake, and Elizabeth Bender for laboratory and field assistance. Our special thanks to Dr. Thomas Hartman (Rutgers University, Mass Spectrometry Support Facility) and Dr. Hans Alborn (USDA-ARS, Center for Medical, Agricultural, and Veterinary Entomology) for their assistance in volatile identification, and to Matthew Kramer (USDA-ARS, Biometrical Consulting Service) for the statistical analyses of gene expression and for generating the graph presented in **Figure 1**. This work was supported by the USDA NIFA Special Grant "Blueberry and Cranberry Disease, Breeding and Insect Management" to Cesar R. Rodriguez-Saona and James Polashock, and by hatch funds to Cesar R. Rodriguez-Saona.

REFERENCES

Abe, H., Shimoda, T., Ohnishi, J., Kugimiya, S., Narusaka, M., Seo, S., et al. (2009). Jasmonate-dependent plant defense restricts thrips performance and preference. *BMC Plant Biol.* 9:97. doi:10.1186/1471-2229-9-97

Agrawal, A. A. (1998). Induced responses to herbivory and increased plant performance. *Science* 279, 1201–1202.

Agrawal, A. A. (2000). Benefits and costs of induced plant defense for *Lepidium virginicum* (Brassicaceae). *Ecology* 81, 1804–1813.

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H., and Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276, 945–949.

Arimura, G.-I., Kost, C., and Boland, W. (2005). Herbivore-induced, indirect plant defences. *Biochim. Biophys. Acta* 1734, 91–111.

Averill, A. L., and Sylvia, M. M. (1998). *Cranberry Insects of the Northeast.* Amherst: University of Massachusetts Extension.

Baldwin, I. T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8113–8118.

Ballhorn, D. J., Kautz, S., and Heil, M. (2013). Distance and sex determine host plant choice by herbivorous beetles. *PLoS ONE* 8:e55602. doi:10.1371/journal.pone.0055602

Bartram, S., Jux, A., Gleixner, G., and Boland, W. (2006). Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves. *Phytochemistry* 67, 1661–1672.

Bates, D., Maechler, M., and Bolker, B. (2011). *lme4: Linear Mixed-effects Models Using S4 Classes. R Package Version 0.999375-42.* Available at: http://CRAN.R-project.org/package=lme4.

Beckwith, C. S. (1938). *Sparganothis sulfureana* Clem., a cranberry pest in New Jersey. *J. Econ. Entomol.* 31, 253–256.

Bell, R. A., Owens, C. D., Shapiro, M., and Tardiff, J. R. (1981).

"Development of mass rearing technology," in *The Gypsy Moth: Research Toward Integrated Pest Management*, eds C. C. Doane and M. L. McManus (Washington: US Department of Agricultural), 599–633.

Birkett, M. A., Campbell, C. A. M., Chamberlain, K., Guerrieri, E., Hick, A. J., Martin, J. L., et al. (2000). New roles for cis-jasmone as an insect semiochemical and in plant defense. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9329–9334.

Boland, W., Gäbler, A., Gilbert, M., and Feng, Z. (1998). Biosynthesis of C11 and C16 homoterpenes in higher plants; stereochemistry of the C-C-bond cleavage reaction. *Tetrahedron* 54, 14725–14736.

Boughton, A. J., Hoover, K., and Felton, G. W. (2006). Impact of chemical elicitor applications on greenhouse tomato plants and population growth of the green peach aphid, *Myzus persicae. Entomol. Exp. Appl.* 120, 175–188.

Bruinsma, M., Ijdema, H., Van Loon, J. J. A., and Dicke, M. (2008). Differential effects of jasmonic acid treatment of *Brassica nigra* on the attraction of pollinators, parasitoids, and butterflies. *Entomol. Exp. Appl.* 128, 109–116.

Bruinsma, M., Posthumus, M. A., Mumm, R., Mueller, M. J., Van Loon, J. J. A., and Dicke, M. (2009). Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *J. Exp. Bot.* 60, 2575–2587.

Bruinsma, M., Van Dam, N. M., Van Loon, J. J., and Dicke, M. (2007). Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores. *J. Chem. Ecol.* 33, 655–668.

Chehab, E. W., Kaspi, R., Savchenko, T., Rowe, H., Negre-Zakharov, F., Kliebenstein, D., et al. (2008). Distinct roles of jasmonates and aldehydes in plant-defense responses. *PLoS ONE* 3:e1904. doi:10.1371/journal.pone.0001904

Cipollini, D. Jr., and Redman, A. (1999). Age-dependent effects of jasmonic acid treatment and wind exposure on foliar oxidase activity and insect resistance in tomato. *J. Chem. Ecol.* 25, 271–281.

Cipollini, D., Purrington, C. B., and Bergelson, J. (2003). Costs of induced responses in plants. *Basic Appl. Ecol.* 4, 79–89.

Cooper, W. R., and Goggin, F. L. (2005). Effects of jasmonate-induced defenses in tomato on the potato aphid, *Macrosiphum euphorbiae. Entomol. Exp. Appl.* 115, 107–115.

De Moraes, C. M., Mescher, M. C., and Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410, 577–580.

Denno, R. F., Peterson, M. A., Gratton, C., Cheng, J., Langellotto, G. A., Huberty, A. F., et al. (2000). Feeding-induced changes in plant quality mediate interspecific competition between sap-feeding herbivores. *Ecology* 81, 1814–1827.

Dicke, M., Gols, R., Ludeking, D., and Posthumus, M. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25, 1907–1922.

Dicke, M., and Vet, L. E. M. (1999). "Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore," in *Herbivores: Between Plants and Predators*, eds H. Olff, V. K. Brown, and R. H. Drent (Oxford: Blackwell Science Ltd), 483–520.

Dudareva, N., Klempien, A., Muhlemann, J. K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytol.* 198, 16–32.

Erbilgin, N., Krokene, P., Christiansen, E., Zeneli, G., and Gershenzon, J. (2006). Exogenous application of methyl jasmonate elicits defenses in norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus. Oecologia* 148, 426–436.

Farmer, E. E., Johnson, R. R., and Ryan, C. A. (1992). Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* 98, 995–1002.

Felton, G. W., and Korth, K. L. (2000). Trade-offs between pathogen and herbivore resistance. *Curr. Opin. Plant Biol.* 3, 309–314.

Georgi, L., Johnson-Cicalese, J., Honig, J., Das, S. P., Rajah, V. D., Bhattacharya, D., et al. (2013). The first genetic map of the American cranberry: exploration of synteny conservation and quantitative trait loci. *Theor. Appl. Genet.* 126, 673–692.

Gols, R., Posthumus, M. A., and Dicke, M. (1999). Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis. Entomol. Exp. Appl.* 93, 77–86.

Gols, R., Roosjen, M., Dijkman, H., and Dicke, M. (2003). Induction of direct and indirect plant responses by jasmonic acid, low spider mite densities, or a combination of jasmonic acid treatment and spider mite infestation. *J. Chem. Ecol.* 29, 2651–2666.

Hare, J. D. (2007). Variation in herbivore and methyl jasmonate-induced volatiles among genetic lines of *Datura wrightii. J. Chem. Ecol.* 33, 2028–2043.

Hare, J. D. (2011). Ecological role of volatiles produced by plants in response to damage by herbivorous insects. *Annu. Rev. Entomol.* 56, 161–180.

Heil, M. (2004). Induction of two indirect defences benefits lima bean (*Phaseolus lunatus*, fabaceae) in nature. *J. Ecol.* 92, 527–536.

Heil, M. (2010). Plastic defence expression in plants. *Evol. Ecol.* 24, 555–569.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometr. J.* 50, 346–363.

Karban, R., and Baldwin, I. T. (1997). *Induced Responses to Herbivory*. Chicago: University of Chicago Press.

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144.

Lichtenthaler, H. K., Rohmer, M., and Schwender, J. (1997). Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol. Plant.* 101, 643–652.

Lu, Y.-B., Liu, S.-S., Liu, Y.-Q., Furlong, M. J., and Zalucki, M. P. (2004). Contrary effects of jasmonate treatment of two closely related plant species on attraction of and oviposition by a specialist herbivore. *Ecol. Lett.* 7, 337–345.

Malo, E. A., Castrejón-Gómez, V. R., Cruz-López, L., and Rojas, J. C. (2004). Antennal 20 sensilla and electrophysiological response of male and female *Spodoptera frugiperda* 21 (Lepidoptera: Noctuidae) to conspecific sex pheromone and plant odors. *Ann. Entomol. Soc. Am.* 97, 1273–1284.

McCallum, E. J., Cunningham, J. P., Lucker, J., Zalucki, M. P., De Voss, J. J., and Botella, J. R. (2011). Increased plant volatile production affects oviposition, but not larval development, in the moth *Helicoverpa armigera. J. Exp. Biol.* 214, 3672–3677.

Meng, Z. J., Yan, S. C., Yang, C. P., Jin, H., and Hu, X. O. (2011). Behavioural responses of *Dendrolimus superans* and *Anastatus japonicus* to chemical defences induced by application of jasmonic acid on larch seedlings. *Scand. J. Forest Res* 26, 53–60.

Omer, A. D., Granett, J., Karban, R., and Villa, E. M. (2001). Chemically-induced resistance against multiple pests in cotton. *Int. J. Pest Manag.* 47, 49–54.

Omer, A. D., Thaler, J. S., Granett, J., and Karban, R. (2000). Jasmonic acid induced resistance in grapevines to a root and leaf feeder. *J. Econ. Entomol.* 93, 840–845.

Ozawa, R., Shiojiri, K., Sabelis, M. W., Arimura, G., Nishioka, T., and Takabayashi, J. (2004). Corn plants treated with jasmonic acid attract more specialist parasitoids, thereby increasing parasitization of the common armyworm. *J. Chem. Ecol.* 30, 1797–1808.

Paré, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–332.

Price, P. W., Bouton, C. E., Gross, P., McPheron, B. A., Thompson, J. N., and Weis, A. E. (1980). Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11, 41–65.

R Development Core Team. (2012). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. ISBN 3-900051-07-0. Available at: URL http://www.R-project.org/

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W., and McDade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination 'syndromes' in *Nicotiana. Phytochemistry* 63, 265–284.

Rodriguez-Saona, C. (2011). Herbivore-induced blueberry volatiles and intra-plant signaling. *J. Vis. Exp.* 58, e3440.

Rodriguez-Saona, C., Blaauw, D. R., and Isaacs, R. (2012). "Manipulation of natural enemies in agroecosystems: Habitat and semiochemicals for sustainable insect pest control," in *Integrated Pest Management and Pest Control – Current and Future Tactics*, eds M. L. Larramendy and S. Soloneski (Rijeka: In Tech), 89–126.

Rodriguez-Saona, C., Crafts-Brandner, S. J., Paré, P. W., and Henneberry, T. J. (2001). Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27, 679–695.

Rodriguez-Saona, C., Poland, T. M., Miller, J. R., Stelinski, L. L., Grant, G. G., De Groot, P., et al. (2006). Behavioral and electrophysiological responses of the emerald ash borer, *Agrilus planipennis*, to induced

volatiles of Manchurian ash, *Fraxinus mandshurica*. *Chemoecology* 16, 75–86.

Rodriguez-Saona, C., Vorsa, N., Singh, A., Johnson-Cicalese, J., Szendrei, Z., Mescher, M., et al. (2011). Tracing the history of plant traits under domestication in cranberries: potential consequences on anti-herbivore defences. *J. Exp. Bot.* 62, 2633–2644.

Rohwer, C. L., and Erwin, J. E. (2008). Horticultural applications of jasmonates: a review. *J. Hortic. Sci. Biotech.* 83, 283–304.

Rohwer, C. L., and Erwin, J. E. (2010). Spider mites (*Tetranychus urticae*) perform poorly on and disperse from plants exposed to methyl jasmonate. *Entomol. Exp. Appl.* 137, 143–152.

Scheiner, S. M. (2001). "MANOVA: multiple response variables and multispecies interactions," in *Design and Analysis of Ecological Experiments*, 2nd Edn, eds S. M. Scheiner and J. Gurevitch (New York: Oxford University Press), 99–155.

Schmelz, E. A., Alborn, H. T., and Tumlinson, J. H. (2003). Synergistic interactions between volicitin, jasmonic acid and ethylene mediate insect-induced volatile emission in *Zea mays*. *Physiol. Plant.* 117, 403–412.

Senthil-Nathan, S., Kalaivani, K., Choi, M.-Y., and Paik, C.-H. (2009). Effects of jasmonic acid-induced resistance in rice on the plant brownhopper, *Nilaparvata lugens* Stål (Homoptera: Delphacidae). *Pestic. Biochem. Physiol.* 95, 77–84.

Staswick, P. E., and Lehman, C. C. (1999). "Jasmonic acid-signaled responses in plants," in *Inducible Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture*, eds A. A. Agrawal, S. Tuzun, and E. Bent (St. Paul: APS Press), 117–136.

Suckling, D. M., Karg, G., Gibb, A. R., and Bradley, S. J. (1996). Electroantennogram and oviposition responses of *Epiphyas postvittana* (Lepidoptera: Tortricidae) to plant volatiles. *New Zeal. J. Crop Hort. Sci.* 24, 323–333.

Tan, C.-W., Chiang, S.-Y., Ravuiwasa, K., Yadav, J., and Hwang, S.-Y. (2012). Jasmonate-induced defenses in tomato against *Helicoverpa armigera* depend in part on nutrient availability, but artificial induction via methyl jasmonate does not. *Arthropod Plant Interact* 6, 531–541.

Thaler, J., Stout, M., Karban, R., and Duffey, S. (1996). Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22, 1767–1781.

Thaler, J. S. (1999a). "Jasmonic acid mediated interactions between plants, herbivores, parasitoids, and pathogens: a review of field experiments in tomato," in *Inducible Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture*, eds A. A. Agrawal, S. Tuzun, and E. Bent (St. Paul: APS Press), 319–334.

Thaler, J. S. (1999b). Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399, 686–688.

Thaler, J. S. (1999c). Induced resistance in agricultural crops: effects of jasmonic acid on herbivory and yield in tomato plants. *Environ. Entomol.* 28, 30–37.

Thaler, J. S. (2002). Effect of jasmonate-induced plant responses on the natural enemies of herbivores. *J. Anim. Ecol.* 71, 141–150.

Thaler, J. S., Stout, M. J., Karban, R., and Duffey, S. S. (2001). Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecol. Entomol.* 26, 312–324.

Tholl, D., and Röse, U. S. R. (2006). "Detection and identification of floral scent compounds," in *Biology of Floral Scent*, eds N. Dudareva and E. Pichersky (Boca Raton: CRC Press), 3–25.

Turlings, T. C. J., Tumlinson, J. H., and Lewis, W. J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250, 1251–1253.

van Poecke, R. M. P., and Dicke, M. (2002). Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway. *J. Exp. Bot.* 53, 1793–1799.

Van Zandt, P. A., and Agrawal, A. A. (2004). Community-wide impacts of herbivore-induced plant responses in milkweed (*Asclepias syriaca*). *Ecology* 85, 2616–2629.

Vet, L. E. M., and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37, 141–172.

Viswanathan, D. V., Narwani, A. J. T., and Thaler, J. S. (2005). Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. *Ecology* 86, 886–896.

Walling, L. L. (2000). The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19, 195–216.

Warabieda, W., and Olszak, R. (2010). Effect of exogenous methyl jasmonate on numerical growth of the population of the two-spotted spider mite (*Tetranychus urticae* Koch.) on strawberry plants and young apple trees. *J. Plant Protect. Res.* 50, 541–544.

Zheng, S.-J., and Dicke, M. (2008). Ecological genomics of plant-insect interactions: from gene to community. *Plant Physiol.* 146, 812–817.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 February 2013; accepted: 11 April 2013; published online: 29 April 2013.

Citation: Rodriguez-Saona CR, Polashock J and Malo EA (2013) Jasmonate-mediated induced volatiles in the American cranberry, Vaccinium macrocarpon: from gene expression to organismal interactions. Front. Plant Sci. ***4****:115. doi: 10.3389/fpls.2013.00115*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Rodriguez-Saona, Polashock and Malo. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

HYPOTHESIS AND THEORY ARTICLE
published: 23 July 2013
doi: 10.3389/fpls.2013.00262

Quantitative patterns between plant volatile emissions induced by biotic stresses and the degree of damage

Ülo Niinemets[1]*, Astrid Kännaste[1] and Lucian Copolovici[1,2]

[1] Estonian University of Life Sciences, Tartu, Estonia
[2] Institute of Technical and Natural Sciences Research-Development, Aurel Vlaicu University, Arad, Romania

Edited by:
Mary Beth Mudgett, Stanford University, USA

Reviewed by:
Ted Turlings, University of Neuchâtel, Switzerland
Violeta Velikova, Bulgarian Academy of Sciences, Bulgaria

***Correspondence:**
Ülo Niinemets, Estonian University of Life Sciences, Kreutzwaldi 1, 51014 Tartu, Estonia
e-mail: ylo.niinemets@emu.ee

Plants have to cope with a plethora of biotic stresses such as herbivory and pathogen attacks throughout their life cycle. The biotic stresses typically trigger rapid emissions of volatile products of lipoxygenase (LOX) pathway (LOX products: various C_6 aldehydes, alcohols, and derivatives, also called green leaf volatiles) associated with oxidative burst. Further a variety of defense pathways is activated, leading to induction of synthesis and emission of a complex blend of volatiles, often including methyl salicylate, indole, mono-, homo-, and sesquiterpenes. The airborne volatiles are involved in systemic responses leading to elicitation of emissions from non-damaged plant parts. For several abiotic stresses, it has been demonstrated that volatile emissions are quantitatively related to the stress dose. The biotic impacts under natural conditions vary in severity from mild to severe, but it is unclear whether volatile emissions also scale with the severity of biotic stresses in a dose-dependent manner. Furthermore, biotic impacts are typically recurrent, but it is poorly understood how direct stress-triggered and systemic emission responses are silenced during periods intervening sequential stress events. Here we review the information on induced emissions elicited in response to biotic attacks, and argue that biotic stress severity vs. emission rate relationships should follow principally the same dose–response relationships as previously demonstrated for different abiotic stresses. Analysis of several case studies investigating the elicitation of emissions in response to chewing herbivores, aphids, rust fungi, powdery mildew, and *Botrytis*, suggests that induced emissions do respond to stress severity in dose-dependent manner. Bi-phasic emission kinetics of several induced volatiles have been demonstrated in these experiments, suggesting that next to immediate stress-triggered emissions, biotic stress elicited emissions typically have a secondary induction response, possibly reflecting a systemic response. The dose–response relationships can also vary in dependence on plant genotype, herbivore feeding behavior, and plant pre-stress physiological status. Overall, the evidence suggests that there are quantitative relationships between the biotic stress severity and induced volatile emissions. These relationships constitute an encouraging platform to develop quantitative plant stress response models.

Keywords: biotic stress, green leaf volatiles, fungal infection, herbivory, quantitative stress dose–response relationships, volatile organic compounds

INTRODUCTION

Plants as sedentary organisms cannot escape from attackers and stressors and have to adjust to surrounding environment and biotic attacks through their life cycle. During evolution, plants have evolved various defense strategies, including release of volatile organic compounds (VOCs) from their above-ground organs (Zhang et al., 1999; Zhang and Schlyter, 2004; Huang et al., 2012; Fineschi et al., 2013) into the ambient atmosphere, and even from roots into the soil air space and water (Hiltpold et al., 2011; Turlings et al., 2012). Numerous VOCs have been described, which nevertheless belong to a few broad compound classes, including volatile isoprenoids, volatile products of shikimic acid pathway (phenylpropanoids, benzenoids, indole), carbohydrate and fatty acid cleavage products (**Figure 1** for some examples of characteristic volatiles released from plants and **Figure 2** for their biosynthetic pathways (Knudsen et al., 1993; Dudareva et al., 2006; Qualley and Dudareva, 2008; Dicke and Baldwin, 2010; Fineschi et al., 2013). In a few cases, specialized volatiles such as sulfur-containing glucosinolate cleavage products in Brassicales and Malpighiales and furanocoumarins and their derivatives in Apiales, Asterales, Fabales, Rosales, and Sapindales are produced (Berenbaum and Zangerl, 2008; Agrawal, 2011).

In most cases, plant odors are complex mixtures of compounds, reflecting upregulation of multiple pathways, and synthesis of multiple compounds within given pathway. Due to significant differences in physico-chemical characteristics of VOCs within and among the different compound classes (Niinemets et al., 2004), the release kinetics, compound life-time in the ambient atmosphere and uptake by neighboring vegetation strongly vary (Baldwin et al., 2006; Arneth and Niinemets, 2010; Holopainen et al., 2013).

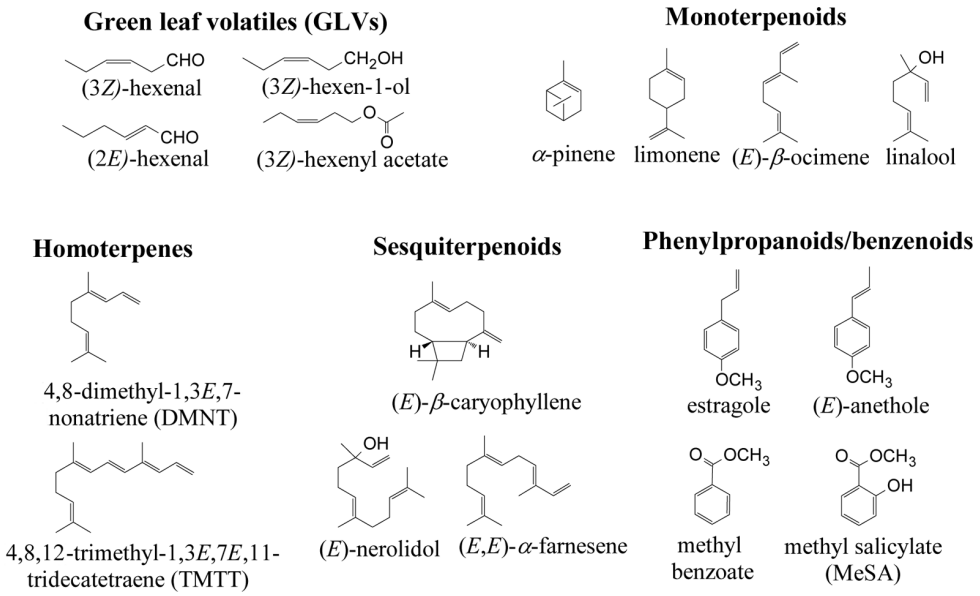


FIGURE 1 | Molecular structures of selected plant volatiles (BVOC) emitted in response to a variety of stress factors. Green leaf volatiles (GLV), also called volatiles of lipoxygenase pathway (LOX) are formed via the lipoxygenase pathway and constitute the ubiquitous stress response (Hatanaka et al., 1978; Hatanaka, 1993; Howe and Schaller, 2008). Terpenoids comprise the largest class of plant secondary metabolites. Various terpenoids are emitted in several constitutive emitters, and emissions of specific terpenoids are elicited in response to different stresses (Degenhardt et al., 2009; Llusià et al., 2010, 2013; Loreto and Schnitzler, 2010). Emissions of benzenoids and phenylpropanoids have been less investigated, but the constitutive emissions of these compounds are often characteristic to flowers, and sometimes to leaves (Gang et al., 2001; Dudareva et al., 2004; Yang et al., 2009; Zhao et al., 2010). Furthermore, methyl salicylate is a characteristic stress-induced volatile (Karl et al., 2008; Zhao et al., 2010), and there is evidence that methyl benzoate may also be released in response to stress (Zhao et al., 2010). Different biochemical pathways are responsible for synthesis of different compound classes (**Figure 2**).

Plant volatile emissions can be constitutive or they can be induced in response to a variety of stresses. Independent of the way of emission, airborne volatiles are thought to be involved in defense reactions elicited by herbivores, pathogens, and even against abiotic stress factors (Dicke and Baldwin, 2010; Fineschi et al., 2013; Possell and Loreto, 2013). These defense responses can be either direct or indirect. In the case of direct responses, emitted volatiles themselves participate in defense or in stress tolerance (Martin and Bohlmann, 2005; Vickers et al., 2009). In the case of indirect defenses, volatiles released serve as infochemical signals eliciting systemic responses within the plant and/or neighboring plants and/or they serves as cues attracting enemies of herbivores (Dicke et al., 1999; Halitschke et al., 2000; Dicke and Bruin, 2001; Heil and Kost, 2006; Heil and Silva Bueno, 2007).

Induced plant defense triggered by plant hormones (Fäldt et al., 2003; Kappers et al., 2010), herbivores (Arimura et al., 2005, 2011), or pathogen attacks (Jansen et al., 2009; Kant et al., 2009; Toome et al., 2010) has been extensively studied. However, much of the work on plant defense responses, in particular on biotic stress responses, has been non-quantitative. Stress-driven plant VOC emission responses have rarely been characterized in relation to the severity of the stress. Yet, this is relevant because mild vs. severe stress might qualitatively alter plant response, either leading to stress priming and adaptation or to hypersensitive response (Heil and Kost, 2006; Frost et al., 2008a; Niinemets, 2010). Quantitative patterns among stress severity and VOC release have been demonstrated for several abiotic stresses including ozone stress (Beauchamp et al., 2005), heat (Karl et al., 2008; Copolovici et al., 2012) and frost stress (Copolovici et al., 2012) and stress induced by diffusely dispersed environmental pollutants such as textile colorants (Copaciu et al., 2013) and antibiotics' residues (Opriş et al., 2013).

Studies of VOC emissions triggered by biotic stresses have been mostly investigated qualitatively (but see e.g., Gouinguené et al., 2003; Schmelz et al., 2003a,b; Copolovici et al., 2011). This reflects the focus of plant–herbivore interactions research on overall stress patterns elicited by severe or moderately severe stress. This research has often been driven by the question of how the elicited compounds participate in communication at different trophic levels. In studies focused on plant responses, lack of quantitative investigations might be related to difficulties in characterizing the severity of biotic stress, and to presence of multiple confounding effects that can result from genotypic differences, plant physiological status, and interactions with environmental drivers. As in the nature plants are under continuous pressure of biotic stresses of differing severity, we argue that the overall lack of quantitative stress dose vs. plant response studies is an important shortcoming. Without knowing the stress dose vs. plant response patterns, plant stress responses in the field under strongly fluctuating stress levels cannot be predicted.

In this paper, we first analyze general patterns of constitutive and induced emissions to clearly define what we consider as an induced emission response and analyze how both types of emissions can benefit plants. Then we analyze mechanisms

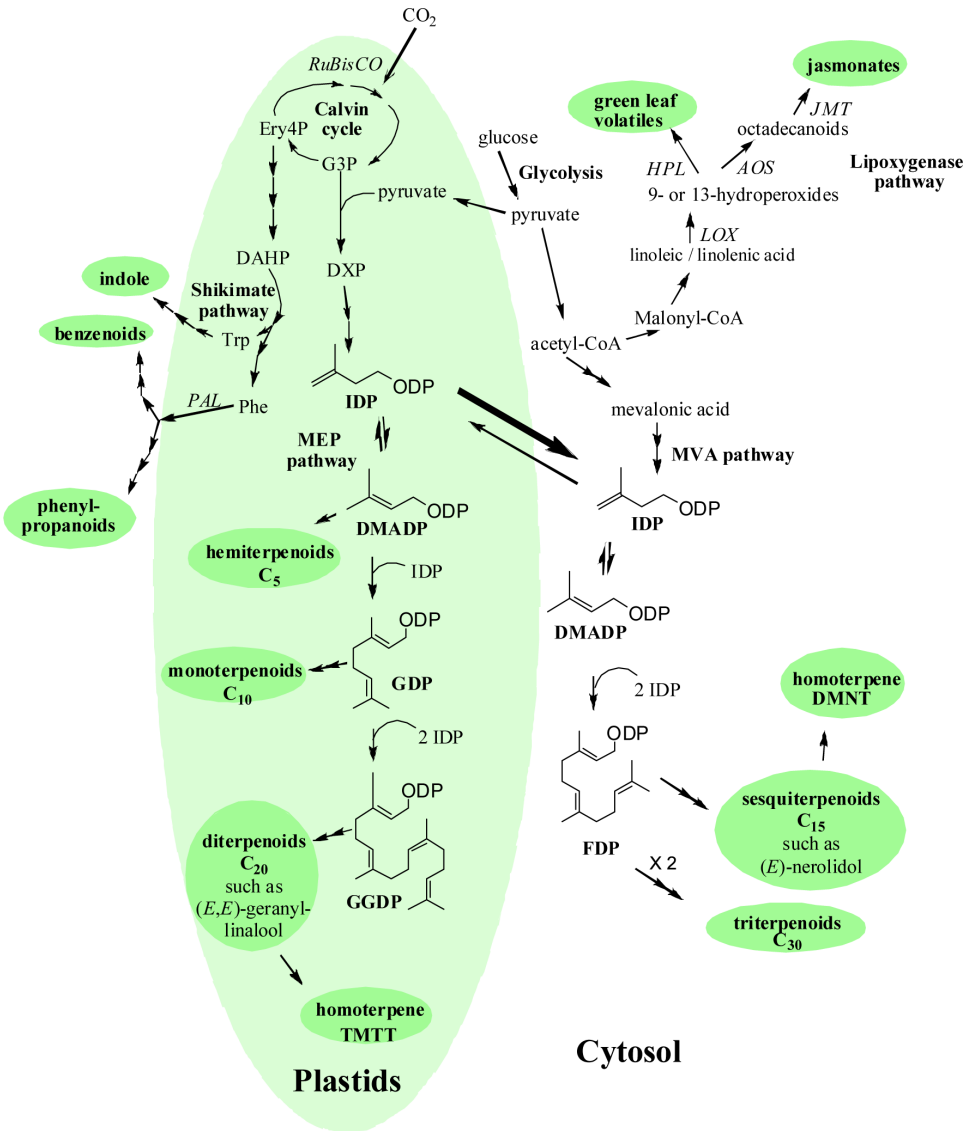


FIGURE 2 | Simplified scheme of the interactions among the biosynthetic pathways responsible for volatile and non-volatile stress metabolites in plants. Pathway names are in italics, volatile compound classes are in bold font inside ellipses, and the key enzymes involved in the biosynthetic pathways are next to the arrows in italics. Abbreviations: acetyl-CoA, acetyl coenzyme A; *AOS*, allene oxide synthase; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DMADP, dimethylallyl diphosphate; DMNT, 4,8-dimethyl-1,3*E*,7-nonatriene; DXP, 1-deoxy-D-xylulose 5-phosphate; Ery4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FDP, farnesyl diphosphate; G3P, glyceraldehyde-3-phosphate; GGDP, geranylgeranyl diphosphate; GDP, geranyl diphosphate; HPL, fatty acid hydroperoxide lyases; IDP, isopentenyl diphosphate; JMT, jasmonic acid carboxyl methyl transferase; LOX, lipoxygenase; MEP-pathway, methylerythritol 4-phosphate pathway; MVA, mevalonic acid; PAL, phenylalanine ammonia lyase; PEP, phosphoenolpyruvate; Phe, phenylalanine; TMTT, 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene. The lipoxygenase pathway starts with the dehydrogenation of linolenic and linoleic acids at C_9 or C_{13} position by lipoxygenases forming 9-hydroperoxy and 13-hydroperoxy derivates of polyenic acids (Hatanaka, 1993; Howe and Schaller, 2008). These compounds are further cleaved by hydroperoxide lyases into oxoacids and C_6-aldehydes. These aldehydes can be converted into the corresponding alcohols by alcohol dehydrogenases (Hatanaka et al., 1978; Hatanaka, 1993; Dudareva et al., 2006). Terpenoids originate from isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMDP), which are synthesized via two different pathways. The cytosolic mevalonate (MVA) pathway starts with the formation of acetoacetyl-CoA (Dewick, 1999), while the plastidial 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) starts with condensation of pyruvate and glyceraldehyde-3-phosphate (Lichtenthaler et al., 1997; Rohmer, 1999). The route in plastids provides precursors for the biosynthesis of isoprene, mono-, and diterpenes, while the cytosol-localized pathway for sesqui- and triterpenes. Precursors of terpenes have been experimentally demonstrated to be transported from plastids to the cytosol (Dudareva et al., 2005; Bartram et al., 2006), referred to as the "cross-talk" between the MEP- and MVA-pathways (for recent reviews on terpenoid synthesis see Li and Sharkey, 2013; Rajabi Memari et al., 2013; Rosenkranz and Schnitzler, 2013). Aromatic volatiles are formed via the shikimic acid pathway, starting by condensation of erythrose 4-phosphate and PEP. After numerous steps via 3-dehydroshikimic acid and chorismate, phenylalanine (Phe) is produced. Phe is further converted to *trans*-cinnamic acid by phenylalanine ammonia lyase. *Trans*-cinnamic acid is a starting point for the synthesis of phenylpropanoids, (e.g., phenylethanol, phenylethylbenzoate) and benzenoids (benzaldehyde, methyl benzoate, methyl salicylate etc.; Boatright et al., 2004; Dudareva et al., 2006). Additionally, tryptophan (Trp), which is the precursor of volatile indole, is biosynthesized via shikimic acid pathway (Paré and Tumlinson, 1996) in chloroplast, while indole itself is synthesized in cytosol (Zhang et al., 2008).

of immediate stress-elicited emissions and systemic responses, review several case studies asking whether biotic stress severity and plant volatile emission responses are quantitatively related, and finally consider some of the difficulties complicating interpretation of stress severity vs. plant emission responses.

ROLE OF CONSTITUTIVE EMISSIONS OF VOLATILE ORGANIC COMPOUNDS IN PLANT DEFENSE REACTIONS

Constitutive emissions are present in all species storing volatiles in specialized storage tissues, e.g., resin ducts in conifers and glandular trichomes in Labiatae. There is continuous release of these volatiles from the storage structures determined by the rate of compound diffusion, and thus, mainly driven by the compound volatility and temperature (for recent reviews see Monson et al., 2012; Grote et al., 2013). In addition, several widespread species lacking specialized storage compartments synthesize volatile isoprenoids, in particular, isoprene or/and monoterpenes, in light- and temperature-dependent manner (for reviews see Grote et al., 2013; Li and Sharkey, 2013; Monson, 2013).

Constitutive emissions occur both during periods when plants do not experience stress, and when they do. However, emission rates of constitutively released compounds can acclimate to variations in environmental conditions, in particular, to average light and temperature, over days to weeks preceding the sampling (Niinemets et al., 2010a; Monson, 2013). Environmental and biotic stress can also alter the rate of constitutive emissions, either increasing or reducing the emission rates depending on stress severity and duration, and plant ontogenetic status (Niinemets et al., 2010a; Monson, 2013; Possell and Loreto, 2013). In the following, we analyze the ways by which constitutive emissions can increase plant resistance to environmental and biotic stresses.

PROTECTION BY NON-STORED VOLATILES

Non-stored constitutively released volatiles can directly participate in abiotic defenses by stabilizing membranes and serving as antioxidants (Sharkey et al., 2008; Chen et al., 2009; Vickers et al., 2009; Possell and Loreto, 2013). The synthesized volatiles partition to leaf liquid and lipid phases according to their equilibrium partition coefficients (Niinemets and Reichstein, 2002; Niinemets et al., 2004; Niinemets et al., 2010b). Lipid solubilization of hydrophobic volatiles possibly enhances lipid–lipid and lipid–protein interactions in membranes at higher temperatures (Sharkey et al., 2008; Vickers et al., 2009; Possell and Loreto, 2013), thereby increasing plant tolerance to elevated temperatures (Sharkey and Singsaas, 1995; Loreto et al., 1998; Copolovici et al., 2005). Enhancement of thermal tolerance has been first demonstrated for isoprene (Sharkey and Singsaas, 1995; Singsaas et al., 1997) and then for monoterpenes (Loreto et al., 1998; Copolovici et al., 2005; Llusià et al., 2005). However, not all monoterpenes appear to be equally effective (Copolovici et al., 2005).

Due to their antioxidative characteristics, solubilized volatiles can also quench stress-generated reactive oxygen species (ROS), production of which becomes enhanced during thermal stress, but also during many other abiotic stresses such as ozone stress (Sharkey et al., 2008; Vickers et al., 2009; Possell and Loreto, 2013). The protective effect of volatile isoprenoids can be particularly relevant under drought when stomata close, resulting in elevated leaf temperatures due to reduced transpiratory cooling of leaves. These are also the conditions that lead to a major buildup of volatiles inside the leaves (Sharkey and Singsaas, 1995; Singsaas et al., 1997).

Apart from involvement in abiotic stress tolerance, constitutively released non-stored volatiles can play an important role in host plant selection by herbivores as well as possible deterrents for herbivores (Zhang et al., 1999; Pichersky and Gershenzon, 2002; Degenhardt et al., 2003; Xugen and Luqin, 2006; Loivamäki et al., 2008; Brilli et al., 2009).

DEFENSES CONFERRED BY STORED VOLATILES

Due to their toxicity, release of compounds stored in specialized storage compartments is known to deter and reduce the feeding activity of herbivores and inhibit biological activity of pathogens (Popp et al., 1995; Ward et al., 1997; Litvak and Monson, 1998; Baier et al., 2002). The emissions of stored volatiles may also serve as important signals in host plant selection (Kelsey and Joseph, 1997; Mita et al., 2002). Involvement of constitutive storage emissions in protecting from abiotic stresses has not been demonstrated, although due to continuous emission, a certain, relatively high, vapor pressure of storage volatiles is maintained in leaf intercellular air space. Depending on compound physicochemical characteristics (Niinemets et al., 2004; Harley, 2013), the vapor pressure supported by storage emissions can result in equilibrium compound concentrations in leaf liquid and lipid phases that are comparable to those observed for non-storage emissions of isoprene and monoterpenes. This suggests that emissions from storage structures can fulfill analogous functions in abiotic stress tolerance as the constitutive emissions in species lacking the storage.

INDUCED VOLATILES IN PLANT DEFENSE RESPONSES: FROM QUALITATIVE TO QUANTITATIVE PATTERNS

While only certain plant species are constitutive emitters, all plant species typically respond to stress by triggering emissions of a variety of characteristic stress volatiles (**Figure 2**, Paré and Tumlinson, 1999; Kessler and Baldwin, 2001; Loreto and Schnitzler, 2010; Niinemets, 2010). Here we briefly consider what are induced emissions, what is emitted, what is the biological role of induced emissions, and by which mechanisms induced emissions could be coupled to stress severity in a dose-dependent manner.

DEFINITION OF INDUCED EMISSIONS

Differently from constitutive emissions, emissions of stress volatiles during periods intervening stress events are only present at very low background levels, often close to the detection limit of analytical systems (e.g., Toome et al., 2010; Copolovici et al., 2011, 2012). Stress leads to amplification of these emissions by several orders of magnitude (Turlings et al., 2004), and after stress relief, the emissions again decrease to the background level (Copolovici et al., 2011; Karban, 2011). Yet, relaxation of emissions after stress typically takes longer than elicitation (Degenhardt and Lincoln, 2006; Karban, 2011). The main difference among constitutive and

induced emissions is not whether or whether not different types of emissions respond to stress. Both types of emissions are stress-responsive, but the stress sensitivity of constitutive and induced emissions is very different, and the level of emission under non-stressed conditions is also different. Detectable induced emissions are only present during stress and during the relaxation period after stress.

We emphasize that induced emissions are generally understood as stress-driven emissions of *de novo* synthesized volatiles (Paré and Tumlinson, 1997; Niinemets et al., 2010b). In constitutively emitting species storing volatiles in specialized compartments, wounding due to herbivory may break the storage compartments, resulting in emission bursts of the stored compounds (e.g., Loreto et al., 2000; Danielsson et al., 2008). Strictly speaking, these emissions should not be called "induced" emissions as wounding results in major enhancement of diffusion of already synthesized compounds rather than a physiological response.

WHAT COMPOUNDS ARE INDUCED?

Various compound classes are induced with differing kinetics, reflecting different emission mechanisms. Induced emissions of some compounds such as green leaf volatiles (volatile products of lipoxygenase (LOX) reaction, LOX products), are emitted within minutes after the start of stress (e.g., Loreto et al., 2006), and reflect activation of already available enzymatic apparatus. In the case of LOX volatiles, rapid emissions are triggered by the release of free fatty acids from cell membranes, and their peroxidation by LOX enzymes (Feussner and Wasternack, 2002; Liavonchanka and Feussner, 2006). In addition, initial stress responses lead to activation of a large number of genes, several of which are responsible for the biosynthesis of defensive plant volatiles, including characteristic monoterpenoids, sesquiterpenoids, and homoterpenes. As the result, emissions of these stress volatiles are induced in hours to days after the start of the sustained stress or following a single stress event (Beauchamp et al., 2005; Copolovici and Niinemets, 2010; Copolovici et al., 2011). (*E*)-β-Ocimene, linalool, methyl salicylate (MeSA), indole, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene and homoterpenes 4,8-dimethyl-1,3*E*,7-nonatriene (DMNT) and 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene (TMTT) are characteristic stress compounds in various plant species (**Figure 1**; Paré and Tumlinson, 1999; Frey et al., 2000; Vuorinen et al., 2007; Toome et al., 2010; Copolovici et al., 2011, 2012; Zhuang et al., 2012). For example in our previous studies, next to LOXpathway products (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-hexanol, and (*Z*)-3-hexenyl acetate, feeding of foliage of temperate deciduous tree *Alnus glutinosa* by larvae of the geometrid moth *Cabera pusaria* induced also the emission of a homoterpene DMNT and a sesquiterpene (*E*,*E*)-α-farnesene (Copolovici et al., 2011), which are not released by the foliage of non-stressed *Alnus glutinosa* (Lindfors et al., 2000).

INFORMATION CARRIED BY INDUCED VOLATILES

Timing, amount, and composition of stress-triggered emissions can carry information about the emitting species, and type of the stress, while timing and amount can reflect the severity of the stress (Llusià et al., 2002; Beauchamp et al., 2005; Niinemets, 2010; Joó et al., 2011; Copolovici et al., 2012; Fatouros et al., 2012). Thus, induced emissions are thought to serve primarily as infochemicals. LOX-compounds released rapidly after stress are known to serve as "messenger-compounds" in plant–plant communication (Shulaev et al., 1997; Arimura et al., 2001; Farag and Paré, 2002) or in triggering systemic response (Farag and Paré, 2002; Park et al., 2007) that can result in triggering volatile emissions in non-stressed leaves of the same plant and in neighboring plants (**Figure 3**, Röse et al., 1996; Halitschke et al., 2000; Heil and Silva Bueno, 2007; Staudt and Lhoutellier, 2007; Dicke and Baldwin, 2010; Peng et al., 2011). Other rapidly elicited volatiles can also potentially serve as messengers (Paré et al., 2005). These primary airborne messengers can further elicit secondary "messengers" such as jasmonic acid or salicylic acid migrating in liquid phase through phloem to the tissues distant from the stressed ones activating defense genes (Park et al., 2007). For example, in lima bean (*Phaseolus lunatus*) plants infested by spider mites (*Tetranychus urticae*), released volatiles activated multifunctional signaling cascades involving the ethylene and jasmonic acid signaling (Arimura et al., 2002).

The question, however, is how informative are the LOX product emissions triggered by various stresses. Plants have multiple LOXs and fatty acid hydroperoxide lyases (Feussner and Wasternack, 2002), and once synthesized, C_6 aldehydes can further be chemically modified resulting in formation of alcohols and esters, collectively generating variations in the emission profiles. The composition of emitted LOX products can be similar for different stresses such as caused by mechanical wounding, herbivory, heat, and frost stress even for different species (Brilli et al., 2011; Copolovici et al., 2011, 2012). Yet, it has been recently demonstrated that herbivores can isomerize LOX products, resulting in different emission blends for mechanical wounding and herbivory and altered attractiveness to predators (Allmann and Baldwin, 2010).

While there is broad evidence of convergent responses for different stresses, in reed (*Phragmites australis*), it was demonstrated that heat stress resulted only in the release of (*E*)-2-hexenal, while wounding caused emissions of (Z)-3-hexenal, (*Z*)-3-hexenol, and (*E*)-2-hexenol (Loreto et al., 2006). Moreover, Bai et al. (2011) demonstrated that even chilling or heating can activate LOX pathway differently. The generality of such changes in emission profiles, and the capacity of different LOX volatiles in eliciting systemic responses clearly need further experimental work.

INDUCED EMISSIONS AS DIRECT DEFENSES

Emissions of isoprenoids triggered in response to stresses are chemically similar to volatiles released in constitutive emitters and could potentially also be involved in direct defense, e.g., serving as antioxidants quenching the ROS formed in plants under stress. Given that the induced emissions are at maximum level when the stress and ROS formation are the greatest, involvement of induced isoprenoids in direct defense is plausible. Such a role could be particularly relevant for the emissions induced by abiotic stresses that likely play a less prominent role in multitrophic signaling. There is currently no evidence of the involvement of induced emissions in direct defense against abiotic stresses, although such a role would be compatible with stress dose-dependent emissions of induced volatiles. However, induced emissions have been demonstrated to serve as repellents of herbivores (Bernasconi et al., 1998).

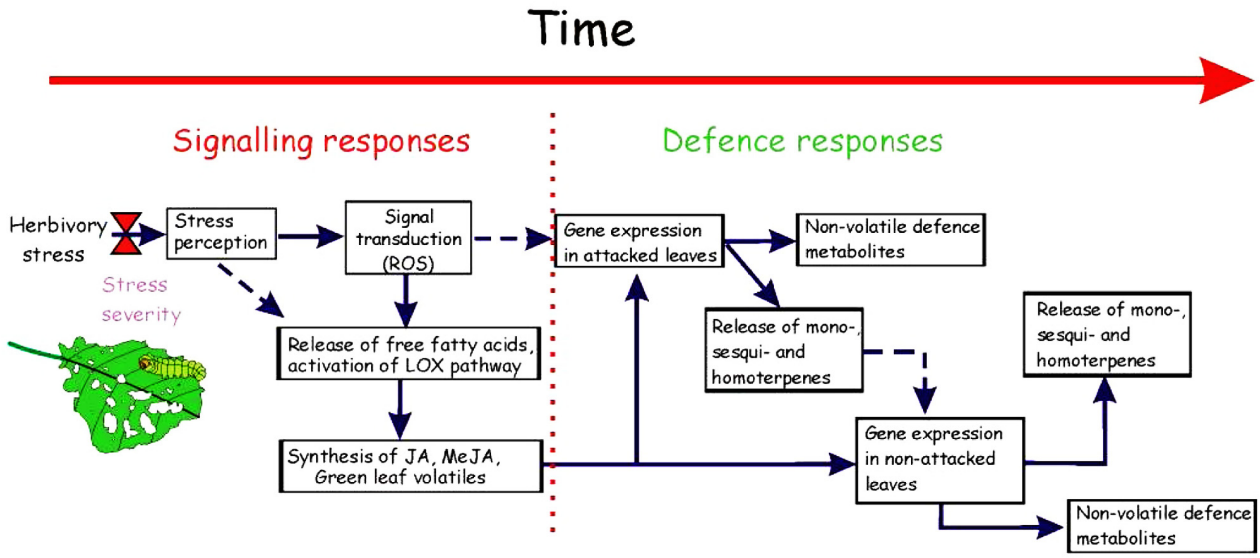


FIGURE 3 | Flow path of time-dependent herbivory-driven signaling and defense responses. Herbivory damage leads to a rapid, within minutes, oxidative burst and release of free fatty acids from plant membranes in the immediate location of damage (Feussner and Wasternack, 2002; Maffei et al., 2007; Arimura et al., 2011; Spinelli et al., 2011). This leads to activation of lipoxygenase pathway that results in release of green leaf volatiles (a variety of C_6 aldehydes) and synthesis of jasmonate and methyl jasmonate (Feussner and Wasternack, 2002). Sometimes, depending on attacking organism, the early signaling responses also include ethylene and methyl salicylate (Maffei et al., 2007; Mithöfer and Boland, 2008; Arimura et al., 2011). Further cascade of events includes activation of defense gene expression that leads to synthesis of a variety of volatile isoprenoids and also production of non-volatile defense compounds such as polyphenols. Gene expression patterns may be directly elicited by reactive oxygen species (ROS) dependent activation of MAP kinases, but these responses more commonly include activation of hormonal pathways (Arimura et al., 2011). Volatile and non-volatile phytohormones released by attacked leaves can elicit defense gene expression in non-attacked leaves (systemic response; Maffei et al., 2007; Mithöfer and Boland, 2008; Arimura et al., 2011). Uncertain or less frequent paths are shown by dashed arrows.

MECHANISMS BY WHICH PLANT DEFENSE RESPONSES CAN BE QUANTITATIVELY MODULATED BY STRESS SEVERITY

The sequence of events leading from initial stress response to release of early stress volatiles, activation of gene responses and specific secondary metabolic pathways, and ultimately to elicitation of emissions of "late" stress-specific volatiles and systemic responses has been studied intensively (Byers et al., 2000; Arimura et al., 2005, 2011; Dudareva et al., 2006; Kant et al., 2009). Nevertheless, there are still significant uncertainties in how the stress signal is received, transduced, and amplified (Niinemets, 2010). While abiotic stresses typically impact the entire plant, the entire organ or multiple organs, biotic stress is characteristically more localized. For instance, depending on species, chewing herbivores start feeding at the margins or form perforations and skeletonized spots within the lamina, while sap-sucking insects typically attack the phloem in the veins. The spread of the damage from the initial localized damage site(s) increases during the course of feeding and depends on the number of insects attacking simultaneously the leaf. Analogously, in plant–pathogen interactions, pathogen spores dispersed by water, wind, or by insects settle on a plant and form hydrophobic interactions with the waxy polymers on leaf surface. Ultimately, the airborne pathogen enters the leaf intracellular space via stomata (El Omari et al., 2001; Prats et al., 2007). The density of pathogen propagules determines the number of stomatal entry points within the given leaf, but the initial response remains characteristically localized unless the pathogen density is very high. Thus, in the case of biotic stresses, the stress severity often increases in time and in spatial coverage.

The key question is how the initial stress localized in the impacted area of the leaf is sensed by the plant and to what extent the stress response is affecting neighboring non-impacted areas and surrounding non-impacted leaves. The other important question is how the overall stress response is associated with the total impacted area (stress dose). In the case of herbivory by chewing insects, chewing damage, i.e., rupture of cell walls, breakage of cellular membranes, and exposure of cell contents to ambient environment, itself can elicit activation of LOX pathway and release of LOX volatiles that can serve as signals for subsequent stress responses (**Figure 3**, Maffei et al., 2007; Howe and Schaller, 2008; Mithöfer and Boland, 2008). There is also evidence that insect-driven elicitors such as β-glucosidase (Mattiacci et al., 1995) or fatty-acid conjugate such as volicitin (Alborn et al., 1997) from the oral secretion of herbivores are triggering the early stress response after becoming in contact with the wounded plant tissue. Such an early stress response includes membrane depolarization, and increases of cytosolic Ca^{2+} level (Dombrowski and Bergey, 2007) that activate calmodulin and other Ca^{2+}-sensing proteins such as mitogen-activated protein kinase (MAPK) pathways (Nakagami et al., 2005; Maffei et al., 2006, 2007; Howe and Schaller, 2008; Mithöfer and Boland, 2008; Vadassery et al., 2012). Localized generation of ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\bullet$; Foyer and Noctor, 2003), is further involved in regulating plant

defense reactions, including activation of MAPK pathways, and elicitation of jasmonic acid or salicylic acid-dependent signaling and gene expression (Desikan et al., 2001; Maffei et al., 2007). It is at this point the LOX pathway is activated leading to emission of LOX products (Maffei et al., 2007).

The non-volatile signal molecules may move from the site of immediate damage to other parts of the leaf and plant through plant apoplast (cellular water and xylem), and through cytosolic path in plasmodesmata and phloem. Furthermore, hypersensitive response in the case of some biotic interactions can "seal off" the damaged area (Lam et al., 2001; Yoda et al., 2003), reducing the propagation of the signal. Volatile airborne signals are more efficiently transmitted over longer distances (Heil and Ton, 2008), but their formation likely requires physical presence of the stressor(s) at the impact sites. In fact, when the stress is relieved, the signal propagation and defense response is silenced as evidenced by reduced activity of expression of defense genes (Hundertmark and Hincha, 2008). Although the expression level of some defense genes may remain high after the stress indicating stress priming (Bruce et al., 2007; Hundertmark and Hincha, 2008), stress-triggered volatile emissions also decrease to the low background level that was observed before the stress (Copolovici et al., 2011). Thus, in the case of biotic stresses, continuous elicitation may be needed to keep the stress-dependent pathways active and maintain induced volatile emissions at high rates. On the other hand, this suggests that more simultaneous sites of damage may be associated with greater emission rates of volatiles, resulting in quantitative stress dose vs. emission relationships. However, this simplified mechanism has difficulties in scaling from localized responses to systemic elicitation of volatile emissions (Röse et al., 1996; Farag and Paré, 2002; Staudt and Lhoutellier, 2007). Furthermore, it is currently unclear how the systemic response is quenched after stress. If sustained systemic elicitation needs a continuous flow of signal molecules from the immediate site of damage, systemic emissions can also depend on the severity of the stress in a dose-dependent manner. It is even plausible that the rate of induction of systemic response depends on the elicitor dose. The higher the concentration of the elicitor in the ambient atmosphere the higher the proportion of elicitor's binding sites that are filled.

EVIDENCE OF DOSE–RESPONSE RELATIONSHIPS UNDER BIOTIC STRESSES FROM CASE STUDIES

As discussed above, the emission rates of key induced volatiles, including LOX products and monoterpenes, have been shown to scale quantitatively with the severity of several abiotic stresses. The evidence also suggests that the propagation of damage and the number of simultaneous stress impact sites can also lead to quantitative relationships between the severity of biotic stress and release of induced volatiles. Here we analyze several case studies that suggest that the biotic stresses can elicit volatiles in dose-dependent manner similarly to abiotic stresses.

HERBIVORY- AND WOUNDING-ELICITED EMISSIONS IN RELATION TO STRESS "SEVERITY"

Caterpillars feeding on leaves typically damage the plant by chewing or tearing off leaf pieces, thereby eliciting the classic release of LOX volatiles, followed by the emissions of terpenoids and shikimic acid pathway products (e.g., Paré and Tumlinson, 1996; Frey et al., 2000; Kessler and Baldwin, 2001; Vuorinen et al., 2004; D'Alessandro et al., 2006). In line with the general patterns, larvae of the geometrid moth, common white wave (*Cabera pusaria*), larvae feeding on the leaves of temperate deciduous tree *Alnus glutinosa* elicited emissions of LOX volatiles, monoterpenes, sesquiterpenes, and homoterpene DMNT (Copolovici et al., 2011). The emission response depended on the number of larvae feeding simultaneously on the given plant and on the degree of leaf damage (**Figure 4**). In particular, the emission rates of the sum of different LOX volatiles and the sum of monoterpenes and sesquiterpene (*E*,*E*)-α-farnesene were quantitatively associated with the severity of herbivory stress (Copolovici et al., 2011). However, differences in emission rates were smaller for some compounds such as homoterpene DMNT, which seemed to be informative of the presence of herbivores, but these differences were not quantitatively associated with the degree of damage (Copolovici et al., 2011).

There is further quantitative and semi-quantitative evidence of dose-dependent release of stress volatiles from wounding, herbivory, or elicitor treatment studies. Brilli et al. (2011) demonstrated that in the grass *Dactylis glomerata*, the amount of LOX volatiles released after mechanical wounding scaled linearly with the length of excision. In another study with *Alnus glutinosa*, there were quantitative relationships between the number of larvae of green alder sawfly (*Monsoma pulveratum*) and the leaf area damaged and the emission of LOX products and monoterpenes (Copolovici et al., unpublished data 2013). In *Zea mays* fed by *Spodoptera littoralis* larvae, the sum of all volatiles emitted (LOX products, indole and mono-, homo-, and sesquiterpenes) was positively correlated with the number of larvae (0–32) feeding (Turlings et al., 2004). In the same plant–herbivore model system,

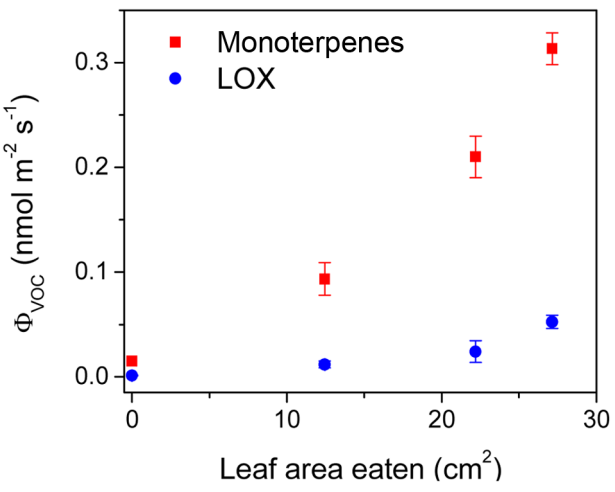


FIGURE 4 | Emissions of monoterpenes and volatile products of lipoxygenase (LOX) pathway from the leaves of temperate deciduous tree *Alnus glutinosa* in relation to the amount of leaf area eaten by the larvae of the geometrid moth *Cabera pusaria*. Different amounts of leaf area correspond to different numbers of larvae feeding on the plant (0, 2, 4, 8 larvae). Modified from Copolovici et al. (2011).

the sum of volatiles increased curvilinearly with the overall degree of plant damage (Gouinguené et al., 2003). In another study with *Z. mays* treated with various elicitors, sesquiterpene emissions increased curvilinearly with the amount of applied *Spodoptera littoralis* elicitor volicitin as well as with the amount of applied jasmonic acid, and with the concentration of ethylene during fumigation; indole emissions also scaled positively with ethylene concentration (Schmelz et al., 2003b). In a further investigation in *Z. mays* fed by larvae of *Spodoptera exigua* (Schmelz et al., 2003a), emissions of indole and sesquiterpenes were positively correlated with the degree of infestation. The degree of infestation was further associated with greater endogenous jasmonic acid levels and greater ethylene production rate, and overall, there were strong positive curvilinear relationships between endogenous jasmonic acid concentration and the rates of emission of sesquiterpenes and indole (Schmelz et al., 2003a). In *Medicago truncatula* infested with aphid *Acyrthosiphon kondoi*, transcript levels for genes characterizing the activity of salicylic acid-dependent signaling strongly increased with the plant infestation score; to some extent, transcripts for several LOX- and jasmonic acid-dependent signaling pathway enzymes also scaled positively with the extent of infestation (Gao et al., 2008). Although the degree of damage was not quantified, there is further evidence of increased emission of stress volatiles with the spread of infestation in *Brassica oleracea* fed by *Pieris brassicae* larvae (Scascighini et al., 2005) and in *Aesculus hippocastanum* infested by *Cameraria ohridella* larvae (Johne et al., 2006). These studies collectively provide conclusive evidence that the stress-dependent elicitation of emissions is linked to the severity of herbivory and mechanical damage or degree of infestation in a dose-dependent manner.

QUANTITATIVE RESPONSES TO PATHOGEN ATTACKS

Attacks by pathogenic fungi such as rust fungi, powdery mildews or *Botrytis cinerea* also lead to emissions of LOX volatiles and release of characteristic terpenoids (Heath, 1997; Steindel et al., 2005; Jansen et al., 2009, 2011; Toome et al., 2010). Leaf rust fungi are biotrophic pathogens and need living host tissue for nutrients and carbon. In contrast, powdery mildews and *Botrytis cinerea* are necrotrophic fungi, which kill the host tissue and adsorb the carbon and nutrients from the dead cells. Both rust fungi and powdery mildews are highly specialized obligate plant parasites (Staples, 2000; Glawe, 2008; Duplessis et al., 2011), while *Botrytis cinerea* is a wide-spectrum plant parasite (Staats et al., 2005).

In the case of rust fungus *Melampsora* infecting hybrid willow (*Salix burjatica* × *S. dasyclados*) foliage, emissions of LOX volatiles, monoterpene (*Z*)-β-ocimene and sesquiterpenes increased with the spread of infection (Toome et al., 2010), indicating that the degree of fungal colonization and volatile emissions were quantitatively related. In the case of oak powdery mildew (*Erysiphe alphitoides*) infecting the leaves of *Quercus robur*, emissions of LOX volatiles and monoterpenes scaled close to linearly with the percentage of leaf area infected with mildew (**Figure 5**). Analogously, Jansen et al. (2009), demonstrated that in tomato (*Solanum lycopersicum*) plants inoculated with *Botrytis cinerea*, the emissions of LOX volatiles and monoterpenes depended on the severity of infection. At larger scale, sesquiterpene emission from Scots pine (*Pinus sylvestris*) correlated with the number of airborne fungal spores incident to vegetation, and the sesquiterpene emissions were suggested to be indicative of plant response to fungal stress (Hakola et al., 2006).

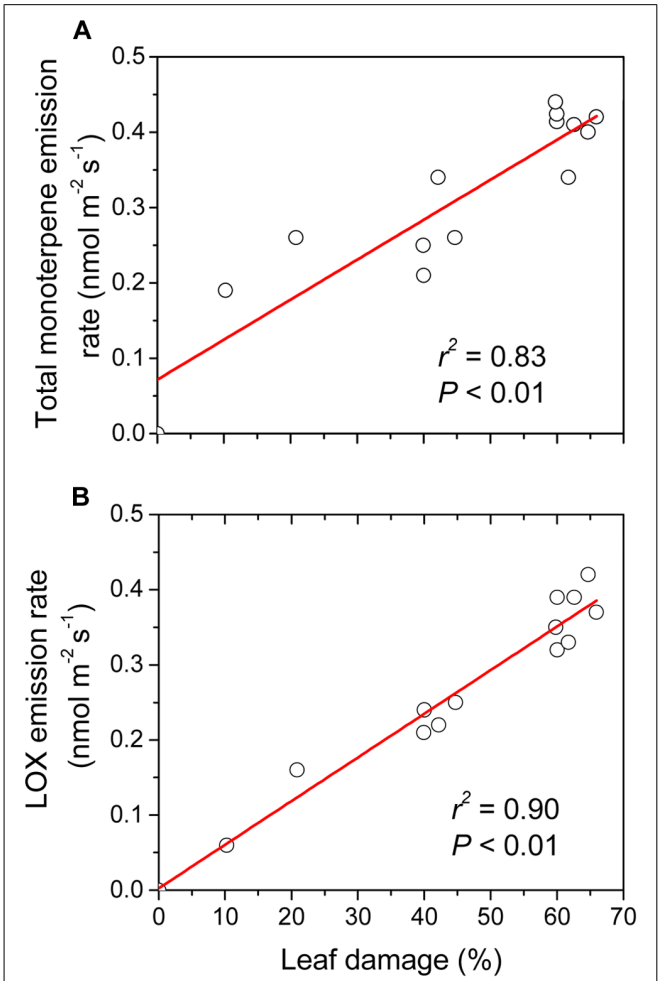


FIGURE 5 | Emissions of monoterpenes (A) and volatile products of LOX pathway (B) from the leaves of temperate deciduous tree *Quercus robur* infected with oak powdery mildew (*Erysiphe alphitoides*) in relation to the percentage of leaf area infected (unpublished data of Copolovici and Niinemets). Volatile collection and analysis from the infected leaves follows the protocol as described in detail in Copolovici et al. (2009, 2011, 2012).

WHY ARE THERE QUANTITATIVE STRESS DOSE VS. PLANT RESPONSE RELATIONS IN NATURE?

As a whole, the outlined evidence suggests that biotic stress severity and emission response are quantitatively related even for that different stresses as herbivory and fungal pathogen attacks. Thus, the rate of induced volatile emission can constitute a reliable indicator for the severity of biotic stress at any moment of time in the vegetation. However, the question is what can be the biological significance of such quantitative relationships? From insect behavioral studies, there seems to be a widespread consensus that emissions induced by biotic stress primarily serve as qualitative signals. In fact, in laboratory olfactometer experiments (Mumm

and Hilker, 2005; Turlings et al., 2005; Fernandes Furtado Michereff et al., 2011), insect performance was only weakly associated with the volatile concentration, if at all. However, under such conditions, there is no relationship or only a weak relationship between volatile concentration and the distance to the "emission source." In field conditions, volatile concentration strongly decreases with the distance from the emission source, especially in reactive atmospheres were the rate of compound destruction might be high (Holopainen et al., 2013), and thus, a greater emission rate also implies a greater spread of the signal. In the field, insect performance does depend on the distance from the emitting plant, underscoring the importance of concentration gradients (e.g., Karban, 2001; de Bruyne and Baker, 2008). Thus, stronger emissions are potentially associated with attraction of herbivore enemies from a wider distance.

Another important issue is the connection between the strength of the emission response and the spread of systemic response. Systemic induction has been shown to be stronger closer to the biotic stress site and gradually decrease with the distance from the site of damage (Tuomi et al., 1998; Frost et al., 2008b; Karban, 2011). Thus, a stronger induced emission reaction in response to a more severe herbivore attack or pathogen infestation would result in a greater spread of systemic elicitation, thereby contributing to mobilization of plant defenses to a greater degree against a more probable biotic attack. This reasoning suggests that the capacity to respond stronger to more severe stress can importantly enhance plant fitness.

COMPLICATIONS IN CHARACTERIZING THE DOSE-DEPENDENCIES OF ELICITED EMISSIONS

Although there is encouraging evidence of quantitative relationships among the severity of biotic stress, the stress "dose," and the emission rate of induced volatiles, the emission time-courses may be complex and the relationships among the stress severity and emission response may vary among genotypes of the given species, among species and depend on the past stress history and other potentially interacting stresses. Effects of interacting and sequential stresses, including stress interactions, stress sequence, and priming have been addressed in several recent reviews (Dicke and Baldwin, 2010; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Niinemets, 2010). Here we highlight modifications in emission rates due differences in elicitation time kinetics, plant genotype and due to variations in plant pre-stress physiological status, substrate availability for production of induced volatiles and physico-chemical constraints on the emission of volatiles.

DIFFERENCES IN ELICITATION TIME KINETICS

Feeding activity of attacking herbivores varies during the day for different herbivores (De Moraes et al., 2001; Fedderwitz et al., 2012; Goodspeed et al., 2012; Jander, 2012). Plant jasmonate-based defense system also has a strong circadian rhythm that can be synchronized with insect circadian behavior (Goodspeed et al., 2012; Jander, 2012). As the result of circadian rhythm of jasmonate-mediated defenses, emission response triggered by given mechanical or herbivory damage or given elicitor treatment can vary depending on the timing of stress event. In addition, the situation can be further complicated by immediate effects of environmental drivers on the rate of induced volatiles. While LOX products are released shortly after damage or elicitor treatments during both the light and dark periods (Arimura et al., 2008), emissions of terpenoids such as (Z)-β-ocimene and linalool are light-dependent (Niinemets et al., 2002; Hansen and Seufert, 2003; Arimura et al., 2008). Thus, in the case of night-time damage, terpenoid emissions are minor during the night period, but jasmonate-dependent defense pathway is activated quickly after damage and transcripts of pertinent terpenoid synthases accumulate during the night (Arimura et al., 2008). As the result, there is a burst of terpenoid emission as soon as the substrate becomes available with the onset of the light period (Arimura et al., 2008). In the case of day-time feeding, the emissions of terpenoids start during the light period as the photosynthetic substrate is available, but emissions are lower than for the night-time damage (Schmelz et al., 2001; Arimura et al., 2008). This reflects the circumstance that accumulation of terpenoid synthase protein is time-consuming and full terpenoid synthesis activity is not reached on the same day of the leaf damage (Arimura et al., 2008).

Bi-phasic emission time-kinetics have also been observed for several volatiles under different biotic stresses. In the case of *Cabera pusaria* caterpillar feeding, (*E*,*E*)-α-farnesene emissions from *Alnus glutinosa* foliage increased bi-phasically during feeding. The emissions were quantitatively related to the degree of damage at the two maxima, but no significant differences among the treatments of varying severity were observed in the intervening period between the two rising phases (Copolovici et al., 2011). In a similar manner, sesquiterpene and (*Z*)-β-ocimene emissions from the rust fungus *Melampsora* infected *Salix burjatica* × *S. dasyclados* (Toome et al., 2010) and LOX product and monoterpene emissions from *Botrytis cinerea* infected *Solanum lycopersicum* (Jansen et al., 2009) increased bi-phasically after infection. Interestingly, in *Salix burjatica* × *S. dasyclados* (Toome et al., 2010) the secondary increase of sesquiterpene emissions was not associated with LOX volatile emissions. It is tempting to speculate that the first peak reflects the immediate signaling response triggered by the biotic elicitor, while the second peak observed in a few days since the initial stress response is indicative of systemic response to airborne volatiles, and may not necessarily originate from the damaged leaf parts. Understanding the bi-phasic nature of the emissions induced by biotic attacks clearly needs further experimental studies independently analyzing the time kinetics of immediate stress-driven and secondary emissions in attacked and non-attacked foliage.

DOSE-DEPENDENCIES IN RELATION TO PLANT GENOTYPE AND PRE-STRESS PHYSIOLOGICAL STATUS

The situation is further complicated by significant genotypic variations in the level of emissions induced in response to given biotic stress (Degen et al., 2004; Turlings et al., 2005; Degenhardt et al., 2008; de Vos et al., 2008; Wu et al., 2008; Fernandes Furtado Michereff et al., 2011). These variations are not fully understood, but such genotypic differences have been associated with the overall degree of elicitation of defense pathways by given stress (Wu et al., 2008). The genotypic variation in elicited emissions can also be dependent on the constitutive resistance to given stress (Turlings et al., 2005; Fernandes Furtado Michereff et al., 2011),

and thus, the degree of damage may vary among genotypes at given stress severity. As the degree of damage has not been routinely reported in studies investigating genotypic differences in stress-elicited induced emissions, further studies are needed to understand whether the observed differences reflect a real variation in plant response or whether they are driven by differences in the degree of damage.

There is ample experimental evidence demonstrating the relevance of pre-stress physiological status in altering the induced emission rates, composition and time kinetics. In *Z. mays*, volicitin-dependent sesquiterpene emissions were much greater under low N nutrition, and the emissions in N-deficient plants were also more sensitive to ethylene (Schmelz et al., 2003b). Overall upregulation in terpenoid synthesis under N-deficiency has also been observed in camphorweed (*Heterotheca subaxillaris*) (Mihaliak and Lincoln, 1989). In contrast, induced emissions were reduced under low nutrient availability in another study with *Z. mays* (Gouinguené and Turlings, 2002). Enhancement of activities of secondary metabolic pathways under limited N have been explained by a variety of hypotheses including "carbon-nutrient balance" (CNB) or "excess carbon" hypotheses, both based on modifications in plant sink–source relations under stress (Bryant et al., 1983; Herms and Mattson, 1992; Peñuelas and Estiarte, 1998). Yet, there is only partial support to these hypotheses (e.g., Llusià et al., 2010; Sardans et al., 2010; Peñuelas et al., 2011; Kännaste et al., 2013) as also the comparisons among different *Z. mays* experiments demonstrate.

Studies on dose–emission relationships should also standardize other environmental drivers. Terpenoid emissions in *Z. mays* elicited by oral secretion of *Spodoptera littoralis* increased with decreasing soil water availability (Gouinguené and Turlings, 2002), and increased curvilinearly with air humidity, light intensity, and temperature (Gouinguené and Turlings, 2002). These environmental responses are analogous to observations in other species (Staudt and Lhoutellier, 2007; Staudt et al., 2010; Staudt and Lhoutellier, 2011), and are consistent with the strong connection of the production of induced terpenoid volatiles and photosynthetic carbon metabolism (see above). In addition, due to high water-solubility of some of the induced compounds such as linalool, methanol and LOX pathway volatiles, variations in stomatal openness during the day and in response to soil drought can directly affect the emissions of water-soluble volatiles (Niinemets et al., 2002; Niinemets et al., 2004; Harley et al., 2007; Harley, 2013). Thus, in assessing the stress dose vs. induced emission responses, it is important to consider the substrate-level and physico-chemical constraints on the rate of induced volatile production and emission.

CONCLUSION

Plants in natural environments are under fluctuating pressure of various abiotic and biotic stressors. Despite differing elicitation mechanisms, various stresses tend to converge at the level of ROS signaling (Fujita et al., 2006), and different stresses elicit release of the same ubiquitous stress volatiles such as volatiles of the LOX pathway as well as more stress-specific mono- and sesquiterpene blends and shikimic acid pathway products. While the emissions of volatiles have been quantitatively related to the severity of abiotic stresses, biotic stress severity is more difficult to quantify, especially because biotic infections typically do not influence the whole organ or plant such that there are impacted and non-impacted regions within the leaf and among the leaves in the given plant, and attacked and non-attacked plants within the vegetation. Also, stress-triggered emissions from impacted areas typically elicit systemic response and lead to secondary emissions from non-damaged plant parts. On the other hand, after the cessation of biotic stress, emissions come rapidly to background level, indicating response silencing.

The evidence summarized here collectively demonstrates that volatile release from plant foliage is quantitatively related to the severity of herbivory and pathogen stresses. However, the patterns can be complex and the responses may not be quantitative at any moment of time through stress development, possibly reflecting combinations of immediate and systemic stress responses, and time-lags between stress and onset of emission. More experimental work is needed to quantify the time-kinetics of emission elicitation by various biotic stresses, separate the contributions of immediate and systemically induced emissions, and also address the degree of silencing and priming of emissions.

ACKNOWLEDGMENTS

Authors' funding for the studies on plant volatiles has been provided by the Estonian Ministry of Science and Education (institutional grant IUT-8-3), the European Commission through the European Regional Fund (the Center of Excellence in Environmental Adaptation), the European Research Council (advanced grant 322603, SIP-VOL+), the European Social Fund (post-doctoral grant MJD14), the European Science Foundation (EUROCORES project A-BIO-VOC), and the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, (project number: PN-II-RU-TE-2011-3-0022).

REFERENCES

Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. *Funct. Ecol.* 25, 420–432. doi: 10.1111/j.1365-2435.2010.01796.x

Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhagen, G., Loughrin, J. H., and Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276, 945–949. doi: 10.1126/science.276.5314.945

Allmann, S., and Baldwin, I. T. (2010). Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science* 329, 1075–1078. doi: 10.1126/science.1191634

Arimura, G., Kost, C., and Boland, W. (2005). Herbivore-induced, indirect plant defences. *Biochim. Biophys. Acta* 1734, 91–111. doi: 10.1016/j.bbalip.2005.03.001

Arimura, G.-I., Köpke, S., Kunert, M., Volpe, V., David, A., Brand, P., et al. (2008). Effects of feeding *Spodoptera littoralis* on lima bean leaves. IV. Diurnal and nocturnal damage differentially initiate plant volatile emission. *Plant Physiol.* 146, 865–973. doi: 10.1104/pp.107.111088

Arimura, G. I., Ozawa, R., Horiuchi, J. I., Nishioka, T., and Takabayashi, J. (2001). Plant–plant interactions mediated by volatiles emitted from plants infested by spider mites. *Biochem. Syst. Ecol.* 29, 1049–1061. doi: 10.1016/S0305-1978(01)00049-7

Arimura, G.-I., Ozawa, R., and Maffei, M. E. (2011). Recent advances in plant early signaling in response to herbivory. *Int. J. Mol. Sci.* 12, 3723–3739. doi: 10.3390/ijms12063723

Arimura, G. I., Ozawa, R., Nishioka, T., Boland, W., Koch, T., Kühnemann, F., et al. (2002).

Herbivore-induced volatiles induce the emission of ethylene in neigbouring lima bean plants. *Plant J.* 29, 87–98. doi: 10.1046/j.1365-313x.2002.01198.x

Arneth, A., and Niinemets, Ü. (2010). Induced BVOCs: how to bug our models? *Trends Plant Sci.* 15, 118–125. doi: 10.1016/j.tplants.2009.12.004

Bai, J., Baldwin, E. A., Imahori, Y., Kostenyuk, I., Burns, J., and Brecht, J. K. (2011). Chilling and heating may regulate C6 volatile aroma production by different mechanisms in tomato (*Solanum lycopersicum*) fruit. *Postharvest Biol. Technol.* 60, 111–120. doi: 10.1016/j.postharvbio.2010.12.002

Baier, P., Führer, E., Kirsitis, T., and Rosner, S. (2002). Defence reactions of Norway spruce against bark beetles and the associated fungus *Ceratocystis polonica* in secondary pure and mixed species stands. *For. Ecol. Manage.* 159, 73–86. doi: 10.1016/S0378-1127(01)00711-3

Baldwin, I. T., Halitschke, R., Paschold, A., Von Dahl, C. C., and Preston, C. A. (2006). Volatile signaling in plant–plant interactions: "talking trees" in the genomics era. *Science* 311, 812–815. doi: 10.1126/science.1118446

Bartram, S., Jux, A., Gleixner, G., and Boland, W. (2006). Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves. *Phytochemistry* 67, 1661–1672. doi: 10.1016/j.phytochem.2006.02.004

Beauchamp, J., Wisthaler, A., Hansel, A., Kleist, E., Miebach, M., Niinemets, Ü., et al. (2005). Ozone induced emissions of biogenic VOC from tobacco: relations between ozone uptake and emission of LOX products. *Plant Cell Environ.* 28, 1334–1343. doi: 10.1111/j.1365-3040.2005.01383.x

Berenbaum, M. R., and Zangerl, A. R. (2008). Facing the future of plant-insect interaction research: le retour à la "raison d'être". *Plant Physiol.* 146, 804–811. doi: 10.1104/pp.107.113472

Bernasconi, M. L., Turlings, T. C. J., Ambrosetti, L., Bassetti, P., and Dorn, S. (1998). Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis. Entomol. Exp. Appl.* 87, 133–142. doi: 10.1046/j.1570-7458.1998.00315.x

Boatright, J., Negre, F., Chen, X. L., Kish, C. M., Wood, B., Peel, G., et al. (2004). Understanding in vivo benzenoid metabolism in *Petunia* petal tissue. *Plant Physiol.* 135, 1993–2011. doi: 10.1104/pp.104.045468

Brilli, F., Ciccioli, P., Frattoni, M., Prestininzi, M., Spanedda, A. F., and Loreto, F. (2009). Constitutive and herbivore-induced monoterpenes emitted by *Populus* x *euramericana* leaves are key volatiles that orient *Chrysomela populi* beetles. *Plant Cell Environ.* 32, 542–552. doi: 10.1111/j.1365-3040.2009.01948.x

Brilli, F., Ruuskanen, T. M., Schnitzhofer, R., Müller, M., Breitenlechner, M., Bittner, V., et al. (2011). Detection of plant volatiles after leaf wounding and darkening by proton transfer reaction "time-of-flight" mass spectrometry (PTR-TOF). *PLoS ONE* 6:e20419. doi: 10.1371/journal.pone.0020419

Bruce, T. J. A., Matthes, M. C., Napier, J. A., and Pickett, J. A. (2007). Stressful "memories" of plants: evidence and possible mechanisms. *Plant Sci.* 173, 603–608. doi: 10.1016/j.plantsci.2007.09.002

Bryant, J. P., Chapin, F. S. III, and Klein, D. R. (1983). Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40, 357–368. doi: 10.2307/3544308

Byers, J. A., Zhang, Q. H., and Birgersson, G. (2000). Strategies of a bark beetle, *Pityogenes bidentatus*, in an olfactory landscape. *Naturwissenschaften* 87, 503–507. doi: 10.1007/s001140050768

Chen, J.-W., Bai, K.-D., and Cao, K.-F. (2009). Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defenses in leaves of rubber tree (*Hevea brasiliensis*). *Acta Physiol. Plant.* 31, 95–101. doi: 10.1007/s11738-008-0205-z

Copaciu, F., Opriș, O., Coman, V., Ristoiu, D., Niinemets, Ü., and Copolovici, L. (2013). Diffuse water pollution by anthraquinone and azo dyes in environment importantly alters foliage volatiles, carotenoids and physiology in wheat (*Triticum aestivum*). *Water Air Soil Pollut.* 224, 1478. doi: 10.1007/s11270-013-1478-4

Copolovici, L., Kännaste, A., and Niinemets, Ü. (2009). Gas chromatography-mass spectrometry method for determination of monoterpene and sesquiterpene emissions from stressed plants. *Stud. Univ. Babes-Bolyai Chem.* 54, 329–339.

Copolovici, L., Kännaste, A., Pazouki, L., and Niinemets, Ü. (2012). Emissions of green leaf volatiles and terpenoids from *Solanum lycopersicum* are quantitatively related to the severity of cold and heat shock treatments. *J. Plant Physiol.* 169, 664–672. doi: 10.1016/j.jplph.2011.12.019

Copolovici, L., Kännaste, A., Remmel, T., Vislap, V., and Niinemets, Ü. (2011). Volatile emissions from *Alnus glutinosa* induced by herbivory are quantitatively related to the extent of damage. *J. Chem. Ecol.* 37, 18–28. doi: 10.1007/s10886-010-9897-9

Copolovici, L., and Niinemets, Ü. (2010). Flooding induced emissions of volatile signalling compounds in three tree species with differing waterlogging tolerance. *Plant Cell Environ.* 33, 1582–1594. doi: 10.1111/j.1365-3040.2010.02166.x

Copolovici, L. O., Filella, I., Llusià, J., Niinemets, Ü., and Peñuelas, J. (2005). The capacity for thermal protection of photosynthetic electron transport varies for different monoterpenes in *Quercus ilex. Plant Physiol.* 139, 485–496. doi: 10.1104/pp.105.065995

D'Alessandro, M., Held, M., Triponez, Y., and Turlings, T. C. J. (2006). The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps. *J. Chem. Ecol.* 32, 2733–2748. doi: 10.1007/s10886-006-9196-7

Danielsson, M., Kännaste, A., Lindstrom, A., Hellqvist, C., Stattin, E., Langstrom, B., et al. (2008). Mini-seedlings of *Picea abies* are less attacked by *Hylobius abietis* than conventional ones: is plant chemistry the explanation? *Scand. J. For. Res.* 23, 299–306. doi: 10.1080/02827580802203560

de Bruyne, M., and Baker, T. C. (2008). Odor detection in insects: volatile codes. *J. Chem. Ecol.* 34, 882–897. doi: 10.1007/s10886-008-9485-4

Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135, 1928–1938. doi: 10.1104/pp.104.039891

Degenhardt, D. C., and Lincoln, D. E. (2006). Volatile emissions from an odorous plant in response to herbivory and methyl jasmonate exposure. *J. Chem. Ecol.* 32, 725–743. doi: 10.1007/s10886-006-9030-2

Degenhardt, D. C., Refi-Hind, S., Stratmann, J. W., and Lincoln, D. E. (2008). Systemin and jasmonic acid regulate constitutive and herbivore-induced systemic volatile emissions in tomato, *Solanum lycopersicum. Phytochemistry* 71, 2024–2037. doi: 10.1016/j.phytochem.2010.09.010

Degenhardt, J., Gershenzon, J., Baldwin, I. T., and Kessler, A. (2003). Attracting friends and feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14, 169–176. doi: 10.1016/S0958-1669(03)00025-9

Degenhardt, J., Koellner, T. G., and Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70, 1621–1637. doi: 10.1016/j.phytochem.2009.07.030

De Moraes, C. M., Mescher, M. C., and Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410, 577–580. doi: 10.1038/35069058

Desikan, R., Mackerness, S. A.-H., Hancock, J. T., and Neill, S. J. (2001). Regulation of the *Arabidopsis* transcriptome by oxidative stress. *Plant Physiol.* 127, 159–172. doi: 10.1104/pp.127.1.159

de Vos, M., Kriksunov, K. L., and Jander, G. (2008). Indole-3-acetonitrile production from indole glucosinolates deters oviposition by *Pieris rapae. Plant Physiol.* 146, 916–926. doi: 10.1104/pp.107.112185

Dewick, P. M. (1999). The biosynthesis of C5–C25 terpenoid compounds. *Nat. Prod. Rep.* 19, 97–130. doi: 10.1039/a708935j

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends Plant Sci.* 15, 167–175. doi: 10.1016/j.tplants.2009.12.002

Dicke, M., and Bruin, J. (2001). Chemical information transfer between plants: back to the future. *Biochem. Syst. Ecol.* 29, 981–994. doi: 10.1016/S0305-1978(01)00045-X

Dicke, M., Gols, R., Ludeking, D., and Posthumus, M. A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25, 1907–1922. doi: 10.1023/A:1020942102181

Dombrowski, J. E., and Bergey, D. R. (2007). Calcium ions enhance systemin activity and play an integral role in the wound response. *Plant Sci.* 172, 335–344. doi: 10.1016/j.plantsci.2006.09.011

Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., et al. (2005). The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proc. Natl. Acad. Sci. U.S.A.* 102, 933–938. doi: 10.1073/pnas.0407360102

Dudareva, N., Negre, F., Nagegowda, D. A., and Orlova, I. (2006). Plant volatiles: recent advances and future perspectives. *Crit. Rev. Plant Sci.* 25, 417–440. doi: 10.1080/07352680600899973

Dudareva, N., Pichersky, E., and Gershenzon, J. (2004). Biochemistry of plant volatiles. *Plant Physiol.* 135, 1893–1902. doi: 10.1104/pp.104.049981

Duplessis, S., Cuomo, C. A., Lin, Y.-C., Aerts, A., Tisserant, E., Veneault-Fourrey, C., et al. (2011). Obligate biotrophy features unraveled by the genomic analysis of rust fungi. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9166–9171. doi: 10.1073/pnas.1019315108

El Omari, B., Fleck, I., Aranda, X., Moret, A., and Nadal, M. (2001). Effect of fungal infection on leaf gas-exchange and chlorophyll fluorescence in *Quercus ilex*. *Ann. For. Sci.* 58, 165–174. doi: 10.1051/forest:2001116

Fäldt, J., Martin, D., Miller, B., Rawat, S., and Bohlmann, J. (2003). Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol. Biol.* 51, 119–133. doi: 10.1023/A:1020714403780

Farag, M. A., and Paré, P. W. (2002). C6-green leaf volatiles trigger local and systemic VOC emissions in tomato. *Phytochemistry* 61, 545–554. doi: 10.1016/S0031-9422(02)00240-6

Fatouros, N. E., Lucas-Barbosa, D., Weldegergis, B. T., Pashalidou, F. G., Van Loon, J. J. A., Dicke, M., et al. (2012). Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. *PLoS ONE* 7:e43607. doi: 10.1371/journal.pone.0043607

Fedderwitz, F., Björklund, N., Ninkovic, V., and Nordlander, G. (2012). "Pine weevil (*Hylobius abietis*) feeding pattern on conifer seedlings," in *Proceedings of Measuring Behavior, Utrecht, The Netherlands, August 28–31, 2012*, eds A. J. Spink, F. Grieco, O. E. Krips, L. W. S. Loijens, L. P. J. J. Noldus and P. H. Zimmerman, (Wageningen: Noldus Information Technology bv). 333–337.

Fernandes Furtado Michereff, M., Laumann, R. A., Borges, M., Michereff-Filho, M., Rezende Diniz, I., Lopes Farias Neto, A., et al. (2011). Volatiles mediating a plant-herbivore-natural enemy interaction in resistant and susceptible soybean cultivars. *J. Chem. Ecol.* 37, 273–285. doi: 10.1007/s10886-011-9917-4

Feussner, I., and Wasternack, C. (2002). The lipoxygenase pathway. *Annu. Rev. Plant Biol.* 53, 275–297. doi: 10.1146/annurev.arplant.53.100301.135248

Fineschi, S., Loreto, F., Staudt, M., and Peñuelas, J. (2013). "Diversification of volatile isoprenoid emissions from trees: evolutionary and ecological perspectives," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 1–20.

Foyer, C. H., and Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant.* 119, 355–364. doi: 10.1034/j.1399-3054.2003.00223.x

Frey, M., Stettner, C., Paré, P. W., Schmelz, E. A., Tumlinson, J. H., and Gierl, A. (2000). An herbivore elicitor activates the gene for indole emission in maize. *Proc. Natl. Acad. Sci. U.S.A.* 97, 14801–14806. doi: 10.1073/pnas.260499897

Frost, C. J., Mescher, M. C., Carlson, J. E., and De Moraes, C. M. (2008a). Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiol.* 146, 818–824. doi: 10.1104/pp.107.113027

Frost, C. J., Mescher, M. C., Carlson, J. E., and De Moraes, C. M. (2008b). Why do distance limitations exist on plant–plant signaling via airborne volatiles? *Plant Signal. Behav.* 3, 466–468. doi: 10.4161/psb.3.7.5675

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., et al. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.* 9, 436–442. doi: 10.1016/j.pbi.2006.05.014

Gang, D. R., Wang, J., Dudareva, N., Hee Nam, K., Simon, J. E., Lewinsohn, E., et al. (2001). An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant Physiol.* 125, 539–555. doi: 10.1104/pp.125.2.539

Gao, L.-L., Klingler, J. P., Anderson, J. P., Edwards, O. R., and Singh, K. B. (2008). Characterization of pea aphid resistance in *Medicago truncatula*. *Plant Physiol.* 146, 996–1009. doi: 10.1104/pp.107.111971

Glawe, D. A. (2008). The powdery mildews: a review of the world's most familiar (yet poorly known) plant pathogens. *Annu. Rev. Phytopathol.* 46, 27–51. doi: 10.1146/annurev.phyto.46.081407.104740

Goodspeed, D., Chehab, E. W., Min-Venditti, A., Braam, J., and Covington, M. F. (2012). *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4674–4677. doi: 10.1073/pnas.1116368109

Gouinguené, S., Alborn, H., and Turlings, T. C. J. (2003). Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. *J. Chem. Ecol.* 29, 145–162. doi: 10.1023/A:1021984715420

Gouinguené, S. P., and Turlings, T. C. J. (2002). The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiol.* 129, 1296–1307. doi: 10.1104/pp.001941

Grote, R., Monson, R. K., and Niinemets, Ü. (2013). "Leaf-level models of constitutive and stress-driven volatile organic compound emissions," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 315–355.

Hakola, H., Tarvainen, V., Bäck, J., Ranta, H., Bonn, B., Rinne, J., and Kulmala, M. (2006). Seasonal variation of mono- and sesquiterpene emission rates of Scots pine. *Biogeosciences* 3, 93–101. doi: 10.5194/bg-3-93-2006

Halitschke, R., Kessler, A., Kahl, J., Lorenz, A., and Baldwin, I. T. (2000). Ecophysiological comparison of direct and indirect defenses in *Nicotiana attenuata*. *Oecologia* 124, 408–417. doi: 10.1007/s004420000389

Hansen, U., and Seufert, G. (2003). Temperature and light dependence of β-caryophyllene emission rates. *J. Geophys. Res.* 108, 4801. doi: 10.1029/2003JD003853

Harley, P., Greenberg, J., Niinemets, Ü., and Guenther, A. (2007). Environmental controls over methanol emission from leaves. *Biogeosciences* 4, 1083–1099. doi: 10.5194/bg-4-1083-2007

Harley, P. C. (2013). "The roles of stomatal conductance and compound volatility in controlling the emission of volatile organic compounds from leaves," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 181–208.

Hatanaka, A. (1993). The biogeneration of green odour by green leaves. *Biochemistry* 37, 1201–1218. doi: 10.1016/0031-9422(91)80003-J

Hatanaka, A., Sekiya, J., and Kajiwara, T. (1978). Distribution of an enzyme system producing cis-3-hexenal and n-hexanal from linolenic and linoleic acids in some plants. *Phytochemistry* 17, 869–872. doi: 10.1016/S0031-9422(00)88636-7

Heath, M. C. (1997). Signalling between pathogenic rust fungi and resistant or susceptible host plants. *Ann. Bot.* 80, 713–720. doi: 10.1006/anbo.1997.0507

Heil, M., and Kost, C. (2006). Priming of indirect defences. *Ecol. Lett.* 9, 813–817. doi: 10.1111/j.1461-0248.2006.00932.x

Heil, M., and Silva Bueno, J. C. (2007). Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5467–5472. doi: 10.1073/pnas.0610266104

Heil, M., and Ton, J. (2008). Long-distance signalling in plant defence. *Trends Plant Sci.* 13, 264–272. doi: 10.1016/j.tplants.2008.03.005

Herms, D. A., and Mattson, W. J. (1992). The dilemma of plants: to grow or defend. *Q. Rev. Biol.* 67, 283–335. doi: 10.1086/417659

Hiltpold, I., Erb, M., Robert, C. A. M., and Turlings, T. C. J. (2011). Systemic root signalling in a belowground, volatile-mediated tritrophic interaction. *Plant Cell Environ.* 34, 1267–1275. doi: 10.1111/j.1365-3040.2011.02327.x

Holopainen, J. K., and Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.* 15, 176–184. doi: 10.1016/j.tplants.2010.01.006

Holopainen, J. K., Nerg, A.-M., and Blande, J. D. (2013). "Multitrophic signalling in polluted atmospheres," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 285–314.

Howe, G. A., and Schaller, A. (2008). "Direct defenses in plants and their induction by wounding and insect herbivores," in *Induced Plant Resistance to Herbivory*, ed. A. Schaller. (Berlin: Springer Science+Business Media B.V.), 7–29.

Huang, M., Sanchez-Moreiras, A. M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., and Tholl, D. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytol.*

193, 997–1008. doi: 10.1111/j.1469-8137.2011.04001.x

Hundertmark, M., and Hincha, D. K. (2008). LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis thaliana. BMC Genomics* 9:118. doi: 10.1186/1471-2164-9-118

Jander, G. (2012). Timely plant defenses protect against caterpillar herbivory. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4343–4344. doi: 10.1073/pnas.1201443109

Jansen, R. M. C., Miebach, M., Kleist, E., Van Henten, E. J., and Wildt, J. (2009). Release of lipoxygenase products and monoterpenes by tomato plants as an indicator of *Botrytis cinerea*-induced stress. *Plant Biol.* 11, 859–868. doi: 10.1111/j.1438-8677.2008.00183.x

Jansen, R. M. C., Wildt, J., Kappers, I. F., Bouwmeester, H. J., Hofstee, J. W., and Van Henten, E. J. (2011). Detection of diseased plants by analysis of volatile organic compound emission. *Ann. Rev. Phytopathol.* 49, 157–174. doi: 10.1146/annurev-phyto-072910-095227

Johne, A. B., Weissbecker, B., and Schütz, S. (2006). Volatile emissions from *Aesculus hippocastanum* induced by mining of larval stages of *Cameraria ohridella* influence oviposition by conspecific females. *J. Chem. Ecol.* 32, 2303–2319. doi: 10.1007/s10886-006-9146-4

Joó, E., Dewulf, J., Amelynck, C., Schoon, N., Pokorska, O., Šimpraga, M., et al. (2011). Constitutive versus heat and biotic stress induced BVOC emissions in *Pseudotsuga menziesii*. *Atmos. Environ.* 45, 3655–3662. doi: 10.1016/j.atmosenv.2011.04.048

Kännaste, A., Pazouki, L., Suhhorutšenko, M., Copolovici, L., and Niinemets, Ü. (2013). Highly variable chemical signatures over short spatial distances among Scots pine (*Pinus sylvestris*) populations. *Tree Physiol.* 33, 374–387. doi: 10.1093/treephys/tpt013

Kant, M. R., Bleeker, P. M., Van Wijk, M., Schuurink, R. C., and Haring, M. A. (2009). "Plant volatiles in defence," in *Plant Innate Immunity*, ed. L. J. C. Van Loon (Waltham: Academic Press), 613–666.

Kappers, I. F., Verstappen, F. W. A., Luckerhoff, L. L. P., Bouwmeester, H. J., and Dicke, M. (2010). Genetic variation in jasmonic acid- and spider mite-induced plant volatile emission of cucumber accessions and attraction of the predator *Phytoseiulus persimilis*. *J. Chem. Ecol.* 36, 500–512. doi: 10.1007/s10886-010-9782-6

Karban, R. (2001). Communication between sagebrush and wild tobacco in the field. *Biochem. Syst. Ecol.* 29, 995–1005. doi: 10.1016/S0305-1978(01)00046-1

Karban, R. (2011). The ecology and evolution of induced resistance against herbivores. *Funct. Ecol.* 25, 339–347. doi: 10.1111/j.1365-2435.2010.01789.x

Karl, T., Guenther, A., Turnipseed, A., Patton, E. G., and Jardine, K. (2008). Chemical sensing of plant stress at the ecosystem scale. *Biogeosciences* 5, 1287–1294. doi: 10.5194/bg-5-1287-2008

Kelsey, R. G., and Joseph, G. (1997). Ambrosia beetle host selection among logs of Douglas fir, western hemlock, and western red cedar with different ethanol and α-pinene concentrations. *J. Chem. Ecol.* 23, 1035–1051. doi: 10.1023/B:JOEC.0000006387.36449.72

Kessler, A., and Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291, 2141–2144. doi: 10.1126/science.291.5511.2141

Knudsen, J. T., Tollsten, L., and Bergstrom, L. G. (1993). Floral scents–a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* 33, 253–280. doi: 10.1016/0031-9422(93)85502-I

Lam, E., Kato, N., and Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* 411, 848–853. doi: 10.1038/35081184

Li, Z., and Sharkey, T. D. (2013). "Molecular and pathway controls on biogenic volatile organic compound emissions," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 119–151.

Liavonchanka, A., and Feussner, N. (2006). Lipoxygenases: occurrence, functions and catalysis. *J. Plant Physiol.* 163, 348–357. doi: 10.1016/j.jplph.2005.11.006

Lichtenthaler, H. K., Schwender, J., Disch, A., and Rohmer, M. (1997). Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Lett.* 400, 271–274. doi: 10.1016/S0014-5793(96)01404-4

Lindfors, V., Laurila, T., Hakola, H., Steinbrecher, R., and Rinne, J. (2000). Modeling speciated terpenoid emissions from the European boreal forest. *Atmos. Environ.* 34, 4983–4996. doi: 10.1016/S1352-2310(00)00223-5

Litvak, M. E., and Monson, R. K. (1998). Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia* 114, 531–540. doi: 10.1007/s004420050477

Llusià, J., Peñuelas, J., Asensio, D., and Munné-Bosch, S. (2005). Airborne limonene confers limited thermotolerance to *Quercus ilex*. *Physiol. Plant.* 123, 40–48. doi: 10.1111/j.1399-3054.2004.00426.x

Llusià, J., Peñuelas, J., and Gimeno, B. S. (2002). Seasonal and species-specific response of VOC emissions by Mediterranean woody plant to elevated ozone concentrations. *Atmos. Environ.* 36, 3931–3938. doi: 10.1016/S1352-2310(02)00321-7

Llusià, J., Peñuelas, J., Sardans, J., Owen, S. M., and Niinemets, Ü. (2010). Measurement of volatile terpene emissions in 70 dominant vascular plant species in Hawaii: aliens emit more than natives. *Glob. Ecol. Biogeogr.* 19, 863–874. doi: 10.1111/j.1466-8238.2010.00557.x

Llusià, J., Sardans, J., Niinemets, Ü., Owen, S. M., and Peñuelas, J. (2013). A screening study of leaf terpene emissions of 43 rainforest species in Danum Valley Conservation Area (Borneo) and their relationships with chemical and morphological leaf traits. *Plant Biosystems* (in press). doi: 10.1080/11263504.2013.770803

Loivamäki, M., Mumm, R., Dicke, M., and Schnitzler, J. P. (2008). Isoprene interferes with the attraction of bodyguards by herbaceous plants. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17430–17435. doi: 10.1073/pnas.0804488105

Loreto, F., Barta, C., Brilli, F., and Nogues, I. (2006). On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. *Plant Cell Environ.* 29, 1820–1828. doi: 10.1111/j.1365-3040.2006.01561.x

Loreto, F., Förster, A., Dürr, M., Csiky, O., and Seufert, G. (1998). On the monoterpene emission under heat stress and on the increased thermotolerance of leaves of *Quercus ilex* L. fumigated with selected monoterpenes. *Plant Cell Environ.* 21, 101–107. doi: 10.1046/j.1365-3040.1998.00268.x

Loreto, F., Nascetti, P., Graverini, A., and Mannozzi, M. (2000). Emission and content of monoterpenes in intact and wounded needles of the Mediterranean pine, *Pinus pinea*. *Funct. Ecol.* 14, 589–595. doi: 10.1046/j.1365-2435.2000.t01-1-00457.x

Loreto, F., and Schnitzler, J.-P. (2010). Abiotic stresses and induced BVOCs. *Trends Plant Sci.* 15, 154–166. doi: 10.1016/j.tplants.2009.12.006

Maffei, M. E., Mithöfer, A., Arimura, G.-I., Uchtenhagen, H., Bossi, S., Bertea, C. M., et al. (2006). Effects of feeding *Spodoptera littoralis* on lima bean leaves. III. Membrane depolarization and involvement of hydrogen peroxide. *Plant Physiol.* 140, 1022–1035. doi: 10.1104/pp.105.071993

Maffei, M. E., Mithöfer, A., and Boland, W. (2007). Before gene expression: early events in plant–insect interaction. *Trends Plant Sci.* 12, 310–316. doi: 10.1016/j.tplants.2007.06.001

Martin, D., and Bohlmann, J. (2005). "Molecular biochemistry and genomics of terpenoid defenses in conifers," in *Recent Advances in Phytochemistry*, ed. J. T. Romeo (Amsterdam: Elsevier), 29–56.

Mattiacci, L., Dicke, M., and Posthumus, M. A. (1995). β-Glucosidase – an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92, 2036–2040. doi: 10.1073/pnas.92.6.2036

Mihaliak, C. A., and Lincoln, D. E. (1989). Changes in leaf mono- and sesquiterpene metabolism with nitrate availability and leaf age in *Heterotheca subaxillaris*. *J. Chem. Ecol.* 15, 1579–1588. doi: 10.1007/BF01012385

Mita, E., Tsitsimpikou, C., Tsiveleka, L., Petrakis, P. V., Ortiz, A., et al. (2002). Seasonal variation of oleoresin terpenoids from *Pinus halepensis* and *Pinus pinea* and host selection of the scale insect *Marchalina hellenica* (Homoptera, Coccoidea, Margarodidae, Coelostonidiinae). *Holzforschung* 56, 572–578. doi: 10.1515/HF.2002.087

Mithöfer, A., and Boland, W. (2008). Recognition of herbivory-associated molecular patterns. *Plant Physiol.* 146, 825–831. doi: 10.1104/pp.107.113118

Monson, R. K. (2013). "Metabolic and gene expression controls on the production of biogenic volatile organic compounds," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 153–179.

Monson, R. K., Grote, R., Niinemets, Ü., and Schnitzler, J.-P. (2012). Tansley review. Modeling the isoprene emission rate from leaves. *New Phytol.*

195, 541–559. doi: 10.1111/j.1469-8137.2012.04204.x

Mumm, R., and Hilker, M. (2005). The significance of background odour for an egg parasitoid to detect plants with host eggs. *Chem. Senses* 30, 337–343. doi: 10.1093/chemse/bji028

Nakagami, H., Pitzschke, A., and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. *Trends Plant Sci.* 10, 339–346. doi: 10.1016/j.tplants.2005.05.009

Niinemets, Ü. (2010). Mild versus severe stress and BVOCs: thresholds, priming and consequences. *Trends Plant Sci.* 15, 145–153. doi: 10.1016/j.tplants.2009.11.008

Niinemets, Ü., Arneth, A., Kuhn, U., Monson, R. K., Peñuelas, J., and Staudt, M. (2010a). The emission factor of volatile isoprenoids: stress, acclimation, and developmental responses. *Biogeosciences* 7, 2203–2223. doi: 10.5194/bg-7-2203-2010

Niinemets, Ü., Monson, R. K., Arneth, A., Ciccioli, P., Kesselmeier, J., Kuhn, U., et al. (2010b). The leaf-level emission factor of volatile isoprenoids: caveats, model algorithms, response shapes and scaling. *Biogeosciences* 7, 1809–1832. doi: 10.5194/bg-7-1809-2010

Niinemets, Ü., Loreto, F., and Reichstein, M. (2004). Physiological and physicochemical controls on foliar volatile organic compound emissions. *Trends Plant Sci.* 9, 180–186. doi: 10.1016/j.tplants.2004.02.006

Niinemets, Ü., and Reichstein, M. (2002). A model analysis of the effects of nonspecific monoterpenoid storage in leaf tissues on emission kinetics and composition in Mediterranean sclerophyllous *Quercus* species. *Global Biogeochem. Cycles* 16, 1110, doi: 10.1029/2002GB001927

Niinemets, Ü., Reichstein, M., Staudt, M., Seufert, G., and Tenhunen, J. D. (2002). Stomatal constraints may affect emission of oxygenated monoterpenoids from the foliage of *Pinus pinea*. *Plant Physiol.* 130, 1371–1385. doi: 10.1104/pp.009670

Opriş, O., Copaciu, F., Soran, M. L., Ristoiu, D., Niinemets, Ü., and Copolovici, L. (2013). Influence of nine antibiotics on key secondary metabolites and physiological characteristics in *Triticum aestivum*: leaf volatiles as a promising new tool to assess toxicity. *Ecotoxicol. Environ. Saf.* 87, 70–79. doi: 10.1016/j.ecoenv.2012.09.019

Paré, P. W., Farag, M. A., Krishnamachari, V., Zhang, H., Ryu, C.-M., and Kloepper, J. W. (2005). Elicitors and priming agents initiate plant defense responses. *Photosyn. Res.* 85, 149–159. doi: 10.1007/s11120-005-1001-x

Paré, P. W., and Tumlinson, J. H. (1996). Plant volatile signals in response to herbivore feeding. *Fla. Entomol.* 79, 93–103. doi: 10.2307/3495807

Paré, P. W., and Tumlinson, J. H. (1997). Induced synthesis of plant volatiles. *Nature* 385, 30–31. doi: 10.1038/385030a0

Paré, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–331. doi: 10.1104/pp.121.2.325

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Peng, J., Van Loon, J. J., Zheng, S., and Dicke, M. (2011). Herbivore-induced volatiles of cabbage (*Brassica oleracea*) prime defence responses in neighbouring intact plants. *Plant Biol.* 13, 276–284. doi: 10.1111/j.1438-8677.2010.00364.x

Peñuelas, J., and Estiarte, M. (1998). Can elevated CO_2 affect secondary metabolism and ecosystem functioning? *Trends Ecol. Evol.* 13, 20–24. doi: 10.1016/S0169-5347(97)01235-4

Peñuelas, J., Sardans, J., Llusià, J., Owen, S. M., and Niinemets, Ü. (2011). Lower P contents and more widespread terpene presence in old Bornean than in young Hawaiian tropical plant species guilds. *Ecosphere* 2, 45. doi: 10.1890/ES10-00185.1

Pichersky, E., and Gershenzon, J. (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* 5, 237–243. doi: 10.1016/S1369-5266(02)00251-0

Popp, M. P., Johnson, J. D., and Lesney, M. S. (1995). Changes in ethylene production and monoterpene concentration in slash pine and loblolly pine following inoculation with bark beetle vectored fungi. *Tree Physiol.* 15, 807–812. doi: 10.1093/treephys/15.12.807

Possell, M., and Loreto, F. (2013). "The role of volatile organic compounds in plant resistance to abiotic stresses: responses and mechanisms," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer Science+Business Media B.V.), 209–235.

Prats, E., Carver, T. L. W., Gay, A. P., and Mur, L. A. J. (2007). Enemy at the gates: interaction-specific stomatal responses to pathogenic challenge. *Plant Signal. Behav.* 2, 275–277. doi: 10.4161/psb.2.4.4153

Qualley, A. V., and Dudareva, N. (2008). "Aromatic volatiles and their involvement in plant defense," in *Induced Plant Resistance to Herbivory*, ed. A. Schaller (Berlin: Springer Science+Business Media B.V.), 409–432.

Rajabi Memari, H., Pazouki, L., and Niinemets, Ü. (2013). "The biochemistry and molecular biology of volatile messengers in trees," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*, eds Ü. Niinemets and R. K. Monson (Berlin: Springer), 47–93.

Rohmer, M. (1999). The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep. 16,* 565–574. doi: 10.1039/a709175c

Röse, U. S. R., Manukian, A., Heath, R. R., and Tumlinson, J. H. (1996). Volatile semiochemicals released from undamaged cotton leaves: a systemic response of living plants to caterpillar damage. *Plant Physiol.* 111, 487–495.

Rosenkranz, M., and Schnitzler, J.-P. (2013). "Genetic engineering of BVOC emissions from trees," in *Biology, Controls and Models of Tree Volatile Organic Compound Emissions,* eds Ü. Niinemets and R. K. Monson (Berlin: Springer), 95–118.

Sardans, J., Llusià, J., Niinemets, Ü., Owen, S., and Peñuelas, J. (2010). Foliar mono- and sesquiterpene contents in relation to leaf economics spectrum in native and alien plant species in Oahu (Hawai'i). *J. Chem. Ecol.* 36, 210–226. doi: 10.1007/s10886-010-9744-z

Scascighini, N., Mattiacci, L., D'Alessandro, M., Hern, A., Rott, A. S., and Dorn, S. (2005). New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction. *Chemoecology* 15, 97–104. doi: 10.1007/s00049-005-0300-1

Schmelz, E. A., Alborn, H. T., Banchio, E., and Tumlinson, J. H. (2003a). Quantitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory. *Planta* 216, 665–673. doi: 10.1007/s00425-002-0898-y

Schmelz, E. A., Alborn, H. T., Engelberth, J., and Tumlinson, J. H. (2003b). Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. *Plant Physiol.* 133, 295–306. doi: 10.1104/pp.103.024174

Schmelz, E. A., Alborn, H. T., and Tumlinson, J. H. (2001). The influence of intact-plant and excised-leaf bioassay designs on volicitin- and jasmonic acid-induced sesquiterpene volatile release in *Zea mays*. *Planta* 214, 171–179. doi: 10.1007/s004250100603

Sharkey, T. D., and Singsaas, E. L. (1995). Why plants emit isoprene. *Nature* 374, 769. doi: 10.1038/374769a0

Sharkey, T. D., Wiberley, A. E., and Donohue, A. R. (2008). Isoprene emission from plants: why and how. *Ann. Bot.* 101, 5–18. doi: 10.1093/aob/mcm240

Shulaev, V., Silverman, P., and Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385, 718–721. doi: 10.1038/385718a0

Singsaas, E. L., Lerdau, M., Winter, K., and Sharkey, T. D. (1997). Isoprene increases thermotolerance of isoprene-emitting species. *Plant Physiol.* 115, 1413–1420. doi: 10.1104/pp.115.4.1413

Spinelli, F., Cellini, A., Marchetti, L., Mudigere Nagesh, K., and Piovene, C. (2011). "Emission and function of volatile organic compounds in response to abiotic stress," in *Abiotic Stress in Plants – Mechanisms and Adaptations*, eds A. K. Shanker and B. Venkateswarlu (Rijeka: InTech), 367–394.

Staats, M., Van Baarlen, P., and Van Kan, J. A. L. (2005). Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. *Mol. Biol. Evol.* 22, 333–346. doi: 10.1093/molbev/msi020

Staples, R. C. (2000). Research on the rust fungi during the twentieth century. *Annu. Rev. Phytopathol.* 38, 49–69. doi: 10.1146/annurev.phyto.38.1.49

Staudt, M., Jackson, B., El-Aouni, H., Buatois, B., Lacroze, J.-P., Poëssel, J.-L., et al. (2010). Volatile organic compound emissions induced by the aphid *Myzus persicae* differ among resistant and susceptible peach cultivars and a wild relative. *Tree Physiol.* 10, 1320–1334. doi: 10.1093/treephys/tpq072

Staudt, M., and Lhoutellier, L. (2007). Volatile organic compound emission from holm oak infested by gypsy moth larvae: evidence for distinct responses in damaged and undamaged leaves. *Tree Physiol.*

27, 1433–1440. doi: 10.1093/treephys/27.10.1433

Staudt, M., and Lhoutellier, L. (2011). Monoterpene and sesquiterpene emissions from *Quercus coccifera* exhibit interacting responses to light and temperature. *Biogeosciences* 8, 2757–2771. doi: 10.5194/bg-8-2757-2011

Steindel, F., Beauchamp, J., Hansel, A., Kesselmeier, J., Kleist, E., Kuhn, U., et al. (2005). Stress induced VOC emissions from mildew infested oak. *Geophys. Res. Abstr.* 7, EGU05-A-03010.

Toome, M., Randjärv, P., Copolovici, L., Niinemets, Ü., Heinsoo, K., Luik, A., et al. (2010). Leaf rust induced volatile organic compounds signalling in willow during the infection. *Planta* 232, 235–243. doi: 10.1007/s00425-010-1169-y

Tuomi, J., Niemelä, P., Rousi, M., Sirén, S., and Vuorisalo, T. (1998). Induced accumulation of foliage phenols in mountain birch: branch response to defoliation? *Am. Nat.* 132, 602–608. doi: 10.1086/284875

Turlings, T. C. J., Davison, A. C., and Tamò, C. (2004). A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. *Physiol. Entomol.* 29, 45–55. doi: 10.1111/j.1365-3032.2004.0362.x

Turlings, T. C. J., Hiltpold, I., and Rasmann, S. (2012). The importance of root-produced volatiles as foraging cues for entomopathogenic nematodes. *Plant Soil* 358, 51–60. doi: 10.1007/s11104-012-1295-3

Turlings, T. C. J., Jeanbourquin, P. M., Held, M., and Degen, T. (2005). Evaluating the induced-odour emission of a Bt maize and its attractiveness to parasitic wasps. *Transgenic Res.* 14, 807–816. doi: 10.1007/s11248-005-0008-6

Vadassery, J., Scholz, S. S., and Mithofer, A. (2012). Multiple calmodulin-like proteins in *Arabidopsis* are induced by insect-derived (*Spodoptera littoralis*) oral secretion. *Plant Signal. Behav.* 7, 1277–1280. doi: 10.4161/psb.21664

Vickers, C. E., Gershenzon, J., Lerdau, M. T., and Loreto, F. (2009). A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nat. Chem. Biol.* 5, 283–291. doi: 10.1038/nchembio.158

Vuorinen, T., Nerg, A.-M., Ibrahim, M. A., Reddy, G. V. P., and Holopainen, J. K. (2004). Emission of *Plutella xylostella*-induced compounds from cabbages grown at elevated CO_2 and orientation behavior of the natural enemies. *Plant Physiol.* 135, 1984–1992. doi: 10.1104/pp.104.047084

Vuorinen, T., Nerg, A.-M., Syrjälä, L., Peltonen, P., and Holopainen, J. K. (2007). *Epirrita autumnata* induced VOC emission of silver birch differ from emission induced by leaf fungal pathogen. *Arthropod Plant Interact.* 1, 159–165. doi: 10.1007/s11829-007-9013-4

Ward, B. B., Courtney, K. J., and Langenheim, J. H. (1997). Inhibition of *Nitrosomonas europaea* by monoterpenes from coastal redwood (*Sequoia sempervirens*) in whole-cell studies. *J. Chem. Ecol.* 23, 2583–2598. doi: 10.1023/B:JOEC.0000006668.48855.b7

Wu, J., Hettenhausen, C., Schuman, M. C., and Baldwin, I. T. (2008). A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. *Plant Physiol.* 146, 927–939. doi: 10.1104/pp.107.114785

Xugen, S., and Luqin, Q. (2006). Effect of volatiles from plants on the selectivity of *Tetranychus viennensis* for different host plants. *Front For. China* 1, 105–108. doi: 10.1007/s11461-005-0018-9

Yang, Z., Sakai, M., Sayama, H., Shimeno, T., Yamaguchi, K., and Watanabe, N. (2009). Elucidation of the biochemical pathway of 2-phenylethanol from shikimic acid using isolated protoplasts of rose flowers. *J. Plant Physiol.* 166, 887–891. doi: 10.1016/j.jplph.2008.11.001

Yoda, H., Yamaguchi, Y., and Sano, H. (2003). Induction of hypersensitive cell death by hydrogen peroxide produced through polyamine degradation in tobacco plants. *Plant Physiol.* 132, 1973–1981. doi: 10.1104/pp.103.024737

Zhang, Q. H., Birgersson, G., Zhu, J. W., Lofstedt, C., Lofqvist, J., and Schlyter, F. (1999). Leaf volatiles from nonhost deciduous trees: variation by tree species, season and temperature, and electrophysiological activity in *Ips typographus*. *J. Chem. Ecol.* 25, 1923–1943. doi: 10.1023/A:1020994119019

Zhang, Q. H., and Schlyter, F. (2004). Olfactory recognition and behavioural avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agric. For. Entomol.* 6, 1–19. doi: 10.1111/j.1461-9555.2004.00202.x

Zhang, R., Wang, B., Jian, O., Li, J., and Wang, Y. (2008). *Arabidopsis* indole synthase, a homolog of tryptophan synthase alpha, is an enzyme involved in the Trp-independent indole-containing metabolite biosynthesis. *J. Integr. Plant Biol.* 50, 1070–1077. doi: 10.1111/j.1744-7909.2008.00729.x

Zhao, N., Guan, J., Ferrer, J.-L., Engle, N., Chern, M., Ronald, P., et al. (2010). Biosynthesis and emission of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol. Biochem.* 48, 279–289. doi: 10.1016/j.plaphy.2010.01.023

Zhuang, X., Fiesselmann, A., Zhao, N., Chen, H., Frey, M., and Chen, F. (2012). Biosynthesis and emission of insect herbivory-induced volatile indole in rice. *Phytochemistry* 73, 15–22. doi: 10.1016/j.phytochem.2011.08.029

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 March 2013; accepted: 27 June 2013; published online: 23 July 2013.

Citation: Niinemets Ü, Kännaste A and Copolovici L (2013) Quantitative patterns between plant volatile emissions induced by biotic stresses and the degree of damage. Front. Plant Sci. 4:262. doi: 10.3389/fpls.2013.00262

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Niinemets, Kännaste and Copolovici. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 30 May 2013
doi: 10.3389/fpls.2013.00165

The rhizosphere revisited: root microbiomics

Peter A. H. M. Bakker*, Roeland L. Berendsen, Rogier F. Doornbos, Paul C. A. Wintermans and Corné M. J. Pieterse

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Philippe Reymond, University of Lausanne, Switzerland
Anton Hartmann, Helmholtz Zentrum München; German Research Center for Environmental Health, Germany

****Correspondence:***
Peter A. H. M. Bakker, Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, Netherlands
e-mail: p.a.h.m.bakker@uu.nl

The rhizosphere was defined over 100 years ago as the zone around the root where microorganisms and processes important for plant growth and health are located. Recent studies show that the diversity of microorganisms associated with the root system is enormous. This rhizosphere microbiome extends the functional repertoire of the plant beyond imagination. The rhizosphere microbiome of *Arabidopsis thaliana* is currently being studied for the obvious reason that it allows the use of the extensive toolbox that comes with this model plant. Deciphering plant traits that drive selection and activities of the microbiome is now a major challenge in which *Arabidopsis* will undoubtedly be a major research object. Here we review recent microbiome studies and discuss future research directions and applicability of the generated knowledge.

Keywords: plant roots, microbial communities, extended phenotype, *Arabidopsis thaliana*, *Pseudomonas* spp

INTRODUCTION

Ever since Lorenz Hiltner, more than a century ago, defined the rhizosphere as the soil compartment influenced by plant roots (Hiltner, 1904; Hartmann et al., 2008), this hotspot for microbial interactions and activities has received ample attention from scientists in different disciplines. Also the above ground plant surface, the so-called phyllosphere, harbors microbial communities that have more recently been studied in detail (Vorholt, 2012). The microbial activity in the rhizosphere is essential for plant functioning as it assists the plant in nutrient uptake and offers protection against pathogen attack (Berendsen et al., 2012). Microbiological studies in the soil environment are hampered by the fact that the largest proportion of soil bacteria as yet cannot be cultured (Amann et al., 1995; Kent and Triplett, 2002; Doornbos et al., 2012). However, developments in metagenomics provide a more complete picture of the rhizosphere microbiome (Leveau, 2007; Sorensen et al., 2009; Hirsch and Mauchline, 2012). Thus the microbial players in the rhizosphere are on their way to be exposed and, perhaps more importantly, transcriptomic studies of the microbiome have been initiated to reveal microbial activities in complex environments (Urich et al., 2008; Gosalbes et al., 2012; Jansson et al., 2012). Unraveling processes that drive selection and activities of the rhizosphere microbiome will open up new avenues to manipulate crop health and yield. In this paper, we review and discuss recent developments in rhizosphere microbiome studies.

THE RHIZOSPHERE EFFECT

Compared to non-rooted bulk soil, the soil compartment directly around the plant root contains much larger populations of microorganisms (Foster et al., 1983). The increased microbial numbers and activities in the rhizosphere are due to the release of large amounts of organic carbon by the plant roots (Walker et al., 2003; Bais et al., 2006; Hartmann et al., 2009). In their extensive review, Jones et al. (2009) describe loss of root cap and border cells, insoluble mucilage, soluble root exudates, volatile organic carbon, flow of carbon to root associated symbionts, and death and lysis of root cells as the major processes of rhizodeposition. Soil microorganisms are chemotactically attracted to the plant root exudates, after which they proliferate in this carbon rich environment (Lugtenberg and Kamilova, 2009). Carbon limitation could be demonstrated in bulk soil but not in the rhizosphere using *Pseudomonas fluorescens* strains carrying carbon-limitation reporter systems (Van Overbeek et al., 1997; Koch et al., 2001). Given the fact that plant root exudates differ between plant species (Rovira, 1969), differences in rhizosphere microbiomes of different plant species are to be expected. Indeed plant-specific microbial communities could be isolated from roots in studies comparing, for example, wheat, ryegrass, bentgrass, and clover (Grayston et al., 1998), or wheat and canola (Germida et al., 1998). Also within a specific bacterial group like fluorescent *Pseudomonas* spp., plant species-specific rhizosphere populations could be isolated (Glandorf et al., 1993; Lemanceau et al., 1995). More recent studies, in which the rhizosphere microbiomes were characterized based on direct extraction of total community DNA, also provide strong evidence for plant species-specific microbiomes (Miethling et al., 2000; Smalla et al., 2001; Kirk et al., 2005; Inceoglu et al., 2013). The roots of wheat, maize, rape, and barrel clover were shown to carry different bacterial communities as a consequence of assimilation of root exudates (Haichar et al., 2008). Bacterial community structures in field grown potato rhizospheres were affected by the growth stage of the plant (Inceoglu et al., 2013). Also at the genotype level within a plant species, specificity of the rhizosphere microbiome has been described (Micallef et al., 2009a,b; Weinert et al., 2011). Micallef et al. (2009b) used *A. thaliana* and showed that the rhizosphere of this model plant mediates a significant change in the bacterial community relative to the bulk soil. To illustrate the rhizosphere effect we compared rhizosphere bacterial

communities of tobacco and *A. thaliana* grown on a potting and a clay soil. In **Figure 1A** total bacterial counts on 1/10 strength tryptic soy agar (TSA) and counts of fluorescent pseudomonads on King's medium B agar (KB) in bulk soil and in the rhizospheres of *A. thaliana* Col-0 and tobacco are presented. The rhizosphere effect is exemplified by the observation that numbers in the rhizosphere are about 10- to 100-fold higher compared to the numbers in bulk soil for both plant species. In **Figures 1B,C**, *Pseudomonas*-specific denaturing gradient gel electrophoresis (DGGE) profiles are shown and compared in a redundancy analysis. For both tobacco and *A. thaliana*, rhizosphere *Pseudomonas* communities are different from those in the bulk soil, and the communities differ between the plant species. In two recent papers the *A. thaliana* root microbiome has been described in detail using pyrosequencing of 16S rRNA gene amplicons (Bulgarelli et al., 2012; Lundberg et al., 2012). Whereas differences between bacterial communities in bulk soil and the rhizosphere were observed in these studies, their focus was on the endophytic compartment. Inside the root, the microbiome clearly differed from the bulk soil and was enriched in Actinobacteria and Proteobacteria (Bulgarelli et al., 2012; Lundberg et al., 2012).

RECRUITMENT OF THE RHIZOSPHERE MICROBIOME

The rhizosphere bacterial community is recruited from the main reservoir of microorganisms present in soil (Normander and Prosser, 2000; De Ridder-Duine et al., 2005; Berg and Smalla, 2009). Thus the soil is an important factor in shaping the rhizosphere microbiome (Garbeva et al., 2008; Lundberg et al.,

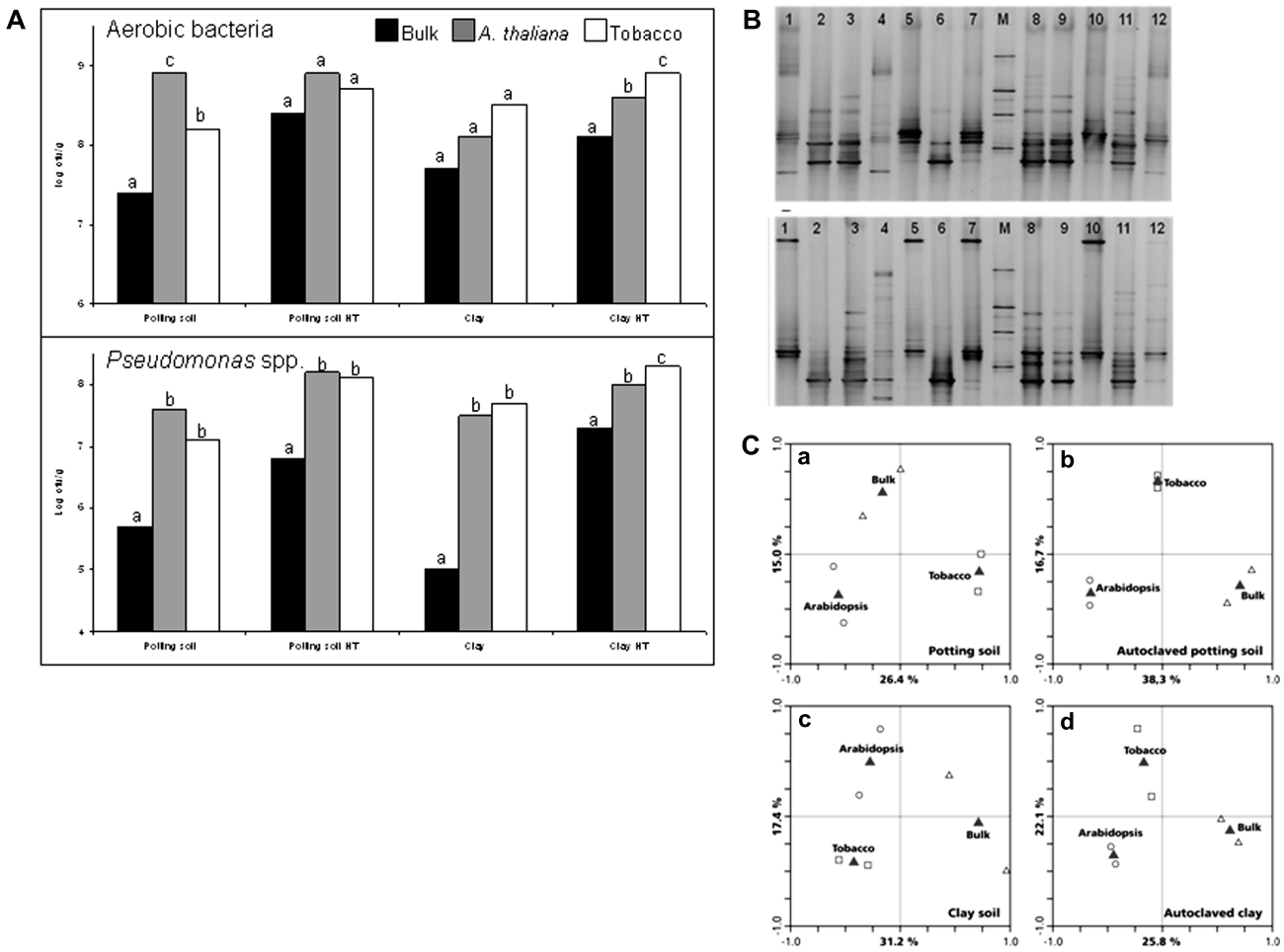


FIGURE 1 | (A) Numbers (log cfu g^{-1}) of culturable aerobic bacteria and *Pseudomonas* spp. in bulk (black bars) and rhizosphere soil of *Arabidopsis* (gray bars) and Tobacco (white bars). Plants were grown for 7 weeks on a potting soil–sand mixture or a clay soil, which were either untreated or autoclaved twice heat treatment (HT) before planting. Different letters indicate significant differences within each soil type. **(B)** Denaturing gradient gel electrophoresis (DGGE) profile showing the *Pseudomonas* spp. community structure from bulk soils (*top gel*: potting soil; *bottom gel*: clay soil), and the rhizospheres of *Arabidopsis* and tobacco grown on these soils. M, reference marker; lanes 1 and 10, *Arabidopsis* rhizosphere grown on non-autoclaved soil; lanes 2 and 6, autoclaved bulk soil; lanes 3 and 9, tobacco rhizosphere grown on autoclaved soil; lanes 4 and 12, non-autoclaved bulk soil; lanes 5 and 7, tobacco rhizosphere grown on non-autoclaved soil; lanes 8 and 11, *Arabidopsis* rhizosphere grown on non-autoclaved soil. **(C)** Ordination biplot generated by redundancy analysis (RDA) of *Pseudomonas*-specific DGGE fingerprints of bulk soil and the rhizospheres of *Arabidopsis* and tobacco grown on **(a)** potting soil–sand mixture; **(b)** autoclaved potting soil–sand mixture; **(c)** clay soil; **(d)** autoclaved clay soil. Open triangles, bulk; open circles, *Arabidopsis* rhizosphere; open squares, tobacco rhizosphere; gray triangles, centroid position of variables.

2012). As described in the previous section, plant genotype is also a driving force for the selection of specific elements from the bulk soil microbial community. Furthermore, when under attack, plants seem to actively select specific elements of their bacterial rhizosphere microflora. This is most clearly observed in so-called disease suppressive soils, in which disease will not develop despite the presence of a virulent pathogen and a susceptible plant. Disease suppressiveness is due to microbial activity and usually needs an outbreak of disease to develop (Mazzola, 2002). A well-studied example is take-all decline (TAD), which develops in continuous wheat cultures after a severe outbreak of the take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Weller et al., 2002). Heat treatment abolishes suppressiveness and the suppressiveness of TAD soil is transferable to a disease conducive soil by mixing small quantities of decline soil through conducive soil. Under continuous wheat cropping, a specific group of fluorescent pseudomonads that produce 2,4-diacetylphloroglucinol (DAPG) is enriched in the rhizosphere and these bacteria appear to be responsible for TAD (Raaijmakers and Weller, 1998). Additional bacterial taxa that may be involved in TAD have more recently been identified using 16S rRNA-based techniques (Sanguin et al., 2009; Schreiner et al., 2010). The specific selection of plant protecting bacteria in the rhizosphere under pathogen attack is supported by a recent study of Mavrodi et al. (2012). They observed that under irrigation the wheat rhizosphere recruits DAPG producing pseudomonads, whereas under dry conditions phenazine producing pseudomonads are recruited. Under irrigated conditions *G. graminis* var. *tritici* is the major soil borne pathogen of wheat, whereas under dry conditions *Rhizoctonia solani* is the main problem. Strikingly *G. graminis* var. *tritici* is more sensitive to DAPG, whereas *R. solani* is more sensitive to phenazines. Thus, under conditions that favor a specific pathogen, antagonists that are most effective against this pathogen are selected by the plant. Also for other disease suppressive soils specific elements of the microbiome have been identified that are associated with suppressiveness. In a *Fusarium* wilt suppressive soil the production of redox-active phenazines by fluorescent pseudomonads and competition for carbon by non-pathogenic *Fusarium oxysporum* have a synergistic effect that may establish suppressiveness (Mazurier et al., 2009). In a soil suppressive to potato common scab a microbial consortium that is associated with suppressiveness was identified (Rosenzweig et al., 2012). For a soil suppressive to black root rot of tobacco, caused by *Thielaviopsis basicola*, several bacterial taxa, including *Pseudomonas*, *Azospirillum*, *Gluconacetobacter*, *Burkholderia*, *Comamonas*, and Sphingomonadaceae, were shown to be more prevalent in the suppressive than in the conducive soil (Kyselkova et al., 2009). To identify bacteria involved in soil suppressiveness against *R. solani*, Mendes et al. (2011) used PhyloChip analysis, which allows simultaneous detection of ∼60,000 bacterial and archaeal operational taxonomic units (OTUs). Over 33,000 OTUs were detected in the rhizospheres of sugar beet grown in *R. solani* suppressive and conducive soil. Taxa that were more abundant in suppressive soil and in a mixture of conducive soil with 10% suppressive soil than in conducive soil, but also more abundant in suppressive soil amended with *R. solani* than in suppressive soil without the pathogen, were identified. Seventeen taxa belonging to the β-proteobacteria, γ-proteobacteria, and the firmicutes were closely associated with disease suppressiveness (Mendes et al., 2011). In all disease suppressive soils mentioned here, consortia of antagonistic microorganisms seem to be recruited by the rhizosphere under pathogen attack.

Not only attack by soil borne pathogens results in the recruitment of beneficial microbes in the rhizosphere. Foliar feeding of aphids on pepper plants reduced disease development caused by the bacterial pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (Lee et al., 2012). Aphid infestation resulted in increased population densities of the plant beneficial *Bacillus subtilis*, whereas it reduced rhizosphere populations of plant pathogenic *Ralstonia solanacearum*. Similar results were found for whitefly infestation of pepper plants, leading to increased resistance to pathogens and to changes in the rhizosphere microbiome (Yang et al., 2011). In the aphid and whitefly systems it would be interesting to investigate possible recruitment of rhizobacteria that produce insecticidal toxins, a feature of certain rhizosphere pseudomonads that was recently reported (Pechy-Tarr et al., 2013; Ruffner et al., 2013) *A. thaliana* plants exposed to methyl jasmonate showed a shift in their bacterial rhizosphere microbiome, including taxa that are associated with disease suppression, based on 16S rRNA gene amplicon pyrosequencing (Carvalhais et al., 2013). However, in a study by Doornbos et al. (2011), leaf application of jasmonic acid did not significantly affect the rhizosphere bacterial community of *A. thaliana*, based on DGGE analysis of 16S rRNA gene amplicons. Rudrappa et al. (2008) showed that *A. thaliana* plants infected by the bacterial leaf pathogen *P. syringae* pv. *tomato*, secrete elevated levels of malic acid in the rhizosphere. Malic acid stimulates binding to roots and biofilm formation on roots by *Bacillus subtilis* strain FB17, a beneficial microbe that can induce systemic resistance against diseases. Thus the plant benefits from protection against disease by the bacteria and in turn provides the bacteria with a more favorable environment. The recruitment of FB17 was recently shown to be mediated by root responses triggered by pathogen-derived microbe-associated molecular patterns in the leaves. Early suppression of defense genes by FB17 was postulated to facilitate colonization of this *Bacillus subtilis* strain on *A. thaliana* roots (Lakshmanan et al., 2012). Induced systemic resistance by *P. putida* KT2440 in *A. thaliana* is related to as yet unknown compounds in the root exudate that are modulated by the bacterium (Matilla et al., 2010). Thus not only pathogenic and symbiotic microorganisms seem to modulate host immunity to their own benefit, but also plant beneficial microorganisms seem to use this strategy (Zamioudis and Pieterse, 2012).

Drought stress is also a shaping factor for the rhizosphere microbiome. Drought-sensitive pepper plants that were grown under desert farming selected for a root microbiome that was enriched for bacteria that can increase photosynthesis and plant biomass production under drought stress (Marasco et al., 2012). Soil nitrogen availability influenced rhizosphere microbial communities of *Medicago truncatula* only in the presence of the plant, and it was suggested that the adaptive strategy of the plant to environmental constraints is a major factor in shaping the rhizosphere microbiome (Zancarini et al., 2012).

Root exudates play an important role in shaping the rhizosphere microbiome. In the rhizosphere of maize, exudation of the benzoxazinone DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) resulted in increased population densities of a *P. putida* strain with plant beneficial characteristics (Neal et al., 2012). In *A. thaliana*, active exudation of phytochemicals mediated by ABC (ATP-binding cassette) transporters was demonstrated (Badri et al., 2012). In the absence of the plant, blends of collected *A. thaliana* root exudates modulated the soil microbiome. Phenolic compounds in the root exudates were suggested to act as specific substrates and signals for soil bacteria (Badri et al., 2013). Plant age affects rhizosphere bacterial communities of *A. thaliana*, suggested to be due to changes in root exudation (Micallef et al., 2009a). In an elegant study by Chaparro et al. (2013), combining metatranscriptomics and metabolomics, a strong correlation was observed between compounds released from the roots at different stages of plant development and the expression of microbial genes involved in metabolism of specific compounds.

Overall, evidence is accumulating that plants shape their rhizosphere microbiome to their own benefit, making sophisticated use of the functional repertoire of the microbiome.

ACTIVATION OF MICROBIOME FUNCTIONS

Next to recruitment of specific soil microbes into the rhizosphere microbiome, plant roots also influence specific functions of the microbiome. Quorum sensing, regulation of microbial gene expression in response to cell density, is an important mechanism to regulate microbial activities. Such activities include antibiotic production, biofilm formation, conjugation, motility, symbiosis, and virulence (Miller and Bassler, 2001). This regulatory mechanism is not only important within a bacterial population but also between bacterial populations (Pierson and Pierson, 2007; Hosni et al., 2011). Interkingdom communication based on quorum sensing signaling molecules, *N*-acyl homoserine lactone (AHL) signals, has also been reported. Proteome analysis revealed that *M. truncatula* responds significantly to AHLs from both symbiotic and pathogenic bacteria (Mathesius et al., 2003). AHL signal molecules produced by *Serratia liquefaciens* and *P. putida* in the rhizosphere of tomato, protected the tomato plants against the fungal leaf pathogen *Alternaria alternata*, through the induction of systemic resistance (Schuhegger et al., 2006). Similarly, growth and disease resistance of *A. thaliana* are modulated by AHLs (Von Rad et al., 2008; Schikora et al., 2011; Liu et al., 2012; Schenk et al., 2012). Interkingdom communication can also involve effects of eukaryotes on bacterial gene expression. Plants can effectively interfere with quorum sensing in bacteria by producing so called AHL mimics (Teplitski et al., 2000; Gao et al., 2003). Thus there seems to be a plant-mediated fine tuning of bacterial gene expression in the rhizosphere. Microarray-based transcriptomic profiling of specific bacteria in response to root exudates of axenically grown plants has been used to identify genes in *Pseudomonas* (Mark et al., 2005) and *Bacillus amyloliquefaciens* (Fan et al., 2012) that are involved in plant microbe interactions. Using a similar approach, effects of phosphate availability on transcriptional responses of *Pseudomonas* in the rhizosphere of *Lolium perenne* was investigated (Zysko et al., 2012). All these studies show that there is a significant impact of root exudates on bacterial gene expression. The studies by Okubara and Bonsall (2008) and Kwak et al. (2012) focused on effects of host cultivar on the production of DAPG by fluorescent pseudomonads. The production of this antifungal metabolite, that plays a central role in TAD, depends on the genotypes of both the plant and the bacterial strain involved in the interaction. Effects of pathogen infection on gene expression and functional diversity has been the focus of several studies. Infection of wheat roots by *G. graminis* var. *tritici* changed gene expression of *P. fluorescens* Pf29Arp (Barret et al., 2009). Strain Pf29Arp was suggested to show an adaptive response to the so called pathorhizosphere of necrotic roots. In the rhizosphere of strawberry, infection with *Verticillium dahliae* increased hydrogen cyanide (HCN) biosynthesis gene expression in *Pseudomonas* sp. LBUM300 (DeCoste et al., 2010). HCN production by beneficial rhizobacteria has been suggested as a mechanism of biological control, and thus this study suggests that upon root pathogen attack such biocontrol activity is stimulated. Even stronger evidence that suggests up-regulation of antifungal activity upon pathogen attack comes from an elegant study by Jousset et al. (2011). They demonstrated in a split root system that infection of barley roots with *Pythium ultimum* on one side of the system, enhanced *phlA* gene expression, required for DAPG production, in *P. fluorescens* CHA0 that colonized the other side of the root system. Root exudation of fumaric acid, *p*-coumaric acid and vanillic acid was increased in *Pythium* infected plants and these phenolic acids increase *phlA* gene expression in a dose-dependent manner (Jousset et al., 2011). Thus plants seem to respond to pathogen infection by systemic signaling leading to enhanced biocontrol activity in the microbiome.

PERSPECTIVE

Exciting new insights in interkingdom signaling in the rhizosphere and the resulting effects on plant performance have emerged during the last decade. *A. thaliana* has been the model system of choice in several recent studies (Doornbos et al., 2011; Schwachtje et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012; Van de Mortel et al., 2012) because a large number of accessions and well characterized mutants are readily available, and transcriptomic and metabolomic analyses are standard procedure for this plant species. Revealing the composition of the microbiome and unraveling the metatranscriptome will certainly help to shed light on the very dark rhizosphere environment. However, the rhizosphere is a dynamic environment in which the microbiome will rapidly evolve in space and time. Obviously, to date many rhizosphere metagenomic studies have focused on a single or a few time points and most studies do not take spatial dynamics into account. Metabolic profiling of living microbial colonies facilitates studying spatiotemporal dynamics of metabolite production in microbial communities (Moree et al., 2012; Watrous et al., 2012). The nanospray desorption electrospray ionization (nano-DESI) mass spectrometry technology used in these studies allows for direct sampling from plant surfaces (Traxler and Kolter, 2012) and is thus a promising development for rhizosphere studies. Given the rapid technological developments, the editors of the classic book "Plant roots: the hidden half" (Eshel and Beeckman, 2013), may want to look for a new title for the next edition.

REFERENCES

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.

Badri, D. V., Chaparro, J. M., Manter, D. K., Martinoia, E., and Vivanco, J. M. (2012). Influence of ATP-binding cassette transporters in root exudation of phytoalexins, signals, and in disease resistance. *Front. Plant Sci.* 3:149. doi: 10.3389/fpls.2012.00149

Badri, D. V., Chaparro, J. H., Zhang, R., Shen, Q., and Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J. Biol. Chem.* 288, 4502–4512. doi: 10.1074/jbc.M112.433300

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Barret, M., Frey-Klett, P., Guillerm-Erckelboudt, A. V., Boutin, M., Guernec, G., and Sarniguet, A. (2009). Effect of wheat roots infected with the pathogenic fungus *Gaeumannomyces graminis* var. *tritici* on gene expression of the biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp. *Mol. Plant Microbe Interact.* 22, 1611–1623. doi: 10.1094/MPMI-22-12-1611

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G., and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi: 10.1111/j.1574-6941.2009.00654.x

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Carvalhais, L. C., Dennis, P. G., Badri, D. V., Tyson, G. W., Vivanco, J. M., and Schenk, P. M. (2013). Activation of the jasmonic acid plant defence pathway alters the composition of rhizosphere bacterial communities. *PLoS ONE* 8:e56457. doi: 10.1371/journal.pone.0056457

Chaparro, J. M., Badri, D. V., Bakker, M. G., Sugiyama, A., Manter, D. K., and Vivanco, J. M. (2013). Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PLoS ONE* 8:e55731. doi: 10.1371/journal.pone.0055731

DeCoste, N. J., Gadkar, V. J., and Filion, M. (2010). Verticillium dahliae alters *Pseudomonas* spp. populations and HCN gene expression in the rhizosphere of strawberry. *Can. J. Microbiol.* 56, 906–915. doi: 10.1139/W10-080

De Ridder-Duine, A. S., Kowalchuk, G. A., Klein Gunnewiek, P. J. A., Smant, W., Van Veen, J. A., and De Boer, W. (2005). Rhizosphere bacterial community composition in natural stands of *Carex arenaria* (sand sedge) is determined by bulk soil community composition. *Soil Biol. Biochem.* 37, 349–357. doi: 10.1016/j.soilbio.2004.08.005

Doornbos, R. F., Geraats, B. P. J., Kuramae, E. E., Van Loon, L. C., and Bakker, P. A. H. M. (2011). Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 24, 395–407. doi: 10.1094/MPMI-05-10-0115

Doornbos, R. F., Van Loon, L. C., and Bakker, P. A. H. M. (2012). Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. *Agron. Sustain. Dev.* 32, 227–243. doi: 10.1007/s13593-011-0028-y

Eshel, A., and Beeckman, T. (2013). *Plant Roots: The Hidden Half*, 4th Edn. Boca Raton, FL: CRC Press.

Fan, B., Cravalhais, L. C., Becker, A., Fedoseyenko, D., Von Wiren, N., and Borriss, R. (2012). Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. *BMC Microbiol.* 12:116. doi: 10.1186/1471-2180-12-116

Foster, R. C., Rovira, A. D., and Cock, T. W. (1983). *Ultrastructure of the Root–Soil Interface*. St. Paul, MN: The American Phytopathological Society.

Gao, M., Teplitski, M., Robinson, J. B., and Bauer, D. W. (2003). Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. *Mol. Plant Microbe Interact.* 16, 827–834. doi: 10.1094/MPMI.2003.16.9.827

Garbeva, P. V., Van Elsas, J. D., and Van Veen, J. A. (2008). Rhizosphere microbial community and its response to plant species and soil history. *Plant Soil* 302, 19–32. doi: 10.1007/s11104-007-9432-0

Germida, J. J., Siciliano, S. D., De Freitas, J. R., and Seib, A. M. (1998). Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiol. Ecol.* 26, 43–50. doi: 10.1111/j.1574-6941.1998.tb01560.x

Glandorf, D. C. M., Peters, L. G. L., Van der Sluis, I., Bakker, P. A. H. M., and Schippers, B. (1993). Crop specificity of rhizosphere pseudomonads and the involvement of root agglutinins. *Soil Biol. Biochem.* 25, 981–989. doi: 10.1016/0038-0717(93)90144-Z

Gosalbes, M. J., Abellan, J. J., Durban, A., Perez-Cobas, A. E., Latorre, A., and Moya, A. (2012). Metagenomics of human microbiome: beyond 16s rDNA. *Clin. Microbiol. Infect. Dis.* 18, 47–49. doi: 10.1111/j.1469-0691.2012.03865.x

Grayston, S. J., Wang, S., Campbell, C. D., and Edwards, A. C. (1998). Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* 30, 369–378. doi: 10.1016/S0038-0717(97)00124-7

Haichar, F. Z., Marol, C., Berge, O., Rangel-Castro, J. I., Prosser, J. I., Balesdent, J., et al. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME J.* 2, 1221–1230. doi: 10.1038/ismej.2008.80

Hartmann, A., Rothballer, M., and Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil* 312, 7–14. doi: 10.1007/s11104-007-9514-z

Hartmann, A., Schmid, M., Van Tuinen, D., and Berg, G. (2009). Plant-driven selection of microbes. *Plant Soil* 321, 235–257. doi: 10.1007/s11104-008-9814-y

Hiltner, L. (1904). Uber neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderden berucksichtigung und Brache. *Arb. Dtsch. Landwirtsch. Gesellschaft* 98, 59–78.

Hirsch, P. R., and Mauchline, T. H. (2012). Who's who in the plant root microbiome? *Nat. Biotechnol.* 30, 961–962. doi: 10.1038/nbt.2387

Hosni, T., Moretti, C., Devescovi, G., Suarez-Moreno, Z. R., Fatmi, M. B., Guarnaccia, C., et al. (2011). Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *ISME J.* 5, 1857–1870. doi: 10.1038/ismej.2011.65

Inceoglu, O., Van Overbeek, L. S., Salles, J. F., and Van Elsas, J. D. (2013). The normal operating range of bacterial communities in soil used for potato cropping. *Appl. Environ. Microbiol.* 79, 1160–1170. doi: 10.1128/AEM.02811-12

Jansson, J. K., Neufeld, J. D., Moran, M. A., and Gilbert, J. A. (2012). Omics for understanding microbial functional dynamics. *Environ. Microbiol.* 14, 1–3. doi: 10.1111/j.1462-2920.2011.02518.x

Jones, D. L., Nguyen, C., and Finlay, R. D. (2009). Carbon flow in the rhizosphere: carbon trading at the soil–root interface. *Plant Soil* 321, 5–33. doi: 10.1007/s11104-009-9925-0

Jousset, A., Rochat, L., Lanoue, A., Bonkowski, M., Keel, C., and Scheu, S. (2011). Plants respond to pathogen infection by enhancing the antifungal gene expression of root-associated bacteria. *Mol. Plant Microbe Interact.* 24, 352–358. doi: 10.1094/MPMI-09-10-0208

Kent, A. D., and Triplett, E. W. (2002). Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annu. Rev. Microbiol.* 56, 211–236. doi: 10.1146/annurev.micro.56.012302.161120

Kirk, J. L., Klironomos, J. N., Lee, H., and Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environ. Pollut.* 133, 455–465. doi: 10.1016/j.envpol.2004.06.002

Koch, B., Worm, J., Jensen, L. E., Hojberg, O., and Nybroe, O. (2001). Carbon limitation induces σs-dependent gene expression in *Pseudomonas fluorescens* in soil. *Appl. Environ. Microbiol.* 67, 3363–3370. doi: 10.1128/AEM.67.8.3363-3370.2001

Kwak, Y. S., Bonsall, R. F., Okubara, P. A., Paulitz, T. C., Thomashow, L. S., and Weller, D. M. (2012). Factors impacting the activity of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* against take-all of wheat. *Soil Biol. Biochem.* 54, 48–56. doi: 10.1016/j.soilbio.2012.05.012

Kyselkova, M., Kopecky, J., Frapolli, M., Defago, G., Sagova-Mareckova, M., Grundmann, G. L., et al. (2009). Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J.* 3, 1127–1138. doi: 10.1038/ismej.2009.61

Lakshmanan, V., Kitto, S. L., Caplan, J. L., Hsueh, Y. H., Kearns, D. B., Wu, Y. S., et al. (2012). Microbe-associated molecular patterns-triggered root responses mediate beneficial rhizobacterial recruitment in *Arabidopsis*. *Plant Physiol.* 160, 1642–1661. doi: 10.1104/pp.112.200386

Lee, B., Lee, S., and Ryu, M. R. (2012). Foliar aphid feeding recruits rhizosphere bacteria and primes plant immunity against pathogenic and non-pathogenic bacteria in pepper. *Ann. Bot.* 110, 281–290. doi: 10.1093/aob/mcs055

Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J.-M., et al. (1995). Effect of two plant species, flax (*Linum usitatissimum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. *Appl. Environ. Microbiol.* 61, 1004–1012.

Leveau, J. H. J. (2007). The magic and menace of metagenomics: prospects for the study of plant growth-promoting rhizobacteria. *Eur. J. Plant Pathol.* 119, 279–300. doi: 10.1007/s10658-007-9186-9

Liu, F., Bian, Z., Jia, Z., Zhao, Q., and Song, S. (2012). The GCR1 and GPA1 participate in promotion of *Arabidopsis* primary root elongation induced by *N*-acyl-homoserine lactones, the bacterial quorum-sensing signals. *Mol. Plant Microbe Interact.* 25, 677–683. doi: 10.1094/MPMI-10-11-0274

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Lundberg, D. S., Lebeis, S. L., Herrera Paredes, S., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Marasco, R., Rolli, E., Ettoumi, B., Vigani, G., Mapelli, F., Borin, S., et al. (2012). A drought resistance-promoting microbiome is selected by root system under desert farming. *PLoS ONE* 7:e48479. doi: 10.1371/journal.pone.0048479

Mark, G. L., Dow, J. M., Kiely, P. D., Higgings, H., Haynes, J., Baysse, C., et al. (2005). Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe–plant interactions. *Proc. Natl. Acad. Sci. U.S.A.* 102, 17454–17459. doi: 10.1073/pnas.0506407102

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B. G., et al. (2003). Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc. Natl. Acad. Sc. U.S.A.* 100, 1444–1449. doi: 10.1073/pnas.262672599

Matilla, M. A., Ramos, J. L., Bakker, P. A. H. M., Doornbos, R. F., Vivanco, J. M., Badri, D. V., et al. (2010). *Pseudomonas putida* KT2440 causes induced systemic resistance and changes in *Arabidopsis* root exudation. *Environ. Microbiol. Rep.* 2, 381–388. doi: 10.1111/j.1758-2229.2009.00091.x

Mavrodi, O. V., Mavrodi, D. V., Parejko, J. A., Thomashow, L. S., and Weller, D. M. (2012). Irrigation differentially impacts populations of indigenous antibiotic-producing *Pseudomonas* spp. in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 78, 3214–3220. doi: 10.1128/AEM.07968-11

Mazurier, S., Corberand, T., Lemanceau, P., and Raaijmakers, J. M. (2009). Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to *Fusarium* wilt. *ISME J.* 3, 977–991. doi: 10.1038/ismej.2009.33

Mazzola, M. (2002). Mechanisms of natural soil suppressiveness to soilborne diseases. *Antonie Van Leeuwenhoek* 81, 557–564. doi: 10.1023/A:1020557523557

Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., Van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Micallef, S. A., Channer, S., Shiaris, M. P., and Colon-Carmona, A. (2009a). Plant age and genotype impact the progression of bacterial community succession in the *Arabidopsis rhizosphere*. *Plant Signal. Behav.* 4, 777–780. doi: 10.4161/psb.4.8.9229

Micallef, S. A., Shiaris, M. P., and Colon-Carmona, A. (2009b). Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *J. Exp. Bot.* 60, 1729–1742. doi: 10.1093/jxb/erp053

Miethling, R., Wieland, G., Backhaus, H., and Tebbe, C. C. (2000). Variation in microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. *Microb. Ecol.* 40, 43–56. doi: 10.1007/s002480000021

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Moree, W. J., Phelan, V. V., Wu, C.-H., Bandeira, N., Cornett, D. S., Duggan, B. M., et al. (2012). Interkingdom metabolic transformations captured by microbial imaging mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 109, 13811–13816. doi: 10.1073/pnas.1206855109

Neal, A. L., Ahmad, S., Gordon-Weeks, R., and Ton, J. (2012). Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PLoS ONE* 7:e35498. doi: 10.1371/journal.pone.0035498

Normander, B., and Prosser, J. I. (2000). Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Appl. Environ. Microbiol.* 66, 4372–4377. doi: 10.1128/AEM.66.10.4372-4377.2000

Okubara, P. A., and Bonsall, R. F. (2008). Accumulation of *Pseudomonas*-derived 2,4-diacetylphloroglucinol on wheat seedling roots is influenced by host cultivar. *Biol. Control* 46, 322–331. doi: 10.1016/j.biocontrol.2008.03.013

Pechy-Tarr, M., Borel, N., Kupferschmied, P., Turner, V., Binggeli, O., Radovanovic, D., et al. (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ. Microbiol.* 15, 736–750. doi: 10.1111/1462-2920.12050

Pierson, L. S. III, and Pierson, E. A. (2007). Roles of diffusible signals in communication among plant-associated bacteria. *Phytopathology* 97, 227–232. doi: 10.1094/PHYTO-97-2-0227

Raaijmakers, J. M., and Weller, D. M. (1998). Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Mol. Plant Microbe Interact.* 11, 144–152. doi: 10.1094/MPMI.1998.11.2.144

Rosenzweig, N., Tiedje, J. M., Quensen, J. F. III, Meng, Q., and Hao, J. J. (2012). Microbial communities associated with potato common scab-suppressive soil determined by pyrosequencing analyses. *Plant Dis.* 96, 718–725. doi: 10.1094/PDIS-07-11-0571

Rovira, A. D. (1969). Plant root exudates. *Bot. Rev.* 35, 17–34. doi: 10.1007/BF02859887

Rudrappa, T., Czymmek, K. J., Paré, P. W., and Bais, H. P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiol.* 148, 1547–1556. doi: 10.1104/pp.108.127613

Ruffner, B., Pechy-Tarr, M., Ryffel, F., Hoegger, P., Obrist, C., Rindlisbacher, A., et al. (2013). Oral insecticidal activity of plant-associated pseudomonads. *Eviron. Microbiol.* 15, 751–763. doi: 10.1111/j.1462-2920.2012.02884.x

Sanguin, H., Sarniguet, A., Gazengel, K., Moenne-Loccoz, Y., and Grundmann, G. L. (2009). Rhizosphere bacterial communities associated with disease suppressiveness stages of take-all decline in wheat monoculture. *New Phytol.* 184, 694–707. doi: 10.1111/j.1469-8137.2009.03010.x

Schenk, S. T., Stein, E., Kogel, K. H., and Schikora, A. (2012). Arabidopsis growth and defense are modulated by bacterial quorum sensing molecules. *Plant Signal. Behav.* 7, 178–181. doi: 10.4161/psb.18789

Schikora, A., Schenk, S. T., Stein, E., Molitor, A., Zuccaro, A., and Kogel, K. H. (2011). *N*-acyl-homoserine lactone confers resistance toward biotrophic and hemibiotrophic pathogens via altered activation of AtMPK6. *Plant Physiol.* 157, 1407–1418. doi: 10.1104/pp.111.180604

Schreiner, K., Hagn, A., Kyselkova, M., Moenne-Loccoz, Y., Welzl, G., Munch, J. C., et al. (2010). Comparison of barley succession and take-all disease as environmental factors shaping the rhizobacterial community during take-all decline. *Appl. Environ. Microbiol.* 76, 4703–4712. doi: 10.1128/AEM.00481-10

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., et al. (2006). Induction of systemic resistance in tomato by *N*-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29, 909–918. doi: 10.1111/j.1365-3040.2005.01471.x

Schwachtje, J., Karojet, S., Thormalen, I., Bernholz, C., Kunz, S., Brouwer, S., et al. (2011). A naturally associated rhizobacterium of *Arabidopsis thaliana* induces a starvation-like transcriptional response while promoting growth. *PLoS ONE* 6:e29382. doi: 10.1371/journal.pone.0029382

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., et al. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* 67, 4742–4751. doi: 10.1128/AEM.67.10.4742-4751.2001

Sorensen, J., Hauberg Nicolaisen, M., Ron, E., and Simonet, P.

(2009). Molecular tools in rhizosphere microbiology – from single-cell to whole-community analysis. *Plant Soil* 321, 483–512. doi: 10.1007/s11104-009-9946-8

Teplitski, M., Robinson, J. B., and Bauer, W. D. (2000). Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol. Plant Microbe Interact.* 13, 637–648. doi: 10.1094/MPMI.2000.13.6.637

Traxler, M. F., and Kolter, R. (2012). A massively spectacular view of the chemical lives of microbes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 10128–10129. doi: 10.1073/pnas.1207725109

Urich, T., Lanzen, A., Qi, J., Huson, D. H., Schleper, C., and Schuster, S. C. (2008). Simultaneous assessment of soil microbial community structure and function through analysis of the meta-transcriptome. *PLoS ONE* 3:e2527. doi: 10.1371/journal.pone.0002527

Van de Mortel, J. E., De Vos, R. C. H., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., et al. (2012). Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiol.* 160, 2173–2188. doi: 10.1104/pp.112.207324

Van Overbeek, L. S., Van Elsas, J. D., and van Veen, J. A. (1997). *Pseudomonas fluorescens* Tn5-B20 mutant RA92 responds to carbon limitation in soil. *FEMS Microbiol. Ecol.* 24, 57–71. doi: 10.1016/S0168-6496(97)00045-7

Von Rad, U., Klein, I., Dobrev, P. I., Kottova, J., Zazimalova, E., Fekete, A., et al. (2008). Response of *Arabidopsis thaliana* to *N*-hexanoyl-DL-homoserine-lactone, a bacterial quorum sensing molecule produced in the rhizosphere. *Planta* 229, 73–85. doi: 10.1007/s00425-008-0811-4

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840. doi: 10.1038/nrmicro2910

Walker, T. S., Bais, H. P., Grotewold, E., and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiol.* 132, 44–51. doi: 10.1104/pp.102.019661

Watrous, J., Roach, P., Alexandrov, T., Heath, B. S., Yang, J. Y., Kersten, R. D., et al. (2012). Mass spectral molecular networking of living microbial colonies. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1743–E1752. doi: 10.1073/pnas.1203689109

Weinert, N., Piceno, Y., Ding, G. C., Meincke, R., Heuer, H., Berg, G., et al. (2011). PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa. *FEMS Microbiol. Ecol.* 75, 497–506. doi: 10.1111/j.1574-6941.2010.01025.x

Weller, D. M., Raaijmakers, J. M., McSpadden Gardener, B. B., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi: 10.1146/annurev.phyto.40.030402.110010

Yang, J. W., Yi, H. S., Kim, H., Lee, B., Lee, S., Ghim, S. Y., et al. (2011). Whitefly infestation of pepper plants elicits defence responses against bacterial pathogens in leaves and roots and changes the below-ground microflora. *J. Ecol.* 99, 46–56. doi: 10.1111/j.1365-2745.2010.01756.x

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Zancarini, A., Mougel, C., Voisin, A. S., Prudent, M., Salon, C., and Munier-Jolain, N. (2012). Soil nitrogen availability and plant genotype modify the nutrition strategies of *M. truncatula* and the associated rhizosphere microbial communities. *PLoS ONE* 7:e47096. doi: 10.1371/journal.pone.0047096

Zysko, A., Sanguin, H., Hayes, A., Wardleworth, L., Zeef, L. A. H., Sim, A., et al. (2012). Transcriptional response of *Pseudomonas aeruginosa* to a phosphate-deficient *Lolium perenne* rhizosphere. *Plant Soil* 359, 25–44. doi: 10.1007/s11104-011-1060-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2013; paper pending published: 19 April 2013; accepted: 10 May 2013; published online: 30 May 2013.

*Citation: Bakker PAHM, Berendsen RL, Doornbos RF, Wintermans PCA and Pieterse CMJ (2013) The rhizosphere revisited: root microbiomics. Front. Plant Sci. **4**:165. doi: 10.3389/fpls.2013.00165*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Bakker, Berendsen, Doornbos, Wintermans and Pieterse. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 14 May 2013
doi: 10.3389/fpls.2013.00122

ISR meets SAR outside: additive action of the endophyte *Bacillus pumilus* INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper

***Hwe-Su Yi*[1†], *Jung Wook Yang*[1†] *and Choong-Min Ryu*[1,2*]**

[1] *Molecular Phytobacteriology Laboratory, Superbacteria Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea*

[2] *Biosystems and Bioengineering Program, School of Science, University of Science and Technology, Daejeon, South Korea*

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Saskia C. M. Van Wees, Utrecht University, Netherlands
Brigitte Mauch-Mani, Université de Neuchâtel, Switzerland

****Correspondence:***
Choong-Min Ryu, Molecular Phytobacteriology Laboratory, Superbacteria Research Center, Korea Research Institute of Bioscience and Biotechnology, 111 Kwahangro Yuseong, Daejeon 305-806, South Korea.
e-mail: cmryu@kribb.re.kr

† Hwe-Su Yi and Jung Wook Yang have contributed equally to this work.

Induced resistance has been recognized as an attractive tool for plant disease management in modern agriculture. During the last two decades, studies on chemically- and biologically elicited induced resistance have revealed previously unknown features of the plant defense response including defense priming. As a biological trigger for induced resistance, plant growth-promoting rhizobacteria (PGPR) are a group of root-associated bacteria that can reduce plant disease severity and incidence, and augment plant growth and yield under greenhouse and field conditions. We evaluated the potential of an endophytic PGPR, *Bacillus pumilus* INR7, to induce systemic resistance against bacterial spot caused by *Xanthomonas axonopodis* pv. vesicatoria in pepper. Trials in the greenhouse showed significantly less symptom development in pepper plants inoculated with strain INR7 compared to a water treatment. Furthermore, a single dipping treatment with INR7 before transplantation of pepper plants into the field elicited an induced systemic resistance response against bacterial spot caused by artificially infiltration of *X. axonopodis* pv. vesicatoria and even against naturally occurring bacterial spot disease. We identified an additive effect on induced resistance after administration of a combination treatment composed of strain INR7 with a chemical inducer, benzothiadiazole (BTH) in the field. The combination treatment stimulated expression of pepper defense marker genes *CaPR1*, *CaTin1*, and *CaPR4* to a greater extent than did treatment with either agent alone. Similar experiments conducted with tobacco revealed no additive effects under field conditions. Interestingly, co-application of plants with INR7 lifted the growth repressing effect of BTH. Application of BTH onto pepper and tobacco did not affect rhizosphere colonization but supported a higher population density inside plant roots when compared to water-treated control plants. Our results indicate that PGPR can be used in combination with BTH for increased induced resistance capacity under field conditions.

Keywords: PGPR, ISR, SAR, defense priming, biological control

INTRODUCTION

Plants establish multiple layers of defense responses, including physical barriers such as the cuticle and cell wall, as well as chemical defenses such as secretion of antimicrobial or anti-insect compounds (Pieterse et al., 2009). Ross reported a novel mechanism of plant defense called systemic acquired resistance (SAR) that was elicited in upper leaves of tobacco plants only after inoculating *Tobacco mosaic virus* (TMV) onto lower leaves of the same tobacco plants (Ross, 1961). Decades of research have identified two common characteristics to the SAR response in several different plant species: (1) broad spectrum effectiveness against diverse pathogens and (2) a long-lasting effect following elicitation (Hammerschmidt, 2009). During SAR responses elicited by necrotrophic pathogens, plants obtain systemic resistance against not only the inducing pathogen but also different classes of pathogens. For instance, TMV-elicited SAR was not limited to TMV but was effective against four different plant viruses and even fungal pathogens (Dempsey et al., 1999). Once SAR was elicited, the response was effective for more than 20 days (Heil and Bostock, 2002). These compelling features of SAR as a defense response have biotechnological applications to manage plant pathogens in crop plants growing under field conditions. Synthetic chemical inducers of SAR such as benzothiadiazole (BTH), known as Actigard® in the USA and BION® in Europe (Tally et al., 1999), have been studied for their role as useful agrochemicals. BTH was found to protect plants very efficiently against pathogens with minimal detrimental effects to either human health or the environment. However, application of BTH was reported to cause a critical negative effect on plant growth (Heil et al., 2000; Van Hulten et al., 2006; Yi et al., 2009). This phenomenon is known as an "allocation fitness

cost" or "trade-off," and describes the requirement for a substantial amount of metabolic resources for the manifestation of SAR in response to chemical elicitors, resulting in reduced plant growth (Heil and Baldwin, 2002). BTH-treated wheat exhibits reduced growth and decreased seed production in response to chemical elicitors, and the reduction in growth is more significant under nitrogen-limiting conditions (Heil et al., 2000; Yang et al., 2011). In addition to allocation fitness cost, the feature of SAR is "priming." Early experiments to elicit SAR revealed that low concentrations of SA failed to trigger plant resistance but augmented defense-related gene expression (Conrath et al., 2006). Defense priming provides an efficient means for plants to acquire immunity against multiple phytopathogens (Conrath et al., 2006). In addition, the primed state can also be prompted by rhizosphere bacteria (rhizobacteria) and entophytes (van Loon, 2007; Van Wees et al., 2008).

In a manner similar to the SAR response, root colonization by certain rhizobacteria induces systemic resistance that is effective against plant pathogens (Kloepper et al., 2004). For instance, the plant growth-promoting rhizobacteria (PGPR) strains *Bacillus pumilus* INR7 and *Serratia marcescens* 90-166 elicited a defense response called induced systemic resistance (ISR) on five and six plant species, respectively (Kloepper and Ryu, 2006). The term ISR describes "activation of the host plant's physical or chemical defenses by an inducing agent" (Kloepper et al., 1992). Interestingly, PGPR induces an ISR response and promotes plant growth at the same time (Kloepper et al., 2004). This is a promising avenue to overcome the allocation fitness cost of BTH and cultivate crops with optimal plant performance and reduced disease potential. ISR has been applied to suppress plant diseases in the greenhouse and field against a broad range of plant pathogens, including viruses, fungi, bacteria, and nematodes (Kokalis-Burelle et al., 2002; Murphy et al., 2003; Zhang et al., 2004; Murphy, 2006; Kang et al., 2007). Among the PGPR candidates for eliciting ISR, research has focused attention on endemic endophytes that were originally isolated inside plant tissues because these were thought to exhibit a stronger interaction with plants than epiphytes (Quadt-Hallmann et al., 1997). Further studies revealed that the endophytes can be used as microbial inoculants to control plant pathogens and promote plant growth (Kloepper and Ryu, 2006). For example, seed or seedling treatment with *B. pumilus* INR7 that was isolated from a surface-sterilized cucumber stem resulted in a significant reduction of the severity of angular leaf spot, cucurbit wilt and the infestation of cucumber beetles in cucumber. Inoculation with INR7 was also effective against diseases caused by *Cucumber mosaic virus* (CMV), *Sclerotium rolfsii*, *Ralstonia solanacearum*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani* in pepper and tomato, and the incidence of Fusiform rust, caused by *Cronartium quercuum* f. sp. *fusiforme*, on loblolly pine (Wei et al., 1996; Enebak and Carey, 2000; Zehnder et al., 2001; Murphy et al., 2003).

To improve the efficacy of ISR, a combined application of inducing agents was employed. In many cases, a mixture of PGPR showed a more robust ISR response than did with single treatment (Raupach and Kloepper, 1998; Jetiyanon and Kloepper, 2002; Jetiyanon et al., 2003). In Thailand, a greenhouse screening of known endophytic *Bacillus* spp. was demonstrated that ISR was elicited in other crops, including a local variety of pepper (Jetiyanon et al., 2003). Multi-species mixtures or single-species treatments of endophytic spore-forming bacteria elicited ISR in the long cayenne pepper (*Capsicum annuum* var. *acuminatum*) and *Colletotrichum gloeosporioides* pathosystem. By contrast, the efficacy of combination treatments between PGPR and chemical inducers is not well understood. A single or two-strain mixture of PGPR was tested for its role in reducing bacterial wilt incidence in tomato along with co-application of BTH (Jetiyanon and Kloepper, 2002). Application of BioYield (two PGPR species and a chitosan mixture) + BTH reduced disease incidence compared to a similar treatment with only a single PGPR, but this effect was only observed in a single experiment, suggesting that the effect may be difficult to reproduce (Jetiyanon and Kloepper, 2002). Moreover, it is possible that the described combination treatment did not involve an ISR response because the site of BioYield application to the root system was the same as the inoculation site for the bacterial wilt pathogen, *Ralstonia solanacearum*.

The primary objective of this study was to investigate the ISR-promoting capacity of an endophyte, *B. pumilus* INR7, against soil-borne and foliar pathogens, including *Ralstonia solanacearum* and *X. axonopodis* pv. vesicatoria, respectively. Due to strong antagonism between *Ralstonia solanacearum* and strain INR7, we focused on ISR against the foliar pathogen *X. axonopodis* pv. vesicatoria. In greenhouse and field trials, we observed a clear additive effect of strain INR7 + BTH treatment compared to treatment with INR7 alone. An additive effect of INR7 and BTH combination treatment was accompanied by the expression of defense priming genes including *CaPR1* for SA signaling, and *CaPR4* for SA/jasmonic acid (JA) signaling, and *CaTin1* for ethylene signaling after 0 and 6 h of pathogen challenge was examined by quantitative RT-PCR (Shin et al., 2003; Yang et al., 2011; Lee et al., 2012; Yi et al., 2012; Song et al., 2013) indicating that the induced resistance may be caused by stimulation of plant defense mechanisms. Co-application of plants with INR7 and BTH overcame the growth suppressing effect of BTH alone. To investigate whether the additive effect of BTH and INR7 on disease resistance was specifically investigated in pepper plants, similar experiments were conducted with tobacco plants, resulting in no additive effect of BTH and INR7. To date, there have been no reports of an additive ISR response by a combination treatment including an endophytic PGPR and a chemical trigger.

MATERIALS AND METHODS

PLANT PREPARATION AND GREENHOUSE EXPERIMENT

Plants were grown and disease assays were carried out as previously described (Kang et al., 2007). Briefly, the seeds of *Capsicum annuum* were surface-sterilized with 6% sodium hypochlorite (NaOCl), washed four times with sterile distilled water (SDW), and then maintained at 25°C for 3 days until germination on Murashige and Skoog medium (Duchefa, Haarlem, the Netherlands). The germinated seeds were then transplanted into soilless media (Punong Horticulture Nursery Media LOW, Punong Co. LTD, Gyeongju, Korea). Plants were grown at 25 ± 2°C under fluorescent light (12 h/12 h day/night cycle, 7000 l× light intensity) in a controlled-environment growth room for seeding growth and transferred to the KRIBB greenhouse facility in Daejeon, South Korea. A *B. pumilus* INR7 suspension was inoculated by drench application at 10^{8-9} colony forming units/ml to the pepper roots,

as described previously (Lee et al., 2012). For pathogen challenge, a culture of the compatible bacterial pathogen *X. axonopodis* pv. vesicatoria for pepper or *Pseudomonas syringae* pv. tabaci for tobacco ($OD_{600} = 0.04$ in 10 mM $MgCl_2$) was pressure-infiltrated into leaves using a needleless syringe 1 week after INR7 application. The severity of symptoms for bacterial spot and wild fire caused by *X. axonopodis* pv. vesicatoria and *P. syringae* pv. tabaci was scored from 0 to 5 as follows: 0, no symptoms; (1), slightly yellow color; (2), chlorosis only; (3), partial necrosis and chlorosis; (4), necrosis of the inoculated area and expanded chlorosis; and (5), complete necrosis of the inoculated area. Similarly, bacterial wilt symptoms were scored using a disease scale at 3 weeks after pathogen challenge: 0, no symptoms; (1), mild wilt on the first 1–3 true leaves, less than 20% of leaves; (2), wilt symptoms on more than 21–50% of leaves; (3), arrested growth and wilt symptoms on more than 51–70% of leaves; (4), wilt symptoms on more than 71% of leaves; and (5), complete whole plant death. *X. axonopodis* pv. vesicatoria, *P. syringae* pv. tabaci, and *Ralstonia solanacearum* were cultured for 2 days at 28°C in LB, King's B, or PGC media, respectively. Chemical treatment of pepper roots was performed as described previously (Yang et al., 2011). As a positive control, plants were drenched with 10 ml of a solution of 0.5 mM benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (benzothiadiazole = BTH; Syngenta, Research Triangle Park, NC, USA). Leaves were harvested at the indicated times and then frozen immediately in liquid nitrogen for total RNA extraction. Untreated pepper leaves were used for non-stress treatments. Following inoculations with pathogens, plants were returned to the growth chamber and leaf tissue was harvested at 0 and 6 h after inoculation with *X. axonopodis* pv. vesicatoria for isolation of total RNA. The experiments were repeated three times.

FIELD TRIAL

The field trial was conducted at Cheongwon-gun, Chungcheongbuk-do, Korea (36° 35′ 32.27″ North, 127° 30′ 34.75″ East) in the second week of April to the second week of September 2009. Pepper and tobacco seeds (*Capsicum annuum* L. cv. Bukwang and *Nicotiana tabaccum*) were surface-sterilized using 5% NaOCl for 10 min, and rinsed five times with SDW. The seeds were then placed on Murashige and Skoog medium (MS, 0.22% MS salt including vitamins, 1.5% sucrose, and 0.8% plant agar, pH 5.8) in a transparent sterile container. The seeds were germinated in a growth chamber at 25°C in the dark. Germinated pepper seeds were transferred to sterilized soil containing a low level of nutrient soilless mixture (Punong Co. Ltd, Gyeongju, Korea) and cultivated for 3 weeks in a greenhouse. For testing ISR and SAR capacity under field conditions, pepper and tobacco seedlings were soaked in an INR7 bacterial suspension at 10^{8-9} cfu/ml and/or 0.5 mM BTH solution for 1 h, and transplanted at a distance of 40 cm apart in the field. For combination treatments, the final concentrations of bacteria and BTH were adjusted to be identical to the individual treatments. Sterilized water was used as a negative control. Before transplanting, each field row was covered with black and white polyethylene plastic film. Treated pepper and tobacco plants were grown in beds 20 cm high and 30 cm × 880 cm in area. Single-row treatment plots were replicated four times in a completely randomized design and consisted of 23 plants. For disease assessment, we evaluated the disease severity (0–5) at 10 and 90 days post transplantation (dpt) for pepper and 21 dpt for tobacco as described above. To assess qRT-PCR analysis, four replications per treatment were used. One replication include eight leaves (two leaves per plant × four plants) from one block.

PLANT GROWTH PARAMETERS

The shoot and root fresh weight was measured at 40 dpt as described previously (Lee et al., 2012).

QUANTIFICATION OF ROOT BACTERIA

Strain INR7 was generated as a spontaneous mutant resistant to 100 μg/ml rifampicin in the TSA media before the root colonization experiment. The number of introduced bacteria isolated from the pepper root surfaces (epiphytes) was counted at 0, 10, 20, 30, and 40 dpt; the number from inside the root structures (endophytes) was counted at 10, 20, 30, and 40 dpt; and at 7, 21, and 42 dpt. Pepper roots were placed in sterile water for 30 min in a shaking incubator at 30°C, and the wash solution was diluted and spread on tryptic soy broth agar containing 100 μg/ml rifampicin for epiphytic bacterial density estimation. For the isolation of endophytic bacteria, the collected roots were surface-sterilized with 6% NaOCl, washed four times with SDW, and then spread on TSA containing 100 μg/ml rifampicin. The bacterial population was calculated from antibiotic-resistant colonies that appeared 2–3 days after spreading.

QUANTITATIVE RT-PCR

Quantitative reverse transcription PCR was performed using a Bio-Rad CFX96 instrument. Total RNA was isolated from pepper leaf tissues using Tri reagent (Molecular Research Inc., Cincinnati, NY, USA) according to the manufacturer's instructions and as per our previous studies (Yang et al., 2009; Lee et al., 2012). First-strand cDNA synthesis was carried out with 2 μg of DNase-treated total RNA, oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Enzynomics, Daejeon, Korea). Reaction mixtures consisted of cDNA, iQ™ SYBR® Green Supermix (BIO-RAD Inc., Hercules, CA, USA) and 10 pM of each primer. Cycling parameters were as follows: initial polymerase activation, 10 min at 95°C; and then 40 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s at 72°C. Conditions were determined by comparing threshold values in a series of dilutions of the RT product with those of a non-RT template control and a non-template control for each primer pair. The expression of candidate priming genes was analyzed using the following primer pairs: 5′-AGCCTGAAATAGAAGAAACGGAGATGGAGATGAGA-3′ (*CaTin1*-F), 5′- GGAACCAGAATTGGTTACTCATGGCTACC-TGAAC-3′ (*CaTin1*-R), 5′-ACTTGCAATTATGATCCACC-3′ (*CaPR1*-F), 5′-ACTCCAGTTACTGCACCATT-3′ (*CaPR1*-R), 5′-AACTGGGATTTGAGAACTGCCAGC-3′ (*CaPR4*-F), and 5′-ATCCAAGGTACATATAGAGCTTCC-3′ (*CaPR4*-R). As a loading control to ensure that equal amounts of RNA were used in each assay, we also analyzed *CaActin* using the primers 5′-CACTGAAGCACCCTTGAACCC -3′ and 5′- GAGACAACAC-CGCCTGAATAGC -3′ (Wang et al., 2013). Relative transcript

quantification was calculated using the 2-ΔΔCT method and standard errors of mean values among replicates were calculated using Bio-Rad manager (version 2.1; Bio-Rad CFX Connect). Student's *t*-test was carried out to determine statistically significant differences between treated and untreated samples. If *P*-values < 0.05, we considered the target genes as differentially expressed. Relative transcript abundance was normalized to levels of *CaActin* mRNA (GenBank accession no. AY572427).

DIAGNOSIS OF VIRAL DISEASE

For viral diagnosis, test samples were selected from areas of the plant that exhibited symptoms of disease. Samples were ground in 50 mM $NaHPO_4$ (pH = 7.0) buffer. To confirm CMV infection, we employed a RT-PCR technique using specific primers for CMV coat protein (CP), 5′-CGTTGCCGCTATCTCTGCTAT-3′ and 5′-GGATGCTGCATACTGACAAACC-3′. As a loading control, *CaActin* was also amplified using the primers 5′-CACTGAAGCACCCTTGAACCC-3′ and 5′-GAGACAACACC-GCCTGAATAGC-3′, which were designed based on the GenBank database sequence (GenBank ID: AY572427.1).

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., Cary, NC, USA). Significant effects of treatment were determined by the magnitude of the *F*-value ($P = 0.05$). When a significant *F*-value was observed, separation of means was accomplished by Fisher's protected least significant difference (LSD) at $P = 0.05$.

RESULTS

BIOLOGICAL CONTROL AND INDUCED RESISTANCE BY STRAIN INR7 IN THE GREENHOUSE

We selected *B. pumilus* strain INR7 as a model endophytic PGPR for the elicitation of ISR (Kloepper and Ryu, 2006). Strain INR7 has been commercialized under the name YieldShield® by Bayer® as a treatment to control soil-borne pathogens including *Rhizoctonia solani* in soybean (Kloepper and Ryu, 2006). Interestingly, the biological control mechanism employed by strain INR7 has been thought to induce systemic resistance in plant tissues since INR7 did not show an inhibitory effect on fungal growth *in vitro* (data not shown). The present study tested whether strain INR7 confers ISR in pepper. The influence of INR7 inoculation on the growth of two pepper pathogens, *X. axonopodis* pv. vesicatoria and *Ralstonia solanacearum*, was tested under greenhouse conditions in Korea. Soil application of strain INR7 reduced disease severity caused by *Ralstonia solanacearum* by 72% compared with the untreated control (**Figure 1B**). Severe wilting symptoms were occurred in the control pepper seedlings, but were rarely observed in plants subjected to INR7 or BTH treatments (**Figure 1A**). We also detected significant growth enhancement on INR7 treatment (**Figure 1A**). However, BTH treatment inhibited seedling growth by allocation fitness cost. The growth reduction on Control treatment was caused by significant infection of *Ralstonia solanacearum* (**Figure 1D**). We found that strain INR7 strongly inhibited the growth of *Ralstonia solanacearum* in an *in vitro* assay on PGC medium, suggesting that the reduction in disease symptoms was caused by direct antagonism between strain

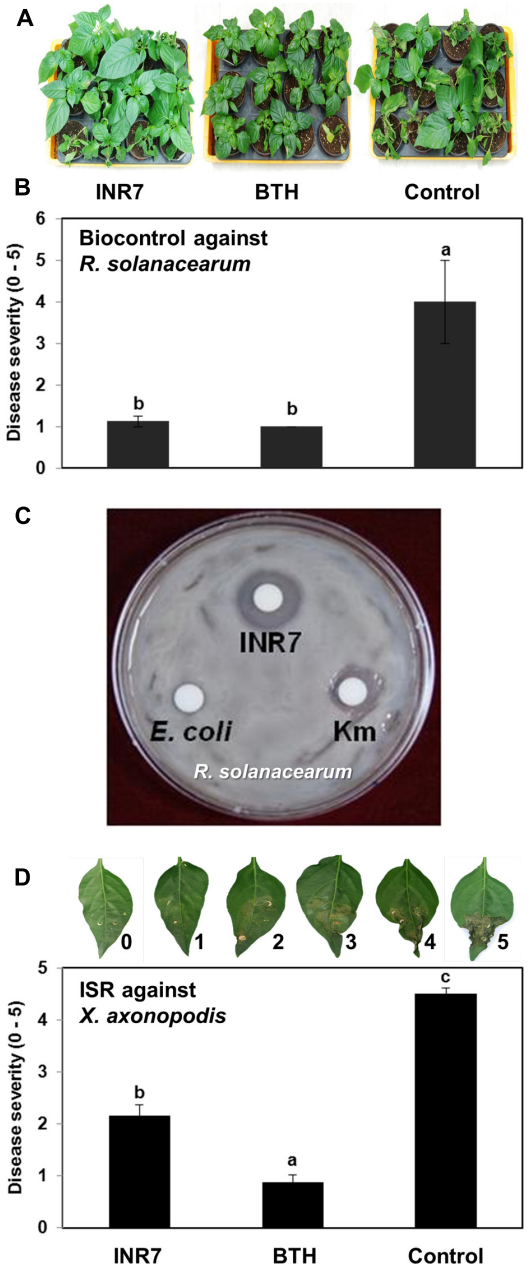


FIGURE 1 | Disease suppression capacity of *Bacillus pumulus* INR7 against *Xanthomoans axonopodis* pv. vesicatoria and *Ralstonia solanacearum* in the greenhouse (A) Presentative photo for biocontrol of bacterial wilt caused by *Ralstonia solanacearum*. The photo was taken at 3 weeks after pathogen challenge into root. **(B)** Biocontrol against *Ralstonia solanacearum* by *B. pumulus* INR7. The disease severity was measured 3 weeks after pathgoen challenge in the soil. Bars represent mean ± SE, sample size $N = 27$ plants per treatment. **(C)** Direct inhibition of *Ralstonia solanacearum* growth on the PGC medium. The photo was taken 2 days after bacteria and kanamycin inoculation on the paper disk. **(D)** Induction of plant resistance against a compatible *X. axonopodis* pv. vesicatoria. Disease severity was measured 7 days after *X. axonopodis* pv. vesicatoria challenge. Bars represent mean ± SEM, sample size $N = 10$ plants per treatment. 0.5 mM BTH was used as positive control. Different letters indicate significant differences between treatments ($P = 0.05$), according to the least significant difference (LSD). The experiments was repeated three times with similar results.

INR7 and the pathogen (**Figure 1C**). We did not conduct further experiments with this pathosystem because the ISR response is characterized by a spatial separation between PGPR and the challenge pathogen, rather than direct antagonism (Kloepper et al., 1992). To overcome this problem, we tested another pathosystem, *X. axonopodis* pv. vesicatoria, a casual pathogen of pepper bacterial spot. In pilot experiments, *X. axonopodis* pv. vesicatoria caused symptoms on pepper leaves, and the growth of this pathogen *in vitro* was not affected by co-culture with strain INR7 (data not shown). Drench application of strain INR7 into root reduced disease severity caused by *X. axonopodis* pv. vesicatoria in the leaf by 52%, compared to untreated controls (**Figure 1D**). Treatment with 0.5 mM BTH also prevented symptom development in pepper plants infected with *X. axonopodis* pv. vesicatoria (**Figure 1D**). However, BTH treatment significantly decreased pepper growth, whereas strain INR7 promoted the growth of pepper plants (**Figure 1A**).

INDUCED RESISTANCE UNDER FIELD CONDITIONS

To evaluate whether strain INR7 induces ISR under field conditions, we examined plants for symptoms of bacterial spot disease 5–10 days after infection. By using a quantitative disease index, we assayed the severity of disease symptoms in infected plants that were either mock-treated or treated with INR7, BTH, or both in combination. At 10 dpt, the disease severity in plants treated with strain INR7, INR7 + BTH, and 0.5 mM BTH was 2.37, 1.17, and 1.69, respectively. Disease severity was 4.09 in mock-treated control plants (**Figure 2A**). Severe leaf disease symptoms appeared in early September and worsened as a consequence of the unusual high temperatures and abundant precipitation in Korea during 2009. Examination of the plants revealed spots, speck, mosaic, and shoe-string symptoms that are characteristic of bacterial spot disease caused by *X. axonopodis* pv. vesicatoria but also may be caused by infection with TMV or CMV. In our field study, biological and biochemical assays and PCR analysis identified the causative agent as *X. axonopodis* pv. vesicatoria, which was based on 16s rRNA data, colony color on LB medium, and morphology on semi-selective agar media, and a pathogenesis test in pepper plants.

At 90 dpt, the bacterial spot symptoms on pepper plants growing in the field were measured again according to the quantitative scale described above. The disease severity was 1.73 for plants treated with strain INR7, 0.74 for plants receiving INR7 + BTH, 1.36 for BTH-treated plants, and 2.79 in the untreated controls (**Figure 2B**). Intriguingly, the combination treatment of INR7 and BTH reduced symptom development significantly ($P = 0.05$) compared to treatments with INR7 or BTH alone at 10 and 90 dpt (**Figure 2A**). The target virus was identified as CMV by enzymatic and virus-specific primer-based PCR (data not shown). For CMV infection, disease symptoms were evaluated based on a similar disease severity scale ranging from 0 to 5. At 90 dpt, plants pre-treated with INR7 + BTH or BTH alone showed a significantly lower disease severity score of 0.91 and 1.2, respectively, compared to control treatment. Strain INR7 alone did not affect ISR against CMV (**Figure 2C**). Taken together, these data suggest that ISR elicited by strain INR7 in pepper plants was dependent on the specific challenge pathogen. Notably, the additive effect of combination treatment with INR7 and BTH was only effective against a bacterial pathogen, *X. axonopodis* pv. vesicatoria, but not against CMV.

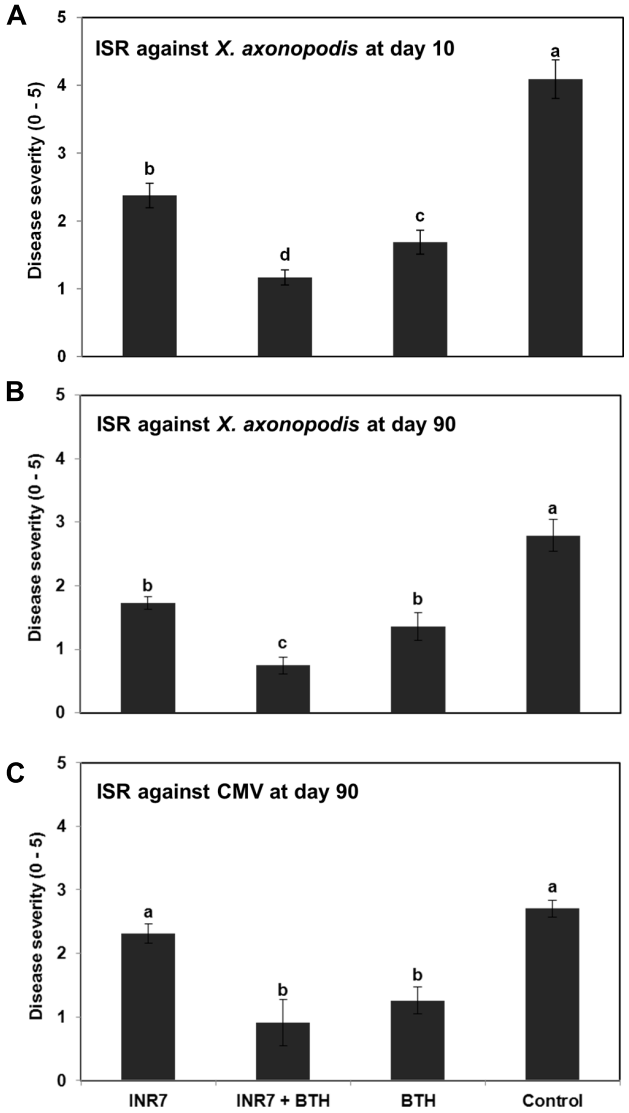


FIGURE 2 | Induction of systemic resistance by *B. pumulis* INR7 and benzothiadiazol in pepper under field conditions. (A,B). Induction of plant resistance against a compatible *X. axonopodis* pv. vesicatoria. Disease severity was measured at 10 and 90 days after infilteration of *X. axonopodis* pv. vesicatoria at 10^6 cfu/ml in plants pretreated with bacterial suspension of strain INR7 (INR7), 0.5 mM BTH (BTH) and the combination (INR7 + BTH). **(C)** Induction of plant resistance against naturally occuring *Cucumber mosaic virus*. Disease severity was measured at 90 days after transplating. Bars represent the mean ± SE (sample size, $N = 40$ replications per treatment). Different letters indicate significant differences between treatments ($P = 0.05$ according to least significant difference).

To further investigate the specificity of INR7 and BTH combination treatment on eliciting an ISR response, we performed similar field trials using the tobacco *P. syringae* pv. tabaci pathosystem. Assessment of ISR and SAR induction under high disease pressure conditions (infiltration of *Pst* at 10^6 cfu/ml) revealed

a disease severity of 2.50 in INR7-treated tobacco, 1.00 in INR7 + BTH-treated plants, 0.16 in BTH-treated plants, and 4.33 in water-treated control plants (**Figure 6A**). In this experiment, BTH in combination with strain INR7 exhibited the capacity to induce resistance in tobacco. However, no additive effect between INR7 and BTH was detected in this pathosystem, suggests that the additive effect was limited to the *X. axonopodis* pv. vesicatoria-pepper system.

EXPRESSION OF DEFENSE-RELATED PRIMING CANDIDATE GENES

Defense priming is an important feature of induced resistance (Van Hulten et al., 2006; Van Wees et al., 2008). To confirm the elicitation of induced resistance and the defense priming, the expression of the defense-related genes *CaPR1* for SA signaling, and *CaPR4* for SA/JA signaling, and *CaTin1* for ethylene signaling after 0 and 6 h of pathogen challenge was examined by qRT-PCR under field conditions.The root application of strain INR7 did not affect notable change of three defense genes (**Figures 3A–C**). PGPR strains INR7 only caused a 1.32-fold upregulation in the transcription of *CaTin1* in pepper seedlings 7 days after PGPR inoculations (**Figure 3A**). In contrast, all three genes showed significant increase transcription by above 2.86-fold in BTH and INR7 + BTH treated plants. To investigate the time-dependent manner of defense gene priming, we normalized the gene expression measurements by dividing the expression level observed at 6 h with that observed at 0 h. From this conversion of the original data, strain INR7 increased *CaTin1* expression by 1.96-fold compared to control treatment (**Figure 3D**). Unexpectedly, we observed clear additive expression of all three genes following INR7 + BTH treatments (**Figures 3D–F**). The normalized values of *CaPR4* at 6 h are 7.59 for INR7, 78.55 for INR7 + BTH, 20.89 for BTH, and 5.64 for control (**Figure 3E**). For *CaPR1*, the values are 1.03, 4.20, 1.80, and 0.63 for INR7, INR7 + BTH, BTH, and the control, respectively (**Figure 3F**). The normalized amount of *CaTin1* are 64.72, 132.78, 91.21, and 32.99 (**Figure 3D**).

PLANT GROWTH MEASUREMENTS

Application of BTH to pepper plants prior to their transplantation into the field caused a significant reduction in shoot and root fresh weight compared to plants treated with either INR7 or the water control at 40 dpt (**Figures 4A,B**). The shoot and root growth of plants receiving the combination treatment (INR7 + BTH) was not different from that of plants treated with only INR7 or BTH at 40 dpt (**Figures 4A,B**).

INFLUENCE OF BTH ON ROOT COLONIZATION BY *B. pumilus* STRAIN INR7

To measure the population density of INR7 bacteria, we assayed for bacteria growing on the rhizosphere (epiphyte) and inside root tissue (endophyte) of pepper and tobacco plants growing under field conditions and treated with either water or BTH. In pepper plants growing at 30 days post INR7 treatment, epiphytic bacterial levels were unchanged in plants treated with only INR7 or INR7 in combination with BTH treatment (**Figure 5A**). At 40 days after root inoculation with INR7, a slight increase in INR7 growth was observed in test plants treated with BTH drench (**Figure 5A**). However, the endophytic bacterial population of BTH-treated pepper plant roots was significantly higher than that of plants receiving no BTH treatment at 42 days after treatment. This results indicate that BTH treatment helped bacterial competence resulting longer surviving until day 42 while no bacteria was detected on treatment without BTH at the same time point (**Figure 6B**).

DISCUSSION

Induced resistance has been recognized as a promising means for managing plant diseases due to the effectiveness of induced resistance against diverse pathogens and insects occurring in actual crop field conditions. The results presented in this study provide additional information for improving the effectiveness of combination treatments composed of a chemical inducer (triggering SAR) and a biological agent (triggering ISR) for stimulating plant defenses. As previously reported for several different crops, treatment with the endophyte strain INR7 alone in pepper plants decreased bacterial spot symptom development in the greenhouse and field. In our experiments, co-treatment with both INR7 and BTH together resulted in decreased bacterial spot disease severity compared to treatment with strain INR7 or BTH alone. However, no additive effect of INR7 and BTH treatment was observed in the response of pepper plants to CMV infection. A similar experiment conducted in tobacco also did not show the additive effect, indicating that enhanced resistance conferred by the combination treatment is dependent on the particular plant and pathogen. The combination treatment led to the stimulation of salicylic acid-mediated plant signaling based on qRT-PCR analysis of pepper defense signaling genes. A higher bacterial population of INR7 was detected within roots of plants treated with BTH in addition to INR7 inoculation. Our result is the first report of additive induced resistance conferred by an endophytic ISR trigger and a chemical SAR trigger under field conditions.

Three similar studies have reported on combination treatments with PGPR and BTH/SA. The first example was described before that chemical induction of SAR elicited by SA and induction of ISR by PGPR can result in enhanced resistance (van Wees et al., 2000). Co-application of 1 mM SA for triggering SAR and *P. fluorescens* strain WCS417r for ISR resulted additive effect on plant protection against *P. syringae* pv. tomato DC3000. Further mechanism study indicates that combination treatment successfully protected *Arabidopsis* against *P. syringae* pv. tomato DC3000 through parallel activation of the SAR and the ISR signaling pathway. However, this is not upon crop species but a model plant. In tomato plants, induced resistance against *Ralstonia solanacearum* and *P. syringae* pv. tomato was investigated (Anith et al., 2004; Obradovic et al., 2005). When BTH was applied in combination with *B. pumilus* SE34, *P. putida* 89B61, or commercial microbial products Equity^{TM} or BioYield^{TM}, the 89B61 + BTH treatment resulted in significantly decreased symptoms of bacterial wilt compared to treatment with BTH alone (Anith et al., 2004). However, the reported reduction in disease severity may have been the result of direct antagonism between strain 89B61 and *Ralstonia solanacearum* arising from competition for the same root ecological niche. In this case, it was therefore not clear whether 89B61 treatment resulted in induced resistance mediated through

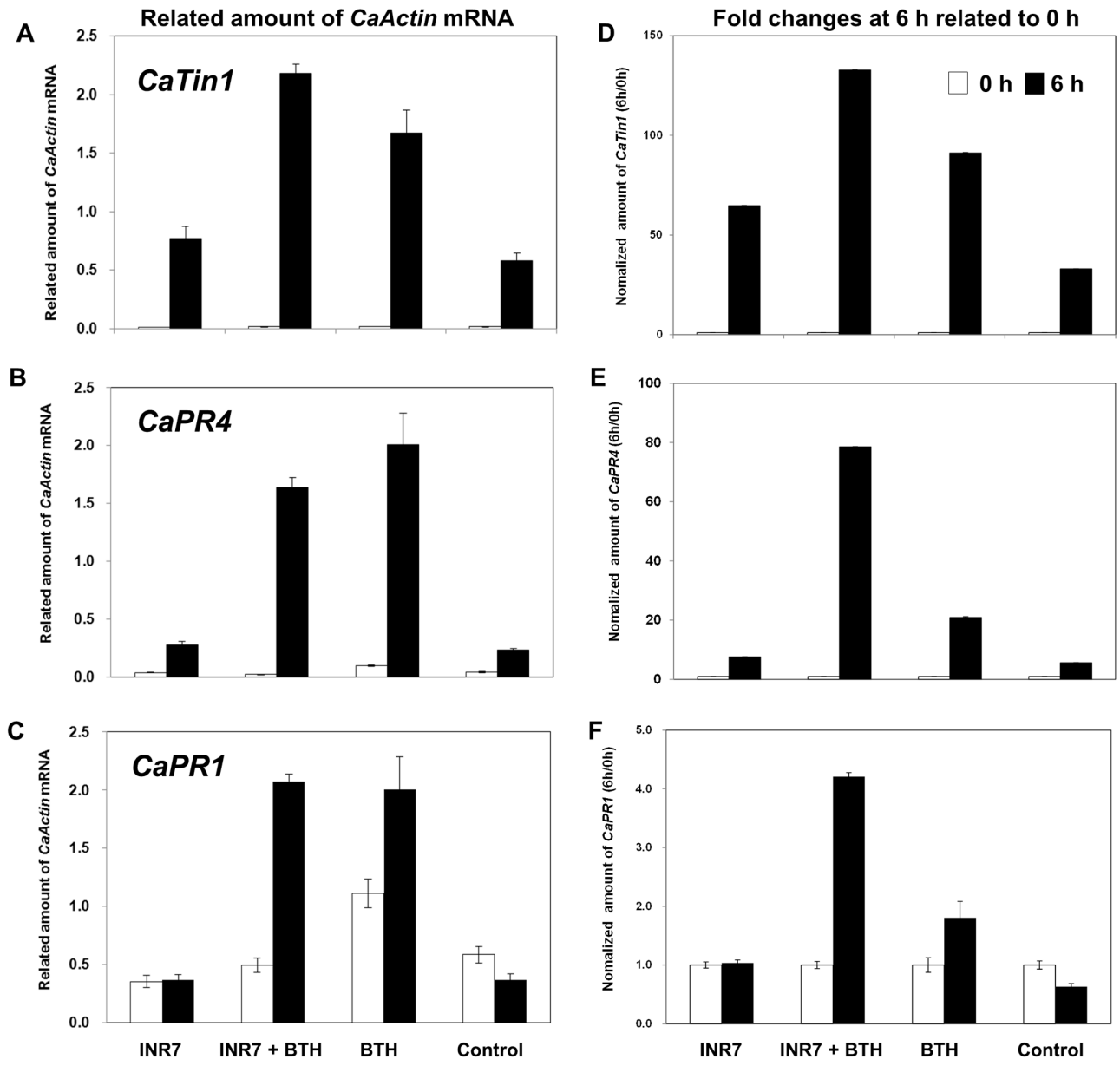


FIGURE 3 | Defense priming of *CaTin1, CaPR4,* and *CaPR1* genes in strain INR7, BTH, INR7 + BTH, and water-treated pepper plants by pathogen challenge under field condition. The expression levels of pepper defense-related genes *CaTin1* **(A)**, *CaPR4* **(B)**, and *CaPR1* **(C)** and their normalized value of *CaTin1* **(D)**, *CaPR4* **(E)**, and *CaPR1* **(F)** were quantified by qRT-PCR at 0 and 6 h after infilteration of *X. axonopodis* pv. vesicatoria at 10^6 cfu/ml 10 days after bacteria and chemical treatments. Bars represent mean ± SEM with four replications per treatment.

plant defense mechanisms. A second study investigated the biocontrol of bacterial spot disease caused by *X. axonopodis* pv. vesicatoria using two PGPR strains and the chemical inducers BTH and harpin (Obradovic et al., 2005). Soil-drench application of *B. pumilus* B122 did not show any additive effect on BTH-mediated induced resistance against bacterial spot pathogen in this study (Obradovic et al., 2005). However, this additive effect of BioYield^{TM} + BTH treatment was evident in only one trial out of three. Molecular markers also did not support the additive effect between BioYield^{TM} and BTH as the expression pattern of tomato *PR-1a* and *Pin2* after challenge with *P. syringae* pv. tomato was not different between the combination treatment and treatment with BTH alone (Obradovic et al., 2005). By contrast, the gene expression profiles of *CaTin1*, *CaPR4,* and *CaPR1* in our study are consistent with our observations of reduced disease severity (**Figures 2** and **3**). The aforementioned two studies also did not report reduced growth of the treated plants, which is an important consequence of BTH-mediated resistance (Heil et al., 2000). In our study, the shoot and root fresh weight of pepper plants treated with INR7 + BTH was not statistically different from those of plants

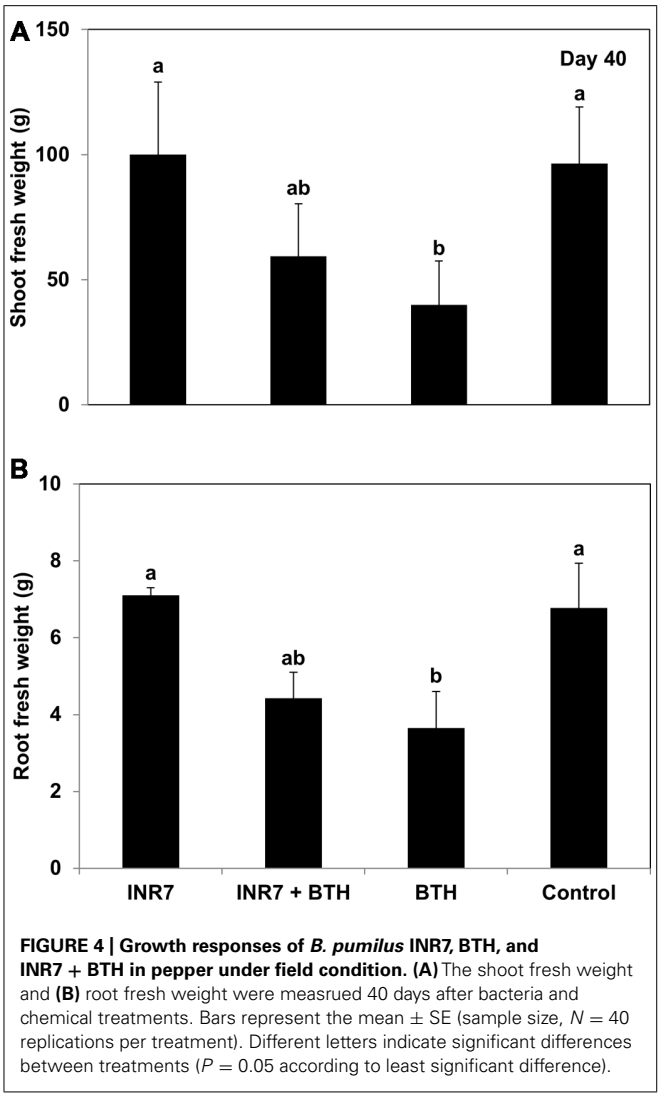


FIGURE 4 | Growth responses of *B. pumilus* INR7, BTH, and INR7 + BTH in pepper under field condition. (A) The shoot fresh weight and **(B)** root fresh weight were measrued 40 days after bacteria and chemical treatments. Bars represent the mean ± SE (sample size, $N = 40$ replications per treatment). Different letters indicate significant differences between treatments ($P = 0.05$ according to least significant difference).

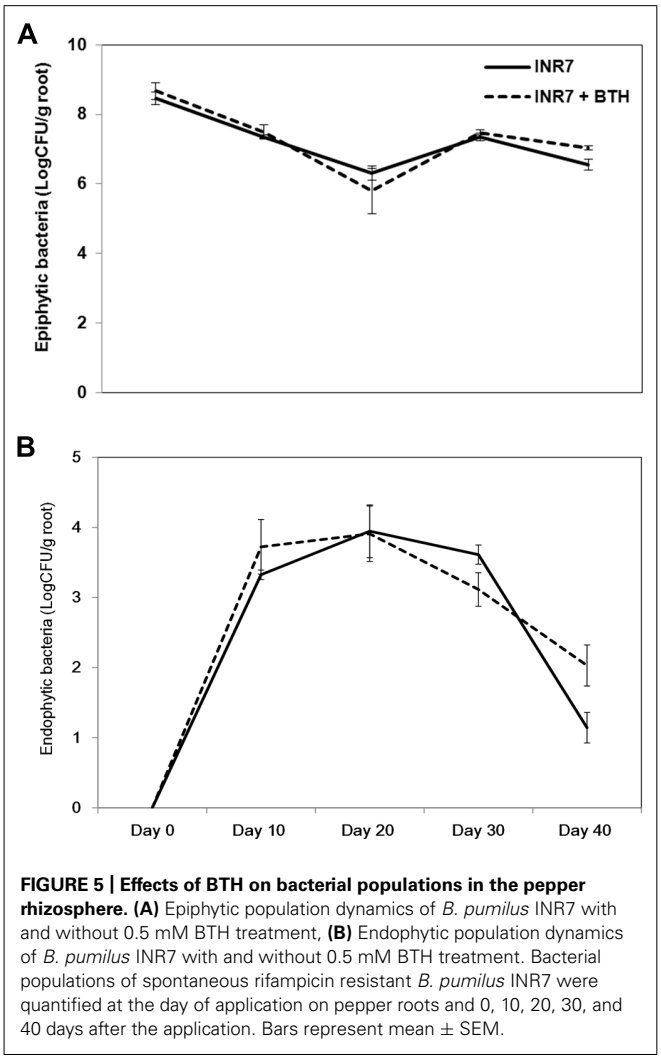


FIGURE 5 | Effects of BTH on bacterial populations in the pepper rhizosphere. (A) Epiphytic population dynamics of *B. pumilus* INR7 with and without 0.5 mM BTH treatment, **(B)** Endophytic population dynamics of *B. pumilus* INR7 with and without 0.5 mM BTH treatment. Bacterial populations of spontaneous rifampicin resistant *B. pumilus* INR7 were quantified at the day of application on pepper roots and 0, 10, 20, 30, and 40 days after the application. Bars represent mean ± SEM.

treated with BTH alone, but the root and shoot fresh weight of plants receiving either treatment was significantly decreased compared with controls (**Figure 4**), which indicates that PGPR strain INR7 acts to recover the reduced plant growth even though minor effect was shown. In two field trials conducted in the United States and Thailand, treatment of cucumber with strain INR7 resulted in significantly increased vegetative growth and yield compared with controls (Wei et al., 1996; Jetiyanon et al., 2003). It is possible that the growth-promoting effect of strain INR7 occurs in a species-specific manner.

In agreement with the results of this study, an additive effect of defense gene expression in pepper has been observed in other experiments. For example, qRT-PCR analysis was employed to investigate the activation of plant defenses against bacterial pathogens in plants simultaneously exposed to sucking insects (Lee et al., 2012) and the synthetic SAR inducer BTH. The BTH + aphid combination treatment had an additive effect on the activation of *CaPR9* in response to a compatible pathogen, *X. axonopodis* pv. vesicatoria, as well as an incompatible pathogen, *X. axonopodis* pv. glycines (Lee et al., 2012). Assessment of the early responses to whitefly + BTH treatment showed that the expression of the SA marker genes *CaPR1* and *CaPR4* in pepper leaves was upregulated compared to plants receiving a single treatment. Expression of the JA-related marker gene, *CaPINII*, was downregulated in the combination treatment, indicating signaling cross-talk typical of the antagonistic interaction between SA and JA pathways (Yang et al., 2011). In our experiments, pepper inoculation with strain INR7 did not increase expression of *CaPR1* and *CaPR4*genes but did upregulate *Capsicum annuum TMV-induced clone* (*CaTin1*), which is induced by ethylene (ET) treatment (Shin et al., 2003). Our results suggest that strain INR7 elicits mainly ET-dependent defense responses but also elevates SA signaling (**Figure 3C**). A more comprehensive analysis will be required for more advanced genetic tools that are difficult to use in pepper.

Although well-studied marker genes in *Arabidopsis* and tobacco are not available as mutants in pepper, mechanisms such as virus-induced gene silencing (VIGS) can be employed to study defense signaling in other species by establishing a knock-down phenotype (Chung et al., 2006). SGT1, a protein that

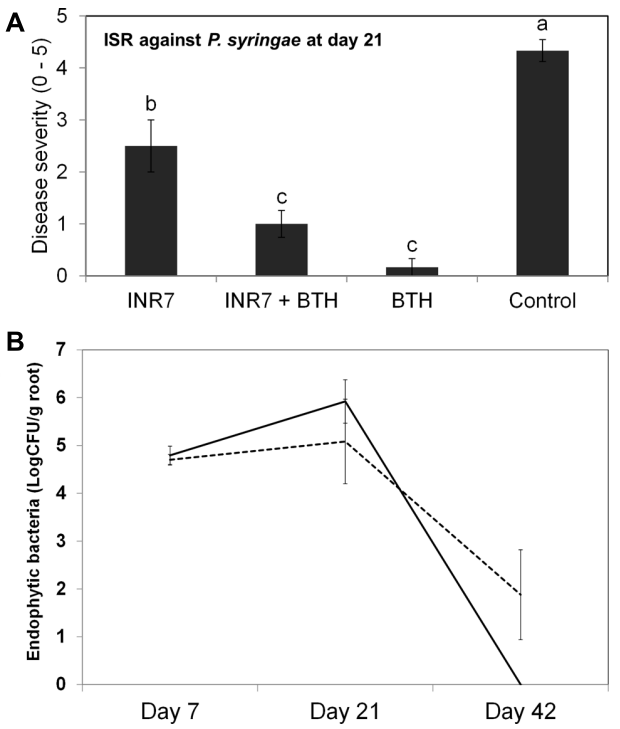


FIGURE 6 | Induction of systemic resistance by *B. pumulis* INR7 and benzothiadiazol and population change by BTH treatment in tobacco under field conditions. (A) Induction of plant resistance against a compatible *P. syringae* pv. tabaci. Disease severity was measured 21 days after infilteration of *P. syringae* pv. tabaci at 10^6 cfu/ml in plants pretreated with bacterial suspension of strain INR7 (INR7), 0.5 mM BTH (BTH), and the combination (INR7 + BTH). Bars represent the mean ± SE (sample size, $N = 40$ replications per treatment). Different letters indicate significant differences between treatments ($P = 0.05$ according to least significant difference). **(B)** Endophytic population dynamics of *B. pumilus* INR7 with and without 0.5 mM BTH treatment. Bacterial populations of spontaneous rifampicin resistant *B. pumilus* INR7 were quantified at the day of application on pepper roots and 0, 7, 21, and 42 days after the application. Bars represent mean ± SEM.

associates with Suppressor of Kinetochore Protein (SKP1)-Cullin-F-box (SCF)–ubiquitin-ligase complexes, plays important roles in defense responses. VIGS of SGT1 caused defects in plant defense when plants were inoculated with non-host pathogens and the shoe-string phenotype on the leaf of *Nicotiana benthamiana* (Peart et al., 2002). Knock-down of *NbSKP1* expression by VIGS did not show an obvious phenotype (data not shown). However, silencing of the homologous gene, *CaSGT1*, or its interacting protein, *CaSKP1*, in pepper resulted in severe dwarfism and final damping-off symptoms when plants were grown in soil, but no phenotype when plants were grown in sterile media. These results suggest that *CaSGT1* and *CaSKP1* play an essential role in basal disease resistance in pepper rather than non-host resistance in tobacco (Chung et al., 2006). In *Arabidopsis*, a double mutant of *ask1* (*Arabidopsis SK*P1-like (ASK1)) and its homologue *ask2* was defective in cell division, cell expansion/elongation and developmental delay during embryogenesis, leading to lethality in the seedling growth stage (Liu et al., 2002).

The correlation between endophytic colonization of PGPR strains and elicitation of induced resistance has not been extensively studied. Examples include a screen of an ISR-defective mutant of *Serratia marcescens* 90-166 against *Colletotrichum orbiculare* in cucumber and *P. syringae* pv. tabaci in tobacco (Press et al., 1997, 2001). Intriguingly, our previous work also showed that the population of *Serratia marcescens* 90-166 and an ISR-defective mutant, *entA*, did not change at any time point in the rhizoplane. Only an endophytic population density of *entA* mutant was significantly reduced, indicating that endophytic colonization by PGPRs plays an important role in ISR (Press et al., 2001). In this case, the mutated gene, *entA,* encodes a siderophore. We originally hypothesized that the lack of enterobactin production in the *entA* mutant may render it more susceptible to reactive oxygen species produced from plant cells, resulting in reduced internal bacterial populations. Further investigation revealed that the *entA* mutant maintained ISR capacity by reducing the virus titre and the symptom development following CMV infection in *Arabidopsis* Col-0 (Ryu et al., 2004). These results suggest that *Serratia marcescens* 90-166 activates different signaling pathways depending on the pathogen or plant species. Another possible explanation is that unknown bacterial determinants mediate ISR using novel mechanisms. As shown in **Figure 5**, BTH treatment supported a population density of endophytic bacteria at above 100 cfu g^{-1} $root^{-1}$ 40 days post-inoculation, while the population of strain INR7 reached 10 cfu g^{-1} $root^{-1}$ (**Figure 5B**). The epiphytic bacterial population on plant roots during both treatments was maintained at approximately 10^6 cfu g^{-1} $root^{-1}$ until day 40 (**Figure 5A**). This level of bacterial density is relatively higher than that reported in other studies (Raaijmakers et al., 1995). The authors suggest that the minimum population required to elicit induced resistance is above 10^5 cfu g^{-1} $root^{-1}$ (Raaijmakers et al., 1995), which is consistent with the results of our study. It remains to be determined why the epiphytic population density is a determinant of PGPR-induced resistance. In our experiments, the epiphytic population of strain INR7 with or without BTH treatment was not different across different time points (**Figure 5A**). To further investigate the additive effect of the bacterial endophyte INR7 and BTH, we conducted similar bacterial growth assays using tobacco plants as a host. We concluded that there was no correlation between induced resistance in tobacco and root colonization with or without BTH treatment (**Figures 6A,B**). Collectively, the root colonization capacity of strain INR7 may not play an important role on induced resistance. This result also indicates that the additive elicitation of induced resistance by INR7 and BTH may be a pepper-specific response.

In conclusion, this study provides new information concerning the additive effect of a combination treatment composed of an endophytic ISR inducer and a synthetic chemical, BTH, on the pepper defense response. An increased biological defense response was also supported by molecular marker data showing increased expression of pepper defense genes *CaTin1*, *CaPR4*, and *CaPR1* after the combination treatment when compared to a single treatment with either agent. The combination treatment also had a mild growth-promoting effect, partially restoring plant growth arrest caused by BTH treatment. Taken together, our data suggest that PGPR-mediated ISR can be applied in a disease management

program when combined with a chemical-based SAR inducer. This regimen has the potential to promote induced resistance and minimize the negative effects of pathogens on plant growth under field conditions.

ACKNOWLEDGMENTS

We thank Drs. Doil Choi and Seon Woo Lee for providing the bacterial strains *X. axonopodis* and *Ralstonia solanacearum*, respectively. We are grateful for financial support from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0011655), the Industrial Source Technology Development Program of the Ministry of Knowledge Economy (TGC0281011) of Korea, the Next-Generation BioGreen 21 Program (SSAC grant #PJ009524), and the KRIBB initiative program, South Korea.

REFERENCES

Anith, K. N., Momol, M. T., Kloepper, J. W., Marios, J. J., Olson, S. M., and Jones, J. B. (2004). Efficacy of plant growth-promoting rhizobacteria, acibenzolar-s-methyl and soil amendment for integrated management of bacterial wilt on tomato. *Plant Dis.* 88, 669–673.

Chung, E., Ryu, C. M., Oh, S. K., and Choi, D. (2006). The essential role of pepper CaSgt1 and CaSkp1 genes in plant development and basal resistance. *Physiol. Plant* 126, 605–612.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant Microbe Interact.* 19, 1062–1071.

Dempsey, D. A., Shah, J., and Klessig, D. F. (1999). Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.* 18, 547–575.

Enebak, S. A., and Carey, W. A. (2000). Evidence for induced systemic protection to fusiform rust in loblolly pine by plant growth-promoting rhizobacteria. *Plant Dis.* 84, 306–308.

Hammerschmidt, R. (2009). Systemic acquired resistance. *Adv. Bot. Res.* 51, 173–222.

Heil, M., and Baldwin, I. (2002). Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends Plant Sci.* 7, 61–67.

Heil, M., and Bostock, R. M. (2002). Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Ann. Bot.* 89, 503–512.

Heil, M., Hilpert, A., Kaiser, W., and Linsenmair, K. E. (2000). Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *J. Ecol.* 88, 645–654.

Jetiyanon, K., Fowler, W. D., and Kloepper, J. W. (2003). Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Dis.* 87, 1390–1394.

Jetiyanon, K., and Kloepper, J. W. (2002). Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol. Control* 24, 285–291.

Kang, S. H., Cho, H. S., Cheong, H., Ryu, C. M., Kim, J. F., and Park, S. H. (2007). Two bacterial endophytes eliciting both plant growth promotion and plant defense on pepper (*Capsicum annuum* L.). *J. Microbiol. Biotechol.* 17, 96–103.

Kloepper, J. W., Tuzun, S., and Kuc, J. A. (1992). Proposed definitions related to induced disease resistance. *Biocon. Sci. Techol.* 2, 349–351.

Kloepper, J. W., Ryu, C. M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259–1266.

Kloepper, J. W., and Ryu, C. M. (2006). "Bacterial Endophytes as Elicitors of Induced Systemic Resistance," in *Who is an Endophyte*? eds Schulz/Boyle/Sieber (Berlin: Springer-Verlag), 33–52.

Kokalis-Burelle, N., Vavrina, C. S., Rosskopf, E. N., and Shelby, R. A. (2002). Field evaluation of plantgrowth-promoting rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant Soil* 238, 257–266.

Lee, B., Lee, S., and Ryu, C. M. (2012). Foliar aphid feeding recruits rhizosphere bacteria and primes plant immunity against pathogenic and non-pathogenic bacteria in pepper. *Ann. Bot.* 110, 281–290.

Liu, X., Schiff, M., Serino, G., Deng, X.-W., and Dinesh-Kumar, S. P. (2002). Role of SCF ubiqutin-ligase and the COP9 signalosome in the N gene-mediated resistance response to tobacco mosaic virus. *Plant Cell* 14, 483–496.

Murphy, J. F. (2006) "Applied aspects of induced resistance to plant virus infection," in *Natural Resistance Mechanisms of Plants to Viruses*, eds. G. Loebenstein and J. P. Carr (Heidelberg: Springer).

Murphy, J. F., Reddy, M., Ryu, C. M., Kloepper, J. W., and Li, R. (2003). Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. *Phytopathology* 93, 1301–1307.

Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., et al. (2005). Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Dis.* 89, 712–716.

Peart, J. R., Lui, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D. C., et al. (2002). Ubiquitin ligase-associated protein SGT1 is required for host and non-host disease resistance in plants. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10865–10869.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316.

Press, C. M., Loper, J. E., and Kloepper, J. W. (2001). Role of iron in rhizobacteria-mediated induced systemic resistance. *Phytopathology* 91, 593–598.

Press, C. M., Wilson, M., Tuzun, S., and Kloepper, J. W. (1997). Salicylic acid produced by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. *Mol. Plant Microbe Interact.* 10, 761–768.

Quadt-Hallmann, A., Hallmann, J., and Kloepper, J. W. (1997). Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. *Can. J. Microbiol.* 43, 254–259.

Raaijmakers, J. M., Leeman, M., vanoorschot, M. M. P., Vander sluis, I., Schippers, B., and Bakker, P. A. H. M. (1995). Dose-response relationships in biological control of fusarium wilt of radish by *Pseudomonas* spp. *Phytopathology* 85, 1075–1081.

Raupach, G. S., and Kloepper, J. W. (1998). Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88, 1158–1164.

Ross, A. F. (1961). Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14, 340–358.

Ryu, C. M., Murphy, J. F., Mysore, K. S., and Kloepper, J. W. (2004). Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against Cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. *Plant J.* 39, 381–392.

Song, G. C., Choi, H. K., and Ryu, C. M. (2013). The folate precursor para-aminobenzoic acid elicits induced resistance against Cucumber mosaic virus and *Xanthomonas axonopodis*. *Ann. Bot.* 111, 925–934.

Shin, R., Kim, M. J., and Paek, K. H. (2003). The CaTin1(*Capsicum annuum* TMV-induced Clone 1) and CaTin1-2 genes are linked head-to-head and share a bidirectional promoter. *Plant Cell Physiol.* 44, 549–554.

Tally, A., Oostendorp, M., Lawton, K., Staub, T., Bassi, B., Agrawal, A., et al. (1999) "Commercial development of elicitors of induced resistance to pathogens" in *Induced Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture*, eds A. A. Agrawal S. Tuzun, and E. Bent (St Paul: APS Press), 357–369.

Van Hulten, M., Pelser, M., Van Loon, L. C., Pieterse, C. M. J., and Ton, J. (2006). Costs and benefits of priming for defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5602–5607.

van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *Eur. J. Plant Pathol.* 119, 243–254.

van Wees, S. C., de Swart, E. A., van Pelt, J. A., van Loon, L. C., and Pieterse, C. M. J. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.*. 97, 8711–8716.

Van Wees, S. C., Van der Ent, S., and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–438.

Wang, Y., Dang, F., Liu, Z., Wang, X., Eulgem, T., Lai, Y., et al. (2013). CaWRKY58, encoding a group I

WRKY transcription factor of *Capsicum annuum*, negatively regulates resistance to *Ralstonia solanacearum* infection. *Mol. Plant Pathol.* 14, 131–144.

Wei, G., Kloepper, J. W., and Tuzun, S. (1996). Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. *Phytopathology* 86, 221–224.

Yang, J. W., Yi, H. S., Kim, H., Lee, B., Lee, S., Ghim, S. Y., et al. (2011). Whitefly infestation of pepper plants elicits defence responses against bacterial pathogens in leaves and roots and changes the below-ground microflora. *J. Ecol.* 99, 46–56.

Yang, J. W., Yu, S. H., and Ryu, C. M. (2009). Priming of defense-related genes confers root-colonizing bacilli-elicited induced systemic resistance in pepper. *Plant Pathol. J.* 25, 303–440.

Yi, H. S., Heil, M., Adame-Alvarez, R. M., Ballhorn, D. J., and Ryu, C. M. (2009). Airborne induction and priming of plant defenses against a bacterial pathogen. *Plant Physiol.* 151, 2152–2161.

Yi, H.-S., Yang, J. W., Choi, H. K., Ghim, S.-Y., and Ryu, C.-M. (2012). Benzothiadiazole-elicited defense priming and systemic acquired resistance against bacterial and viral pathogens of pepper under field conditions. *Plant Biotechnol. Rep.* 6, 373–380.

Zehnder, G. W., Murphy, J. F., Sikora, E. J., and Kloepper, J. W. (2001). Application of rhizobacteria for induced resistance. *Eur. J. Plant Pathol.* 107, 39–50.

Zhang, S., Reddy, M. S., and Kloepper, J. W. (2004). Tobacco growth enhancement and blue mold protection by rhizobacteria: relationship between plant growth promotion and systemic disease protection by PGPR strain 90-166. *Plant Soil* 262, 277–288.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 March 2013; accepted: 16 April 2013; published online: 14 May 2013.

Citation: Yi H-S, Yang JW and Ryu C-M (2013) ISR meets SAR outside: additive action of the endophyte Bacillus pumilus INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper. Front. Plant Sci. ***4****:122. doi: 10.3389/fpls.2013.00122*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Yi, Yang and Ryu. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 10 April 2013
doi: 10.3389/fpls.2013.00081

Getting the ecology into interactions between plants and the plant growth-promoting bacterium *Pseudomonas fluorescens*

W. H. Gera Hol*, T. Martijn Bezemer and Arjen Biere

Department of Terrestrial Ecology, Netherlands Institute of Ecology, Wageningen, Netherlands

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Choong-Min Ryu, Korea Research Institute of Bioscience and Biotechnology, South Korea
Ana Pineda, Wageningen University, Netherlands

****Correspondence:***
W. H. Gera Hol, Department of Terrestrial Ecology, Netherlands Institute of Ecology, Droevendaalsesteeg 10, 6708 PB Wageningen, Netherlands.
e-mail: g.hol@nioo.knaw.nl

Plant growth-promoting rhizobacteria (PGPR) are increasingly appreciated for their contributions to primary productivity through promotion of growth and triggering of induced systemic resistance in plants. Here we focus on the beneficial effects of one particular species of PGPR (*Pseudomonas fluorescens*) on plants through induced plant defense. This model organism has provided much understanding of the underlying molecular mechanisms of PGPR-induced plant defense. However, this knowledge can only be appreciated at full value once we know to what extent these mechanisms also occur under more realistic, species-diverse conditions as are occurring in the plant rhizosphere. To provide the necessary ecological context, we review the literature to compare the effect of *P. fluorescens* on induced plant defense when it is present as a single species or in combination with other soil dwelling species. Specifically, we discuss combinations with other plant mutualists (bacterial or fungal), plant pathogens (bacterial or fungal), bacterivores (nematode or protozoa), and decomposers. Synergistic interactions between *P. fluorescens* and other plant mutualists are much more commonly reported than antagonistic interactions. Recent developments have enabled screenings of *P. fluorescens* genomes for defense traits and this could help with selection of strains with likely positive interactions on biocontrol. However, studies that examine the effects of multiple herbivores, pathogens, or herbivores and pathogens together on the effectiveness of PGPR to induce plant defenses are underrepresented and we are not aware of any study that has examined interactions between *P. fluorescens* and bacterivores or decomposers. As co-occurring soil organisms can enhance but also reduce the effectiveness of PGPR, a better understanding of the biotic factors modulating *P. fluorescens*–plant interactions will improve the effectiveness of introducing *P. fluorescens* to enhance plant production and defense.

Keywords: PGPR-bacteria, *Pseudomonas fluorescens*, species interactions, complexity, herbivores, decomposers, bacterivores, mutualists

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are a diverse group of microorganisms that are increasingly appreciated for their contributions to primary productivity through promotion of growth and triggering of induced systemic resistance (ISR) in plants. By triggering plant defense, PGPR can make an important contribution to biocontrol of pests and pathogens of plants. However, the effectiveness of PGPR-triggered plant defense depends on a variety of genetic and biotic/abiotic environmental factors. PGPR naturally occur within a complex community of soil organisms inhabiting the rhizosphere. Hence, in order to understand the role of PGPR in influencing a plant's defense against pests and pathogens, it is important to understand how biotic interactions with these rhizosphere organisms will affect the ability of PGPR to enhance plant defense. The aim of this review is to examine how the impact of PGPR on plant defense is modulated by the presence of other organisms in the rhizosphere. Other reviews have focused on particular interactions, e.g., between PGPR and aboveground insects (Pieterse and Dicke, 2007; Pineda et al., 2010) or type of defense, e.g., volatiles (Dicke and Baldwin, 2010). Those reviews have taken a plant centric approach (but see Whipps, 2001). In this paper, we will review how biotic interactions between PGPR and other rhizosphere- or plant-associated organisms affect the ability of PGPR to enhance plant defenses. We use *Pseudomonas fluorescens*, a very common and well-studied PGPR, as a model species. To test the dependence of PGPR–plant interactions on direct and indirect biotic interactions with other rhizosphere biota, we compare studies in which effects of *P. fluorescens* on plant defense are examined for a single *P. fluorescens* isolate with studies in which these effects are examined for a *P. fluorescens* isolate in combination with other isolates and/or species. We will discuss these interactions in increasing order of complexity, starting with single introductions of *P. fluorescens* with introductions of multiple *P. fluorescens* isolates, then with other PGPR, with other plant growth-promoting fungi, bacterivores, and finally with decomposing organisms. The basic interaction in all these studies is formed by a plant, *P. fluorescens* and a herbivore or pathogen. The latter is necessary to judge whether plant defense was changed. In

addition, studies without herbivore or pathogens but that measure plant defense genes are included. Before we review these interactions we provide a brief introduction to PGPR and *P. fluorescens* in particular. Moreover, as we argue that the effect of PGPR on induced plant defense cannot be considered in isolation from the effects of other organisms that are also present in the soil such as nematodes, fungi, earthworms, or protozoa on the PGPR or on the plant, we also provide a brief overview of interactions between bacteria and other soil dwelling organisms in the rhizosphere.

INTERACTIONS BETWEEN BACTERIA AND OTHER SOIL ORGANISMS IN THE RHIZOSPHERE

Live roots and root exudates provide a diverse range of resources to soil organisms. As a result, the zone around plant roots, the rhizosphere, is a highly diverse habitat. It consists of root herbivores, such as nematodes and insect larvae, their natural enemies, and a wide variety of soil microbes, including symbiotic, pathogenic, and saprophytic fungi and protozoa. The vast majority of soil organisms in the rhizosphere are bacteria (including PGPR), with densities as high as 10^9 cells per gram of soil. The abundance and composition of these soil bacteria depends on abiotic conditions such as soil pH, temperature, and moisture (Bardgett, 2005). However, in the rhizosphere of plants, the density and activity of bacteria is fuelled largely by root-derived carbon. Bacteria compete with each other and other soil microorganisms for these carbon resources.

In the rhizosphere, bacteria can have direct beneficial or harmful effects on the plant. However, there are also important indirect feedback interactions between plant roots, soil bacteria, and other microorganisms (Berendsen et al., 2012). For example, root-released exudates promote bacterial growth (Bais et al., 2001). These bacteria are consumed by protozoa and bacterivorous nematodes, and these consumers generally cause strong top-down control of bacteria. Via bacterial grazing, these bacterivores liberate nutrients, which in turn, stimulate plant growth (Bonkowski, 2004). The quality and quantity of root-derived carbon sources vary temporally, between plant species and between individual plants that belong to the same plant species. This variation can be attributed, at least partly, to interactions between plants and other organisms. Foliar herbivory, but also interactions between roots and soil organisms such as root herbivores or mycorrhizal fungi (Jones et al., 2004; Bais et al., 2006), often causes an increase in the rate of carbon and nitrogen exudation from roots which then leads to enhanced microbial activity in the rhizosphere (Holland et al., 1996; Bardgett and Wardle, 2010). Hence, bacterial growth and activity will depend on the direct and indirect interactions of the plant with other (soil) organisms. Apart from consumption, movement of larger soil fauna also affects the dispersal of soil microorganisms such as bacteria. Thus, plant roots, bacteria, and other microbes interact in complex food webs and in order to understand the interactions between plants and bacteria it is important to consider them in a multitrophic context.

PGPR AND PLANT DEFENSE

Rhizobacteria with growth-promoting capacity occur in a number of bacterial phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria) with as best known members *Pseudomonas* spp. and *Bacillus* spp. (Compant et al., 2005). Studies on model organisms like *Pseudomonas fluorescens* have provided considerable understanding of the underlying molecular mechanisms of PGPR-induced plant defense. The type of defense triggered by microorganisms differs among pathogenic and non-pathogenic microbes (van Loon, 2007; Pieterse et al., 2012). Biotrophic pathogens generally induce systemic acquired resistance (SAR). SAR is dependent on salicylic acid (SA) signaling and results in enhanced expression of pathogenesis-related (PR) proteins (Durrant and Dong, 2004). By contrast, PGPR trigger ISR. ISR is generally independent of the SA signaling pathway and is not associated with major alterations in the expression of defense-related genes, but with priming of defenses (Verhagen et al., 2004). PGPR-primed plants do not have elevated expression of defense genes. Instead, they show more rapid or stronger activation of defenses once they are attacked by pathogens and herbivores, a response that is often dependent on a functional JA pathway (van der Ent et al., 2009). ISR is a systemic response, expressed in both roots and shoots, that can affect a wide range of organisms, including above- and belowground pathogens, herbivores, and their natural enemies (Pineda et al., 2010, 2012), a spectrum that only partly overlaps with that of SAR (van Oosten et al., 2008).

The effect of PGPR on plant growth promotion and on plant defense against attackers depends on many factors, including the plant species or genotype, the pathogen species, and the abiotic conditions, such as nutrient availability. In some cases variation in these factors can even lead to opposite effects of PGPR on plant traits. For instance, *P. fluorescens* addition stimulated nitrogen mineralization in one crop species and decreased it in another (Brimecombe et al., 1999). Similar variation in the effects of PGPR among plant species has been observed for their effects on plant defense against pathogens and herbivores. For instance, Tétard-Jones et al. (2007) showed that supplementing the rhizobacterial community with a *Pseudomonas aeruginosa* strain influenced the fitness of the cereal aphid (*Sitobion avenae*) on barley (*Hordeum vulgare*) either positively or negatively (increased or decreased population size) depending on plant and aphid genotype. In a later study (Tétard-Jones et al., 2012) they identified genomic regions (QTLs) underlying the differential plant-mediated responses to the rhizobacterium. This linking of differential responses to genomic regions is exceptional; often studies can only indicate that factors such as genotype may be relevant for the effectiveness of PGPR-triggered plant defense responses, without further mechanistic explanation. Many studies focusing on PGPR and induced defense have been carried out under relatively sterile conditions in laboratories or greenhouses. Studies on induced defense of *P. fluorescens* under field conditions are relatively rare. In this review we do include references to biocontrol studies but want to stress that biocontrol can be the result of many mechanisms of which ISR is only one. Studies testing whether effects of particular PGPR strains on plant defense observed under sterile greenhouse conditions can be observed in the field as well have yielded mixed results (e.g., Guo et al., 2004; Akila et al., 2011). When differences are observed, these could be due to the several biotic and abiotic factors which differ between greenhouse and the field. When

spatial variation in the disease suppressive effects of PGPR are observed in field trials on multiple locations, abiotic factors such as fertility, temperature, and moisture are usually discussed as explanation for the variable results (Guo et al., 2004; Ji et al., 2006; Maeder et al., 2011). Remarkably, surprisingly little attention has been paid so far to the role of biotic factors such as local plant mutualists, predators, decomposers, other pathogens, or herbivores which might interfere with the PGPR effects on plant defense.

Pseudomonas COMBINED WITH OTHER *Pseudomonas* STRAINS OR WITH OTHER PLANT MUTUALISTS

Most plant species can have myriad mutualistic interactions, which provide benefits such as increasing nutrients, producing hormones, increasing tolerance to abiotic stresses (water, temperature, heavy metals), or biotic stresses (pests and pathogens). These benefits can be provided by both bacteria and fungi; depending on the mutualist species, the association can be on the root or leaf surface or inside the plants (endophytes). *P. fluorescens* is mostly known as a root colonizer. Many studies have examined the effects of adding several mutualistic organisms simultaneously to soils on plant defense (e.g., Jaderlund et al., 2008; Saravanakumar et al., 2009; Senthilraja et al., 2010). A general conclusion that can be drawn from these studies is that multiple microbial introductions typically are more effective than single introductions for biocontrol (Whipps, 2001).The combinations of species that have been added range from multiple *Pseudomonas* isolates (Saravanakumar et al., 2009; Seenivasan et al., 2012) to adding other mutualistic bacteria (Domenech et al., 2006) or mutualistic fungi (Jaderlund et al., 2008). The addition of multiple agents enhances the chance that at least one is well adapted to the local environment where the organisms are introduced. Disease suppression by the plant can also be improved when the introduced mutualists differ in their effects on induced defense responses (Domenech et al., 2006). Moreover, interactions between mutualists may lead to different gene expression and secondary metabolite production in the bacterium and this can result in synergistic effects of mutualists on the plant (Combes-Meynet et al., 2011; Garbeva et al., 2011). The studies by Garbeva et al. (2011) and Combes-Meynet et al. (2011) illustrate both the potential of bacteria species interactions to alter gene expression in *P. fluorescens*, and the potential effects that such changes in gene expression can have on the interactions with the plant. Even without altered gene expression in the bacteria, the plant may respond synergistically to the microbial-associated molecular patterns (MAMPs) of multiple plant mutualists (Desaki et al., 2012). MAMPs are molecules from pathogenic and mutualistic microbes which trigger plant immune response (van Wees et al., 2008). PGPR may vary their phenotype in order to avoid stimulation of the plants' immune system (Zamioudis and Pieterse, 2012). It is unknown how effective such phase variation is when other PGPR are also colonizing the plant, each species with their specific MAMPs, but triggering pathways that at some point converge (van Wees et al., 2008; Zamioudis and Pieterse, 2012). Currently not enough is known about the MAMPs of *P. fluorescens* and other mutualists to speculate about synergistic effects between more different MAMPs.

Pseudomonas COMBINED WITH OTHER *P. fluorescens* STRAINS

A number of studies have applied multiple strains of *P. fluorescens* to achieve better biocontrol of plant pests and pathogens, with the aim to find combinations of strains with complementary effects on plant defense. A recent study by Loper et al. (2012) on the genomes of 10 *P. fluorescens* strains shows that these strains vary considerable in their defense traits. This offers ample room for selection of complementary strains and different lifestyles. Agusti et al. (2011) selected two *P. fluorescens* strains which differed in secondary metabolite production and found that dual inoculations lead to better control of *Phytophthora cactorum* in strawberry, as well as to a reduction of the within-experiment variability, compared to single introductions. Several studies have reported that a combination of introductions of *P. fluorescens* isolates Pf1, TDK1, and PY15 is very effective in controlling pests and diseases. Introduction of the combination of the three *P. fluorescens* isolates, for example, is very effective in reducing populations of the root-feeding nematode *Meloidogyne graminicola* (Seenivasan et al., 2012) as well as in controlling sheath rot *Sarocladium oryzae* in rice (Saravanakumar et al., 2009). The explanation for the effectiveness of this particular combination is that these three isolates do not compete for space and together colonize the root surface more effectively than single isolates. This is important for the direct nematicidal effects of the isolates. However, plants inoculated with *P. fluorescens* mixtures also had higher activities of peroxidase and chitinase enzymes than single inoculations (Saravanakumar et al., 2009; Seenivasan et al., 2012), suggesting that higher activation of defense-related enzymes may play a role in addition to a more efficient occupation of the root surface.

Pseudomonas COMBINED WITH OTHER SPECIES OF PGPR

The most commonly investigated combination of *P. fluorescens* and other PGPR is with *Bacillus* spp., but also combined introductions with *Burkholderia* spp., *Rhizobium* spp., and *Serratia* spp. are frequently studied. For *Bacillus* spp., as far as we are aware, no antagonistic effects on control of bacteria, fungi, and viruses have been reported. García-Gutiérrez et al. (2012) tested suppression of both fungal and bacterial pathogens by *P. fluorescens* in combination with *Bacillus*; combinations were equally effective as single introductions of *P. fluorescens.* The improved control of *Fusarium* disease by a combination of *P. fluorescens* and *Bacillus* was associated with the induction of the defense-related enzymes peroxidase and polyphenol oxidase (Akila et al., 2011; Sundaramoorthy et al., 2012). Most studies on the effects of combined introductions of *Pseudomonas* and other PGPR have reported effects on improved biological control. Combes-Meynet et al. (2011) hypothesized that during evolution PGPR have developed mechanisms to affect and respond to each other and that it is likely that the secondary metabolites from *P. fluorescens* will affect other PGPR. The authors tested the effect of 2,4-diacetylphloroglucinol (2,4-DAPG), a secondary metabolite from *P. fluorescens*, on *Azospirillum* gene expression and found that genes involved in several traits related to root colonization and growth promotion were upregulated. Co-inoculation of *P. fluorescens* and *Azospirillum* stimulated root growth in spring wheat (Combes-Meynet et al., 2011). Garbeva et al. (2011) studied changes in gene expression in *P. fluorescens* when exposed to three

other rhizobacteria: *Bacillus* sp., *Brevundimonas* sp., or *Pedobacter* sp. Interestingly, *P. fluorescens* had specific responses to the different competitors; two species increased antimicrobial metabolite production by *P. fluorescens*, but *Bacillus* did not (Garbeva et al., 2011). There are also studies where inoculation with *P. fluorescens* alone was more effective than inoculations in which *Pseudomonas* was combined with other PGPR (Anwar-ul-Haq et al., 2011; Stockwell et al., 2011). *P. fluorescens* A506 proved incompatible with two other biological control agents *Pantoea vagans* and *Pantoea agglomerans* since proteases from *P. fluorescens* A506 degrade the antibiotics from the *Pantoea* spp. that play an important role in the control fire blight in pear (Stockwell et al., 2011).

Pseudomonas COMBINED WITH FUNGI

Fungi are introduced together with *P. fluorescens* with three main aims: improved nutrition or plant growth (mycorrhizal fungi), improved disease control (e.g., *Trichoderma* spp.) or improved insect pest control (*Beauveria* spp.). So far, there are only a few papers that have examined the effectiveness of combined introductions of *Pseudomonas* with the entomopathogenic fungus *Beauveria.* Entomopathogenic fungi can be found as plant endophyte and may have plant growth-promoting properties (Vega et al., 2009). The majority of papers report increased control of pests or diseases when the entomopathogenic fungus *Beauveria* is applied in combination with *P. fluorescens*. Senthilraja et al. (2010) used combinations of *P. fluorescens* and *Beauveria bassiana* and found the three-strain combination of two *P. fluorescens* strains with one *Beauveria bassiana* to be more effective than single or two-strain inoculations for controlling both a leafminer and collar rot. The explanation is that *P. fluorescens* affects plant metabolism, and this, in turn, makes the insects more vulnerable to *Beauveria*. Similarly, Karthiba et al. (2010) combined *P. fluorescens* with *Beauveria bassiana*, and found simultaneous control of pests and pathogens on rice.

Pseudomonas fluorescens is known to control pathogens including fungi, and thus we may anticipate that combined effects of *P. fluorescens* and mutualistic fungi on plant resistance will be less than additive because mutualistic fungi will suffer from *P. fluorescens*. On the other hand, *Pseudomonas fluorescens* is identified as one of the mycorrhiza helper bacteria for both ecto- and arbuscular mycorrhiza (Frey-Klett et al., 2007). Mycorrhiza helper bacteria are bacteria associated with mycorrhiza that promote the symbiosis between fungus and plant by stimulating fungal growth or protecting the fungus against other fungal competitors. There are many examples where *P. fluorescens* combined with mutualistic fungi was more successful than single inoculations of either bacteria or fungi (Tayal et al., 2011; Walker et al., 2012), and antagonistic interactions have rarely been reported (but see Sukhada et al., 2011). Inoculation of the mycorrhizal fungus *Glomus intraradices* has a positive effect on *P. fluorescens* survival on maize; it is unclear whether this is a plant-mediated or direct effect (Walker et al., 2012). Sukhada et al. (2011) found both under controlled conditions and in the field that the tripartite inoculation of *P. fluorescens* with the arbuscular mycorrhizal fungi *G. mosseae* and with *T. harzianum* was not as good in reducing *Phytophthora* disease incidence as the dual inoculations. It is unknown whether the predominantly positive results of mutualistic fungi with *P. fluorescens* indicates that natural selection has favored traits that result in interactions between mutualistic fungi and *P. fluorescens* that are neutral or positive for the plant or whether this reflects the research bias towards studies using candidates with good prospects for positive interactions in their effects on biocontrol. Another major group of mutualistic fungi are the Class I endophytes *Neotyphodium* spp. and *Acremonium* spp. but little is known about their effects on belowground processes, except for a stimulation of root exudation (Omacini et al., 2012). Recently, Wicklow and Poling (2009) showed that there are negative effects of antibiotics from *Acremonium zeae* on *P. fluorescens*, but apart from that, we are not aware of any study examining the effects of plant – endophyte – *P. fluorescens* interactions on plant defense.

INTERACTIONS BETWEEN *Pseudomonas* AND BACTERIVORES AND DECOMPOSERS

Pseudomonas fluorescens may also interact with two other groups of rhizosphere organisms that have less close relations with the plant: organisms which feed on bacteria (bacterivores) and organisms which break down organic material (decomposers).

Pseudomonas fluorescens AND BACTERIVORES

Pseudomonas fluorescens are grazed by predatory bacteria, protozoa, and bacterivorous nematodes (Elsherif and Grossmann, 1996). For bacterivores there is a clear potential direct effect on *P. fluorescens* abundance via grazing. However, whether grazing will affect plant defense is dependent on the selectivity of grazing and whether induction of plant defense is density-dependent. A threshold density of *P. fluorescens* is known for effective suppression of take-all decline (Raaijmakers and Weller, 1998) but it is unclear if the same applies to other pests and diseases. Selective grazing can change bacterial competition (Pedersen et al., 2009) and bacterivores avoiding *P. fluorescens* due to the secondary metabolite production by *P. fluorescens* can improve the competitive advantage of *P. fluorescens* over other bacteria (Jousset et al., 2008; Jousset, 2012). *Pseudomonas* can produce hydrogen cyanide and this repels bacterivorous nematodes. Also 2,4-DAPG, an antibiotic compound produced by *P. fluorescens*, acts as nematicide (Neidig et al., 2011). However, even when *P. fluorescens* is grazed upon by predators, a reduction in ISR response is not self-evident, because the reduction in abundance by predation may be accompanied by other changes in the PGPR that enhance ISR. For instance, grazing by amoebae was found to upregulate 2,4-DAPG synthesis in *P. fluorescens* (Jousset and Bonkowski, 2010). This compound is also known to be directly involved in ISR in plants (Weller et al., 2012). Bacterivorous nematodes can also stimulate PGPR effects on plant growth (Jiang et al., 2012). Addition of bacterivorous nematodes together with *Burkholderia* or *Pseudomonas* to plants growing in natural soil increased microbial biomass (Jiang et al., 2012), indicating a stimulation of bacterial abundance by grazing. Both nematode addition and *Burkholderia* addition increased the number of root tips, but their combined effect was significantly higher than their single effects (Jiang et al., 2012). There is an urgent need for experiments, that include bacterivores, *P. fluorescens*, plants, and pathogens in which the expression of plant defense or defense genes are measured. We are not aware of any of such studies.

Pseudomonas fluorescens AND DECOMPOSERS

For decomposers more and more evidence is accumulating that they can affect induced defense responses of plants. For instance, Collembola induce auxin-responsive genes and defense genes in shoots of *Arabidopsis* (Endlweber et al., 2011), although in this case Collembola may act as herbivores instead of decomposers. Earthworms reduced the damage by plant parasitic nematodes in rice, without directly affecting nematode abundance (Blouin et al., 2005). The exact mechanism is unknown, but earthworms did modulate expression of three stress-related genes and they also improved the photosynthetic capacity of the plant (Blouin et al., 2005). The presence of earthworms in the soil can also cause an increase or decrease in defensive glucosinolates in Brassicaceous plants (Wurst et al., 2006; Lohmann et al., 2009; González Megías and Müller, 2010). These results clearly show that decomposers can affect plant defense and therefore, decomposers may interact with *P. fluorescens*-mediated ISR. Indirect effects of decomposers on *P. fluorescens*–plant outcomes may occur via changes in nutrient availability and substrate quality, and several studies have indicated that soil nutritional conditions are crucial for ISR (e.g., Hoitink and Boehm, 1999). It is also possible that decomposers affect plant growth and root exudation, and that this in turn affects *P. fluorescens* abundance and ultimately plant defense. However, we are not aware of any study describing effects of decomposers on *P. fluorescens*–plant interactions. For other PGPR it has been shown that earthworm casts increased PGPR abundances (Wu et al., 2012). Jana et al. (2010) investigated the effects of earthworms on *Arabidopsis thaliana* in nutrient poor and rich soil. Since earthworms affected several plant parameters independent of soil nutrient conditions, the authors suggested that earthworms stimulate nutrient mineralization but also stimulate phytohormone-producing bacteria (Jana et al., 2010). In another study, earthworms increased the abundance of fluorescent pseudomonads in the rhizosphere of three plant species (Elmer, 2009), and therefore these results suggest that this may be a general phenomenon. The mechanism of stimulation is unknown, but Troxler et al. (2012) observed that earthworms provide survival hotspots for *P. fluorescens* in soil.

SYNTHESIS, APPLICATION, AND OUTLOOK

The effect of other soil dwelling organisms on the impact of *P. fluorescence* on plant defense responses will depend on whether there is a threshold density and whether the effects are (linear or non-linear) density-dependent. If other PGPR organisms target the same ISR mechanism then one could easily imagine additive interactions if there is a linear relationship between density at introduction and the effect on the plant. If the relation between density and plant response is non-linear there is room for synergistic reactions. There are several mechanisms by which the presence of other organisms can influence interactions between *P. fluorescens*, host plants, and herbivores or pathogens (**Figure 1**). The other species can act directly via affecting abundance or effectiveness of *P. fluorescens* and indirectly via plant-mediated effects. The direct effects can be separated into quantitative and qualitative effects: the quantitative effects are those that determine the number of *P. fluorescens* cells in the rhizosphere. The qualitative effects determine the effectiveness of *P. fluorescens* in triggering plant defense, e.g., by changing 2,4-DAPG production. The number of *P. fluorescens* cells can be decreased due to predation by bacterivores, such as predatory bacteria, nematodes, and protozoa (**Figure 1**; Pedersen et al., 2009). *Pseudomonas fluorescens* is a suitable food source for bacterivores (Elsherif and Grossmann, 1996), but *P. fluorescens* can produce defense compounds to avoid predation. The overall effect of grazing on *P. fluorescens* population dynamics will depend on the defense levels of *P. fluorescens*, the availability of alternative food sources and the selectivity of the grazers. We do not foresee an immediate application of combining *P. fluorescens* inoculation with bacterivores to increase root colonization.

Competition for nutrients or space with other PGPR or rhizobacteria is another factor that will negatively affect *P. fluorescens* numbers (**Figure 1**; Prieto et al., 2011). Other PGPR could also produce secondary metabolites which inhibit *P. fluorescens* (**Figure 1**; Gu, 2009). Many efforts have been made to isolate and screen *P. fluorescens* strains and to select successful combinations of multiple *P. fluorescens* strains or other PGPR. Recent developments have now enabled screenings of *P. fluorescens* genomes for defense traits and this could help with selection of compatible and potential synergistic strains. Synergistic interactions between *P. fluorescens* and other plant mutualists are much more common than antagonistic interactions, but this may be due to a bias in experimental studies to use species with prospects of positive interactive effects on biocontrol. Only the most promising strains are selected and these are first tested for compatibility, i.e., absence of *in vitro* inhibition (e.g., Sundaramoorthy et al., 2012) or competition during root colonization (Prieto et al., 2011). This approach probably provides a biased view of the effects of interactions between mutualist species on plant defense. For inoculation approaches this screening approach is very efficient, but for understanding interactions between introduced *P. fluorescens* with the resident biocontrol agents knowledge of a broader range of species interactions is necessary. Application of single *P. fluorescens* requires knowledge on potential interactions with resident *P. fluorescens* and other organisms. At least 22 genotypes of 2,4-DAPG-producing *P. fluorescens* have been detected thus far; multiple isolates are found together in soils (Weller et al., 2007) and thus interactions between resident and inoculated *P. fluorescens* are likely to occur. Also other PGPR are widespread and they can interact with introduced *P. fluorescens*. Those groups that negatively affect *P. fluorescens* abundance (bacterivores and other PGPR) may also trigger secondary metabolite production in *P. fluorescens* (qualitative effect). Those secondary metabolites such as 2,4-DAPG serve as defense compounds against predators but are also involved in ISR in plants (Weller et al., 2012). Upregulation of such inducing compounds probably lowers the threshold density necessary to induce ISR in plants. Garbeva et al. (2011) showed how some PGPR increased secondary metabolites production in *P. fluorescens* while other PGPR did not change secondary metabolite production. This variation in interactions allows for selection of compatible PGPR combinations, but prediction of field effects due to interactions with resident species will remain problematic. For most field situations there is no knowledge of the resident *P. fluorescens* and other PGPR. The fast developments in molecular techniques continuously improve

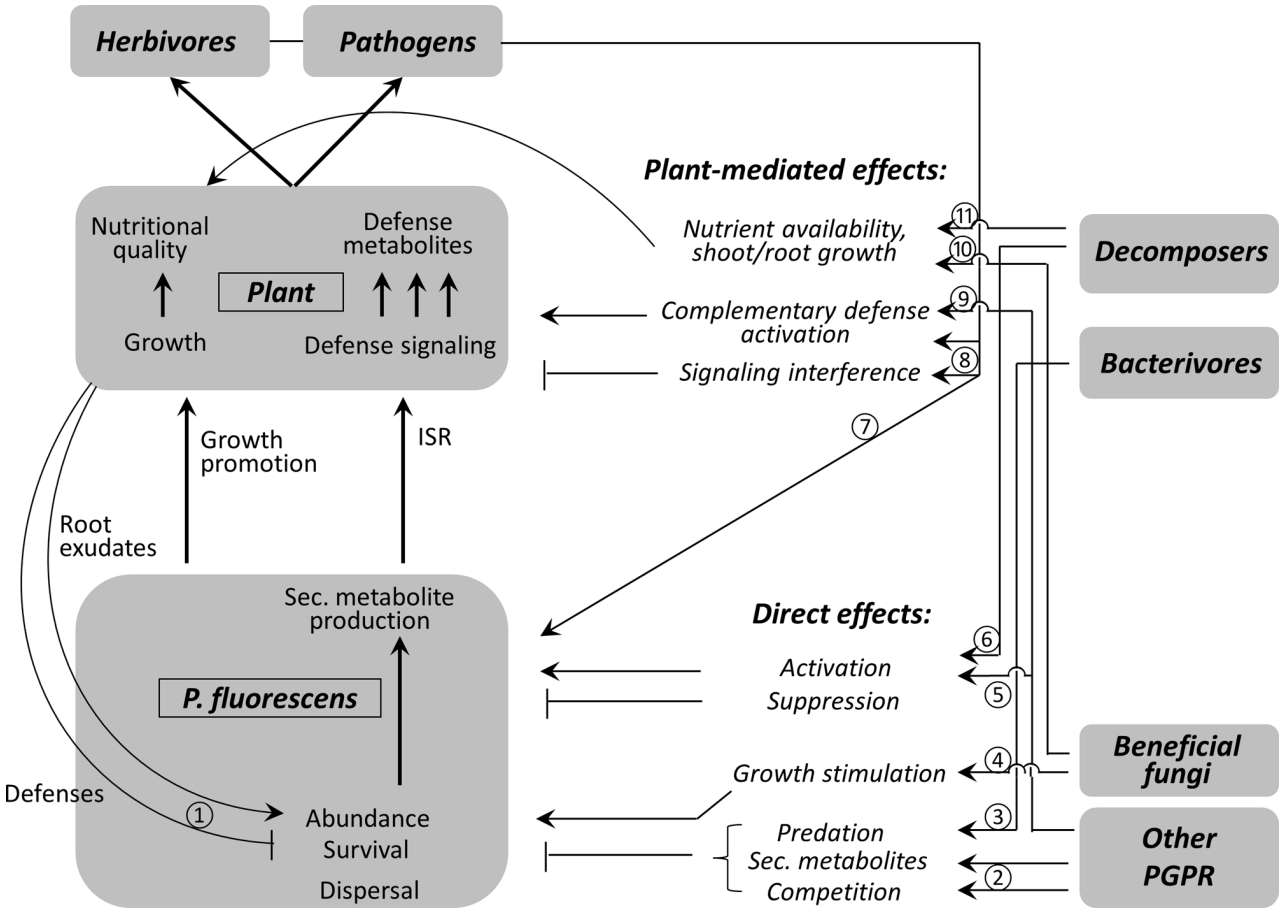


FIGURE 1 | Simplified scheme of direct and indirect (plant-mediated) effects of rhizosphere- and plant-associated organisms on interactions between *Pseudomonas fluorescens* and host plant defenses. Arrows: enhancing (positive) effects, lines ending with small vertical bars: suppressive (negative) effects. For simplicity, reciprocal effects (effects of *P. fluorescens* on other organisms) have not been included. Circled numbers refer to articles describing the interactions: (1) Jin et al. (2010), (2) Gu (2009); Prieto et al. (2011), (3) Elsherif and Grossmann (1996); Pedersen et al. (2009), (4) Walker et al. (2012), (5) Combes-Meynet et al. (2011); Garbeva et al. (2011), (6) Elmer (2009); Troxler et al. (2012), (7) Jousset et al. (2011), (8) Dicke et al. (2009); Zhang et al. (2013), (9) Elbadry et al. (2006), (10) Jones et al. (2004), and (11) Wurst (2010).

the resolution at which microbial community composition can be assessed. Even when composition of the local bacterial community is known, this knowledge would be of little use when for most species nothing is known about their potential interaction with introduced *P. fluorescens.* Metagenomics and transcriptomics will offer insight in activity and function of the microbiome, especially the recruitment and activation of beneficials (Berendsen et al., 2012).

Both decomposers (Elmer, 2009; Troxler et al., 2012) and mycorrhizal fungi (Walker et al., 2012) can have positive effects on the number of *P. fluorescens* cells in the rhizosphere. However, there is only a single report for stimulation of *P. fluorescens* survival by mycorrhizal fungi and hence the generality of this phenomenon remains unclear. The exact mechanism of *P. fluorescens* stimulation by decomposers and mycorrhiza is still unknown. However, regardless of the mechanism, the positive effect of decomposers and possibly mycorrhizal fungi on *P. fluorescens* abundance and dispersal could be exploited by adapting management practices to, e.g., stimulate earthworms by organic amendments. Tillage and fertilization could be adapted to favor arbuscular mycorrhizal fungi. Apart from promoting PGPR, it is clear that there are many other reasons why decomposers or arbuscular mycorrhizal fungi are stimulated in agricultural soils, although such management practises are often not yet adopted in intensive agricultural systems.

Indirect effects via plant feedback comprise a multitude of interactions. Decomposers and beneficial fungi may increase nutrient availability and increase shoot and root growth (Laakso and Setälä, 1999; Jones et al., 2004). The increased growth may change plant defense and root exudation patterns and this, in turn, can affect *P. fluorescens* populations (Jin et al., 2010). With the recently increased awareness of the indirect effects of decomposers on plant defense (e.g., Wurst, 2010), the interaction between *P. fluorescens,* plants, and plant–decomposer is a research area waiting to be explored.

Although there certainly is interest in the control of multiple pests or pathogens simultaneously (Karthiba et al., 2010; Senthilraja et al., 2010), most experiments thus far have tested

the effect of *P. fluorescens* on control of pathogens separately. Experiments, where the effect of one pathogen on the interaction with another pathogen and *P. fluorescens* has been studied, are scarce. Pathogens, mutualists, and pests with intimate relationships with the plant such as aphids or cyst nematodes might be able to synergize or antagonize the ISR-triggered responses through interference with defense signaling or activation/repression of downstream defenses. The pathogen *Pythium ultimum* can change 2,4-DAPG production by *P. fluorescens* (Jousset et al., 2011), but it is currently unknown how that would affect a second attacker. For insects, interspecific asymmetrical competition is frequently found (Kaplan and Denno, 2007). Therefore, control of one insect pest could result in an increase in abundance of another. Some plant–nematode combinations are sensitive to *P. fluorescens*, but not all (Timper et al., 2009). Thus, similar to insects, controlling one nematode pest might affect the abundance of other nematode species for example by changing competition for root space (Brinkman et al., 2004). A single plant which is attacked by multiple herbivores, pathogens or herbivores and pathogens would have to deal with a number of possible conflicting signals. A second attacker could activate or repress downstream defenses induced by a previous attacker (Dicke et al., 2009; Zhang et al., 2013). In this respect the order by which a plant is attacked is crucial for plant defense induction. Priority effects are receiving increasingly more attention recently, also in the context of plant–soil interactions, but mostly from the plant perspective. However, there is very little empirical work on the interactions between *P. fluorescens*, a host plant, and multiple attackers. In fact, empirical studies that examine how interactions between herbivores, pathogens, mutualists, decomposers, or bacterivores affect plant–*P. fluorescens* interactions in a full-factorial design are non-existing and can only be addressed by individual-based models of plant-based multitrophic species interactions, such as in Meyer et al. (2009). We conclude that other rhizosphere inhabitants can greatly influence *P. fluorescens* and its interactions with the plant. However, there is still a dearth of information about the effects of other species on interactions between *P. fluorescens* and plant defense. Insight into these interactions will contribute to improved performance of biocontrol agents in the field.

ACKNOWLEDGMENTS

We thank Prof. Wim van der Putten for critically reading earlier drafts of the manuscript. This is publication 5434 of NIOO-KNAW.

REFERENCES

Agusti, L., Bonaterra, A., Moragrega, C., Camps, J., and Montesinos, E. (2011). Biocontrol of root rot of strawberry caused by *Phytophthora cactorum* with a combination of two *Pseudomonas fluorescens* strains. *J. Plant Pathol.* 93, 363–372.

Akila, R., Rajendran, L., Harish, S., Saveetha, K., Raguchander, T., and Samiyappan, R. (2011). Combined application of botanical formulations and biocontrol agents for the management of *Fusarium oxysporum* f. sp. *cubense* (Foc) causing *Fusarium* wilt in banana. *Biol. Control* 57, 175–183.

Anwar-ul-Haq, M., Anwar, S. A., Shahid, M., Javed, N., Khan, S. A., and Mehamood, K. (2011). Management of root knot nematode *Meloidogyne incognita* by plant growth-promoting rhizobacteria on tomato. *Pak. J. Zool.* 43, 1027–1031.

Bais, H. P., Loyola-Vargas, V. M., Flores, H. E., and Vivanco, J. M. (2001). Root-specific metabolism: the biology and biochemistry of underground organs. *In Vitro Cell. Dev. Plant* 37, 730–741.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 233–266.

Bardgett, R. D. (2005). *The Biology of Soil: a Community and Ecosystem Approach*. Oxford: Oxford University Press.

Bardgett, R. D., and Wardle, D. A. (2010). *Above-Belowground Linkages: Biotic Interactions, Ecosystem Processes, and Global Change*. Oxford: Oxford University Press.

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486.

Blouin, M., Zuily-Fodil, Y., Pham-Thi, A. T., Laffray, D., Reversat, G., Pando, A., et al. (2005). Belowground organism activities affect plant aboveground phenotype, inducing plant tolerance to parasites. *Ecol. Lett.* 8, 202–208.

Bonkowski, M. (2004). Protozoa and plant growth: the microbial loop in soil revisited. *New Phytol.* 162, 617–631.

Brimecombe, M. J., De Leij, F., and Lynch, J. M. (1999). Effect of introduced *Pseudomonas fluorescens* strains on the uptake of nitrogen by wheat from N-15-enriched organic residues. *World J. Microbiol. Biotechnol.* 15, 417–423.

Brinkman, E. P., van Veen, J. A., and van der Putten, W. H. (2004). Endoparasitic nematodes reduce multiplication of ectoparasitic nematodes, but do not prevent growth reduction of *Ammophila arenaria* (L.) Link (marram grass). *Appl. Soil Ecol.* 27, 65–75.

Combes-Meynet, E., Pothier, J. F., Moenne-Loccoz, Y., and Prigent-Combaret, C. (2011). The *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. *Mol. Plant Microbe Interact.* 24, 271–284.

Compant, S., Duffy, B., Nowak, J., Clement, C., and Barka, E. A. (2005). Use of plant-growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959.

Desaki, Y., Otomo, I., Kobayashi, D., Jikumaru, Y., Kamiya, Y., Venkatesh, B., et al. (2012). Positive crosstalk of MAMP signaling pathways in rice cells. *PLoS ONE* 7:e51953. doi: 10.1371/journal.pone.0051953

Dicke, M., and Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends Plant Sci.* 15, 167–175.

Dicke, M., van Loon, J. J. A., and Soler, R. (2009). Chemical complexity of volatiles from plants induced by multiple attack. *Nat. Chem. Biol.* 5, 317–324.

Domenech, J., Reddy, M. S., Kloepper, J. W., Ramos, B., and Gutierrez-Manero, J. (2006). Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soilborne diseases in pepper and tomato. *Biocontrol* 51, 245–258.

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209.

Elbadry, M., Tahaa, R. M., Eldougdoug, K. A., and Gamal-Eldin, H. (2006). Induction of systemic resistance in faba bean (*Vicia faba* L.) to bean yellow mosaic potyvirus (BYMV) via seed bacterization with plant growth promoting rhizobacteria. *J. Plant Dis. Prot.* 113, 247–251.

Elmer, W. H. (2009). Influence of earthworm activity on soil microbes and soilborne diseases of vegetables. *Plant Dis.* 93, 175–179.

Elsherif, M., and Grossmann, F. (1996). Role of biotic factors in the control of soil-borne fungi by fluorescent pseudomonads. *Microbiol. Res.* 151, 351–357.

Endlweber, K., Krome, K., Welzl, G., Schaffner, A. R., and Scheu, S. (2011). Decomposer animals induce differential expression of defence and auxin-responsive genes in plants. *Soil Biol. Biochem.* 43, 1130–1138.

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007). The mycorrhiza helper bacteria revisited. *New Phytol.* 176, 22–36.

Garbeva, P., Silby, M. W., Raaijmakers, J. M., Levy, S. B., and de Boer, W. (2011). Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors. *ISME J.* 5, 973–985.

García-Gutiérrez, L., Romero, D., Zeriouh, H., Cazorla, F. M., Torés, J. A., DeVicente, A., et al. (2012). Isolation and selection of plant growth-promoting rhizobacteria as inducers of systemic resistance in melon. *Plant Soil* 358, 201–212.

González Megías, A., and Müller, C. (2010). Root herbivores and detritivores shape above-ground multitrophic assemblage through plant-mediated effects. *J. Anim. Ecol.* 79, 923–931.

Gu, W. (2009). Bioactive metabolies from *Alternaria brassicicola* ML-P08, an endophytic fungus residing in *Malus halliana*. *World J. Microbiol. Biotechnol.* 25, 1677–1683.

Guo, J. H., Qi, H. Y., Guo, Y. H., Ge, H. L., Gong, L. Y., Zhang, L. X., et al. (2004). Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biol. Control* 29, 66–72.

Hoitink, H. A. J., and Boehm, M. J. (1999). Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu. Rev. Phytopathol.* 37, 427–446.

Holland, J. N., Cheng, W. X., and Crossley, D. A. (1996). Herbivore-induced changes in plant carbon allocation: assessment of below-ground C fluxes using carbon-14. *Oecologia* 107, 87–94.

Jaderlund, L., Arthurson, V., Granhall, U., and Jansson, J. K. (2008). Specific interactions between arbuscular mycorrhizal fungi and plant growth-promoting bacteria: as revealed by different combinations. *FEMS Microbiol. Lett.* 287, 174–180.

Jana, U., Barot, S., Blouin, M., Lavelle, P., Laffray, D., and Repellin, A. (2010). Earthworms influence the production of above- and belowground biomass and the expression of genes involved in cell proliferation and stress responses in *Arabidopsis thaliana*. *Soil Biol. Biochem.* 42, 244–252.

Ji, P., Campbell, H. L., Kloepper, J. W., Jones, J. B., Suslow, T. V., and Wilson, M. (2006). Integrated biological control of bacterial speck and spot of tomato under field conditions using foliar biological control agents and plant growth-promoting rhizobacteria. *Biol. Control* 36, 358–367.

Jin, C. W., Li, G. X., Yu, X. H., and Zheng, S. J. (2010). Plant Fe status affects the composition of siderophore-secreting microbes in the rhizosphere. *Ann. Bot.* 105, 835–841.

Jiang, Y., Wu, Y., Xu, W. S., Cheng, Y. H., Chen, J. D., Xu, L., et al. (2012). IAA-producing bacteria and bacterial-feeding nematodes promote *Arabidopsis thaliana* root growth in natural soil. *Eur. J. Soil Biol.* 52, 20–26.

Jones, D. L., Hodge, A., and Kuzyakov, Y. (2004). Plant and mycorrhizal regulation of rhizodeposition. *New Phytol.* 163, 459–480.

Jousset, A. (2012). Ecological and evolutive implications of bacterial defences against predators. *Environ. Microbiol.* 14, 1830–1843.

Jousset, A., and Bonkowski, M. (2010). The model predator *Acanthamoeba castellanii* induces the production of 2,4-DAPG by the biocontrol strain *Pseudomonas fluorescens* Q2-87. *Soil Biol. Biochem.* 42, 1647–1649.

Jousset, A., Rochat, L., Lanoue, A., Bonkowski, M., Keel, C., and Scheu, S. (2011). Plants respond to pathogen infection by enhancing the antifungal gene expression of root-associated bacteria. *Mol. Plant Microbe Interact.* 24, 352–358.

Jousset, A., Scheu, S., and Bonkowski, M. (2008). Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. *Funct. Ecol.* 22, 714–719.

Kaplan, I., and Denno, R. F. (2007). Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. *Ecol. Lett.* 10, 977–994.

Karthiba, L., Saveetha, K., Suresh, S., Raguchander, T., Saravanakumar, D., and Samiyappan, R. (2010). PGPR and entomopathogenic fungus bioformulation for the synchronous management of leaffolder pest and sheath blight disease of rice. *Pest Manag. Sci.* 66, 555–564.

Laakso, J., and Setälä, H. (1999). Sensitivity of primary production to changes in the architecture of belowground food webs. *Oikos* 87, 57–64.

Lohmann, M., Scheu, S., and Müller, C. (2009). Decomposers and root feeders interactively affect plant defence in *Sinapis alba*. *Oecologia* 160, 289–298.

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis, E. W., Lim, C. K., Shaffer, B. T., et al. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* 8:e1002784. doi: 10.1371/journal.pgen.1002784

Maeder, P., Kaiser, F., Adholeya, A., Singh, R., Uppal, H. S., Sharma, A. K., et al. (2011). Inoculation of root microorganisms for sustainable wheat–rice and wheat–black gram rotations in India. *Soil Biol. Biochem.* 43, 609–619.

Meyer, K. M., Vos, M., Mooij, W. M., Hol, W. H. G., Termorshuizen, A. J., Vet, L. E. M., et al. (2009). Quantifying the impact of above- and belowground higher trophic levels on plant and herbivore performance by modeling. *Oikos* 118, 981–990.

Neidig, N., Paul, R. J., Scheu, S., and Jousset, A. (2011). Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microb. Ecol.* 61, 853–859.

Omacini, M. Semmartin, M., Perez, L. I., and Gundel, P. E. (2012). Grass endophyte symbiosis: a neglected aboveground interaction with multiple belowground consequences. *Appl. Soil Ecol.* 61, 273–279.

Pedersen, A. L., Nybroe, O., Winding, A., Ekelund, F., and Bjornlund, L. (2009). Bacterial feeders, the nematode *Caenorhabditis elegans* and the flagellate *Cercomonas longicauda*, have different effects on outcome of competition among the *Pseudomonas* biocontrol strains CHA0 and DSS73. *Microb. Ecol.* 57, 501–509.

Pieterse, C. M. J., and Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends Plant Sci.* 12, 1360–1385.

Pieterse, C. M. J., van der Does, D., Zamioudis, C., Leon-Reyes, A., and van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521.

Pineda, A., Zheng, S. J., van Loon, J. J. A., and Dicke, M. (2012). Rhizobacteria modify plant–aphid interactions: a case of induced systemic susceptibility. *Plant Biol.* 14, 83–90.

Pineda, A., Zheng, S. J., van Loon, J. J. A., Pieterse, C. M. J., and Dicke, M. (2010). Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends Plant Sci.* 15, 507–514.

Prieto, P., Schiliro, E., Maldonado-Gonzalez, M. M., Valderrama, R., Barroso-Albarracin, J. B., and Mercado-Blanco, J. (2011). Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. *Microb. Ecol.* 62, 435–445.

Raaijmakers, J. M., and Weller, D. M. (1998). Natural plant protection by 2,4-diacetylphloroglucinol – producing *Pseudomonas* spp. in take-all decline soils. *Mol. Plant Microbe Interact.* 11, 144–152.

Saravanakumar, D., Lavanya, N., Muthumeena, K., Raguchander, T., and Samiyappan, R. (2009). Fluorescent pseudomonad mixtures mediate disease resistance in rice plants against sheath rot (*Sarocladium oryzae*) disease. *Biocontrol* 54, 273–286.

Seenivasan, N., David, P. M. M., Vivekanandan, P., and Samiyappan, R. (2012). Biological control of rice root-knot nematode, *Meloidogyne graminicola* through mixture of *Pseudomonas fluorescens* strains. *Biocontrol Sci. Technol.* 22, 611–632.

Senthilraja, G., Anand, T., Durairaj, C., Raguchander, T., and Samiyappan, R. (2010). Chitin-based bioformulation of *Beauveria bassiana* and *Pseudomonas fluorescens* for improved control of leafminer and collar rot in groundnut. *Crop Prot.* 29, 1003–1010.

Stockwell, V. O., Johnson, K. B., Sugar, D., and Loper, J. E. (2011). Mechanistically compatible mixtures of bacterial antagonists improve biological control of fire blight of pear. *Phytopathology* 101, 113–123.

Sukhada, M., Manjula, R., and Rawal, R. D. (2011). Evaluation of arbuscular mycorrhiza and other biocontrol agents against *Phytophthora parasitica* var. *nicotianae* infecting papaya (*Carica papaya* cv. Surya) and enumeration of pathogen population using immunotechniques. *Biol. Control* 58, 22–29.

Sundaramoorthy, S., Raguchander, T., Ragupathi, N., and Samiyappan, R. (2012). Combinatorial effect of endophytic and plant growth-promoting rhizobacteria against wilt disease of *Capsicum annum L.* caused by *Fusarium solani*. *Biol. Control* 60, 59–67.

Tayal, P., Kapoor, R., and Bhatnagar, A. K. (2011). Functional synergism among *Glomus fasciculatum*, *Trichoderma viride* and *Pseudomonas fluorescens* on *Fusarium* wilt in tomato. *J. Plant Pathol.* 93, 745–750.

Tétard-Jones, C., Kertesz, M. A., Gallois, P., and Preziosi, R. F. (2007). Genotype-by-genotype interactions modified by a third species in a plant–insect system. *Am. Nat.* 170, 492–499.

Tétard-Jones, C., Kertesz, M. A., and Preziosi, R. F. (2012). Identification of plant quantitative trait loci modulating a rhizobacteria-aphid indirect effect. *PLoS ONE* 7:e41524. doi: 10.1371/journal.pone.0041524

Timper, P., Kone, D., Yin, J. F., Ji, P. S., and Gardener, B. B. M. (2009). Evaluation of an antibiotic-producing strain of *Pseudomonas fluorescens* for suppression of plant-parasitic nematodes. *J. Nematol.* 41, 234–240.

Troxler, J., Svercel, M., Natsch, A., Zala, M., Keel, C., Moenne-Loccoz, Y., et al. (2012). Persistence of a biocontrol *Pseudomonas* inoculant as high populations of culturable and non-culturable cells in 200-cm-deep soil profiles. *Soil Biol. Biochem.* 44, 122–129.

van der Ent, S., van Wees, S. C. M., and Pieterse, C. M. J. (2009). Jasmonate signaling in plant interactions with resistance-inducing

beneficial microbes. *Phytochemistry* 70, 1581–1588.

van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *Eur. J. Plant Pathol.* 119, 243–254.

van Oosten, V. R., Bodenhausen, N., Reymond, P., van Pelt, J. A., van Loon, L. C., Dicke, M., et al. (2008). Differential effectiveness of microbially induced resistance against herbivorous insects in *Arabidopsis*. *Mol. Plant Microbe Interact.* 21, 919–930.

van Wees, S. C. M., van der Ent, S., and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., et al. (2009). Fungal entomopathogens: new insights in their ecology. *Fung. Ecol.* 2, 149–159.

Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H. S., van Loon, L. C., and Pieterse, C. M. J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.* 17, 895–908.

Walker, V., Couillerot, O., Von Felten, A., Bellvert, F., Jansa, J., Maurhofer, M., et al. (2012). Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum*, *Pseudomonas* and *Glomus* consortium under field conditions. *Plant Soil* 356, 151–163.

Weller, D. M., Landa, B. B., Mavrodi, O. V., Schroeder, K. L., De La Fuente, L., Bankhead, S. B., et al. (2007). Role of 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. in the defence of plant roots. *Plant Biol.* 9, 4–20.

Weller, D. M., Mavrodi, D. V., van Pelt, J. A., Pieterse, C. M. J., van Loon, L. C., and Bakker, P. (2012). Induced systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato by 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. *Phytopathology* 102, 403–412.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511.

Wicklow, D. T., and Poling, S. M. (2009). Antimicrobial activity of pyrrocidines from *Acremonium zeae* against endophytes and pathogens of maize. *Phytopathology* 99, 109–115.

Wu, F. Y., Wan, J. H. C., Wu, S. C., and Wong, M. H. (2012). Effects of earthworms and plant growth-promoting rhizobacteria (PGPR) on availability of nitrogen, phosphorus, and potassium in soil. *J. Plant Nutr. Soil Sci.* 175, 423–433.

Wurst, S. (2010). Effects of earthworms on above- and belowground herbivores. *Appl. Soil Ecol.* 45, 123–130.

Wurst, S., Langel, R., Rodger, S., and Scheu, S. (2006). Effects of belowground biota on primary and secondary metabolites in *Brassica oleracea*. *Chemoecology* 16, 69–73.

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150.

Zhang, P. J., Broekgaarden, C., Zheng, S. J., Snoeren, T. A., van Loon, J. J., Gols, R., et al. (2013). Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defence in *Arabidopsis thaliana*. *New Phytol.* 197, 1291–1299.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 December 2012; accepted: 19 March 2013; published online: 10 April 2013.

*Citation: Hol WHG, Bezemer TM and Biere A (2013) Getting the ecology into interactions between plants and the plant growth-promoting bacterium Pseudomonas fluorescens. Front. Plant Sci. **4**:81. doi: 10.3389/fpls.2013.00081*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Hol, Bezemer and Biere. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 04 July 2013
doi: 10.3389/fpls.2013.00235

Plant growth in *Arabidopsis* is assisted by compost soil-derived microbial communities

Lilia C. Carvalhais, Frederico Muzzi, Chin-Hong Tan, Jin Hsien-Choo and Peer M. Schenk*

Plant-Microbe Interactions Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia

Edited by:
Marcel Dicke, Wageningen University, Netherlands

Reviewed by:
Maria J. Pozo, Consejo Superior de Investigaciones Científicas, Spain
Christos Zamioudis, Utrecht University, Netherlands

Correspondence:
Peer M. Schenk, Plant-Microbe Interactions Laboratory, School of Agriculture and Food Sciences, The University of Queensland, John Hines Building 62, Mansfield Place, Brisbane, QLD 4072, Australia
e-mail: p.schenk@uq.edu.au

Plants in natural and agricultural environments are continuously exposed to a plethora of diverse microorganisms resulting in microbial colonization of roots and the rhizosphere. This process is believed to be accompanied by an intricate network of ongoing simultaneous interactions. In this study, we examined *Arabidopsis thaliana* roots and shoots in the presence or absence of whole microbial communities extracted from compost soil. The results show a clear growth promoting effect on *Arabidopsis* shoots in the presence of soil microbes compared to plants grown in microbe-free soil under otherwise identical conditions. Element analyses showed that iron uptake was facilitated by these mixed microbial communities which also led to transcriptional downregulation of genes required for iron transport. In addition, soil microbial communities suppressed the expression of marker genes involved in nitrogen uptake, oxidative stress/redox signaling, and salicylic acid (SA)-mediated plant defense while upregulating jasmonate (JA) signaling, cell wall organization/biosynthesis and photosynthesis. Multi-species analyses such as simultaneous transcriptional profiling of plants and their interacting microorganisms (metatranscriptomics) coupled to metagenomics may further increase our understanding of the intricate networks underlying plant-microbe interactions.

Keywords: *Arabidopsis*, gene expression, iron deficiency, plant growth promotion, plant-microbe interactions, soil microbial communities

INTRODUCTION

Microbes and plants can establish a multitude of interactions with one another. From an agronomic perspective, soil microorganisms can exert beneficial or detrimental effects on plant growth and productivity. Many beneficial microbes were extensively studied. The classical examples are mycorrhizal fungi and rhizobia. Mycorrhizae associate with roots and provide phosphate, nitrogen (N) and water at the expense of photosynthates (Parniske, 2008; Feddermann et al., 2010; Garg and Chandel, 2010). Rhizobia fix N in exchange of carbon (C) sources especially in leguminous plant species (Raposeiras et al., 2006; Franche et al., 2009; Masson-Boivin et al., 2009). Other interactions involve root and rhizosphere-colonizing fungi and bacteria that are typically attracted by root exudates (Dennis et al., 2010; Carvalhais et al., 2011) and exert beneficial effects on plants by a number of mechanisms. These microbes are known as plant growth promoting microorganisms (PGPM) and they typically promote plant growth and/or improve health by a variety of mechanisms, including phosphate solubilization (Richardson et al., 2009), IAA production (Spaepen and Vanderleyden, 2011), siderophore biosynthesis (Dey et al., 2004), antibiotics production (Chen et al., 2009; Scholz et al., 2011), ACC deaminase activity (Glick et al., 2007; Siddikee et al., 2010), and can increase photosynthetic efficiency (Zhang et al., 2008) and induce systemic resistance (Wang et al., 2009; Phi et al., 2010; Zamioudis and Pieterse, 2012) in plants.

The effects of individual beneficial microbial isolates on plant growth and health have been widely reported (Scotti et al., 2007; Burkett-Cadena et al., 2008; Gulati et al., 2010; Hayat et al., 2010; Niu et al., 2011). The best documented bacterial genera of PGPM are *Pseudomonas* spp. (Jan et al., 2011; Yang et al., 2011) and *Bacillus* spp. (Idris et al., 2007; Fan et al., 2012; Perez-Garcia et al., 2011). Inoculation of mixtures of different strains has been also applied in attempts to produce synergistic results (Ryu et al., 2007; Zachow et al., 2010; Gupta et al., 2011). Plant gene expression during such interactions has also been evaluated in several instances. For example, transcriptome analyses of *Arabidopsis* plants colonized by the endophytic plant growth promoting rhizobacteria (PGPR) *Pseudomonas fluorescens* FPT9601-T5 (Wang et al., 2005), *Pseudomonas thivervalensis* (strain MLG45) (Cartieaux et al., 2003) as well as *Bradyrhizobium* sp. strain ORS278 and the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Cartieaux et al., 2008) were performed. In another instance, gene expression profiles of cotton plants treated with the PGPR *Bacillus subtilis* UFLA285 were evaluated (Medeiros et al., 2011).

Pathogen/microbial associated molecular patterns (PAMPs or MAMPS) are invariant microbial epitopes that are recognized by plants. Cell surface elements including components of fungal cell wall (glucan, chitosan), lipopolysaccharides and peptidoglycanes, as well as flagellins are PAMPs/MAMPs that are recognized by receptors on the root cell surface and trigger a basal immune response, also known as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Millet et al., 2010; Torres, 2010). It has been shown that early responses to infection by symbiotic organisms or pathogenic microbes are rather similar. Plants produce reactive

oxygen species (ROS) in early stages of symbiosis with bacteria and fungi, and this is believed to be reminiscent of the oxidative burst generally triggered by pathogens (Torres, 2010). A defense response is initially produced, but then interrupted at a later stage (Zamioudis and Pieterse, 2012). Microbial molecules released extracellularly, such as siderophores (Meziane et al., 2005; Ran et al., 2005), antibiotics (Weller et al., 2002; Ongena et al., 2007), N-alkylated benzylamine (Ongena et al., 2005), N-acyl-l-homoserine lactone (Schuhegger et al., 2006), and volatiles (Ryu et al., 2004) have also been reported to elicit resistance. A body of evidence indicates that these systemic responses induced by beneficial rhizobacteria are typically mediated by JA as well as ethylene and can lead to induced systemic resistance (ISR) (Van Wees et al., 2008; Van der Ent et al., 2009; Niu et al., 2011). These induced systemic responses confer an enhanced defensive capacity on plants to subsequent pathogen infections and is also known as "priming." JA also modulates responses against necrotrophic pathogens, which feed on dead tissues (Thomma et al., 2000) and JA limits the production of ROS in plants, contributing to resistance against necrotrophs (Ton et al., 2002; Pieterse et al., 2009). Alternatively, biotrophic pathogens that feed on living tissues induce salicylic acid (SA)-mediated responses when recognized, typically leading to a hypersensitive response, characterized by the production of ROS (Jones and Dangl, 2006; Pieterse et al., 2009). In most natural and agricultural environments, however, an intricate network of interactions between plants and their associated microbes takes place simultaneously and often successful pathogens hijack a defense pathway that worsens the infection or are falsely recognized as beneficial (Grant et al., 2013).

A number of studies have comprehensively characterized the root microbiome (Mendes et al., 2011; Bulgarelli et al., 2012; Lundberg et al., 2012). However, to our knowledge, there is currently no information available on transcriptional profiles of roots and shoots affected by whole microbial communities. In this study, we investigated transcriptional responses in roots and shoots of *Arabidopsis thaliana* ecotype Col-0 cultivated in sterile soil or soil inoculated with whole microbial communities extracted from compost soil. The results demonstrate that the combined effect of mixed microbial soil communities provides clear benefits to *Arabidopsis* nutrition and growth.

MATERIALS AND METHODS

PLANT CULTIVATION

Seeds of *A. thaliana* ecotype Col-0 were surface-sterilized using the vapour-phase sterilization method. Briefly, seeds were exposed to chlorine fumes in a desiccator jar for 4 h. Chlorine fumes were generated by adding concentrated hydrochloric acid to commercial bleach (minimum 10.5% available chlorine) up to a final concentration of 1%. Seeds were then placed onto half-strength Murashige and Skoog (MS) medium (pH 5.7) containing 0.8% agarose and incubated at 4°C in the dark for 72 h to break the dormancy. Plates with seeds were then transferred to a tissue culture room with a photoperiod of 16 h of light/8 h darkness and light intensity of 60–100 μmol m^2 s^{-1} at 22°C for 14 days. Five seedlings each were then transplanted into 7.5 cm-diameter clear transparent tissue culture jars which contained 50 g of a 1:1 mixture of University of California mix and commercial compost soil (Greenfingers B2 potting mix, Nerang, Australia) that had undergone one of the soil treatments described below on the same day. This soil blend provided optimized water drainage for cultivation of *Arabidopsis* plants in tissue culture jars. Before planting, jars were filled with soil and γ-irradiated by using a ^{60}Co source at a dose of 25 (KiloGray) kGy and a rate of 20 kGy/h. Sterile soils were tested for microbial contamination by incubating soil samples in Luria-Bertani and Potato Dextrose broth for 7 days at 30°C.

Treatments consisted of three biological replicates containing 10 jars each (50 plants per replicate). Four treatments were applied to sterile soil: (1) addition of non-sterile compost soil extract, which constituted a source of soil microbial communities, (2) filter-sterilized compost soil extract, (3) a sterile solution of Na-Fe-EDTA (13 μM) and (4) and sterile water. The compost soil extract was prepared by adding compost soil to sterile water up to a final concentration of 3.3% (w/v). After stirring, large particles were removed by filtering through Whatman grade 1 filter paper (11 μm). For the control, microbes were removed by passing this extract through a 0.22 μm filter. Each of the five seedlings in the tissue culture jars received 1.2 mL of the corresponding treatment solution. An additional experiment was carried out to compare autoclavation and γ-irradiation as soil sterilization methods. Soils were autoclaved twice at 121°C for 30 min and tested for microbial contamination as described above. This experiment also comprised three biological replicates per treatment with 50 plants per replicate (200 plants in total).

The use of compost soil extract as the inoculum allowed the addition of both culturable and unculturable soil microbes, as it is widely known that the vast majority of soil microbes cannot be cultivated in standard culture media (Kellenberger, 2001). A preliminary analysis on culturable bacteria from this compost soil showed that the majority came from the genera *Pseudomonas*, *Burkholderia*, *Pusillimonas,* and *Achromotobacter*. To also account for unculturable microbes, we performed 16 S rRNA gene amplicon pyrosequencing analysis using DNA extracted from a soil sample that has been inoculated with non-filtered compost soil extract (Carvalhais et al., unpublished results). This analysis targets both culturable and unculturable bacterial and archaea populations and a considerably high Operational Taxonomic Unit richness was found (∼500).

PLANT HARVEST, TOTAL RNA EXTRACTION AND cDNA SYNTHESIS

Four weeks after germination (including 2 weeks of soil treatments), plants were evaluated for phenotypic differences before harvesting. Soil was removed by washing and blotting on a tissue paper before fresh weights of roots and shoots were quickly measured, snap-frozen in liquid N, and stored at −80°C. Total RNA from roots and shoots was extracted independently with the SV Total RNA Isolation System (Promega, Madison, WI, USA) using 70 mg of ground tissue pooled from 50 plants per replicate as a representative sample. RNA concentrations were measured using a NanoDrop ND-1000

UV-Vis Spectrophotometer (Nanodrop Technologies, Rockland, DE). A total of 272 ng of RNA was used for cDNA synthesis using the SuperScript III reverse transcriptase for quantitative reverse transcriptase PCR (Invitrogen, Carlsbad, CA, US) following the manufacturer's instructions.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE PCR

Primers used in qRT-PCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA; **Table S1**). Each reaction was performed in a final volume of 10 μL, and contained 2 μL of cDNA, 1 μL of each primer (1 μM), 5 μL of SYBR Green using the 7900 HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative expression (*n*-fold) of the normalized target gene in both treatments was determined as proposed by Pfaffl (2001). *Arabidopsis* transcript levels in shoots and roots were normalized to the expression of a mixture of three genes encoding β-*ACTIN2*, *ACTIN7,* and *ACTIN8* (Schenk et al., 2005). Thermal cycling conditions consisted of 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C prior to 2 min at 25°C.

cDNA MICROARRAY ANALYSIS

Total RNA for microarray hybridizations was isolated from shoots and roots from an independent experiment with three biological replicates (50 plants each) as detailed above using γ-sterilized soil. Plant growth conditions were the same as the ones used for qRT-PCR. Three microarrays were used for the three replicate shoot samples and one microarray was used for a preliminary study on roots using combined RNA samples from three replicate root samples to obtain sufficient RNA. Total RNA was reverse-transcribed into cDNA and labeled with either Cy3 or Cy5 fluorescent dyes, mixed and used for subsequent hybridization onto 4 × 44K Agilent *Arabidopsis* GeneChip arrays (Agilent Technologies, Santa Clara, CA, USA). Labeling and hybridization of cDNA, including scanning of the chips were performed by the Australian Genome Research Facility (AGRF, Victoria, Australia). Signal intensities for each feature were extracted from scanned microarray images using Agilent Feature Extraction version 10.5.1.1 software (Agilent Technologies). Extracted data were analyzed using Integromics Biomarker Discovery (Integromics, Granada, Spain) and then normalized within arrays with the Loess algorithm and between arrays using the quantile method (Bolstad et al., 2003). Microarray data sets were deposited at Gene Expression Omnibus (accession GSE44984).

Significantly differentially expressed genes were selected based on the following criteria. Firstly, genes with signals that had high signal intensities, as well as higher than background signals based on the Agilent Feature Extraction in both Cy3 and Cy5 channels were selected. Secondly, genes with *P*-values lower than 0.05 using a parametric based test (Welch *t*-test) were considered statistically significant. Finally, genes that presented a signal difference of equal or greater than 1.5 fold-change in shoots and equal or greater than 2.5 fold-change in roots between the treatments (non-sterile vs. sterile) were considered as significant. A statistical analysis for overrepresentation of Gene Ontology (GO) terms in differentially expressed genes in shoots and roots in the presence of microbes was carried out for a simplified overview of affected functions. Gene IDs significantly associated with specific GO-terms ($P < 0.05$) were downloaded from the GO browser AmiGO (http://amigo.geneontology.org). Statistically significant microarray data was generally consistent with qRT-PCR data. However, differences were observed for three genes (*CAT3*, *AT2G43150* and *NIA1*). Out of these, *NIA1* was independently shown to be downregulated in the presence of microbes by qRT-PCR. Differences observed between microarray data and qRT-PCR may be caused by cross-hybridization, impurities in hybridization buffers causing deposition of dust on some spots and the fact that qRT-PCR and microarray data are derived from independent experiments where microbial communities may have slightly differed (although great care was taken to ensure that conditions were kept consistent).

PLANT TISSUE AND RHIZOSPHERE SOIL ELEMENT ANALYSIS

From each treatment, 28-day-old plants were harvested by careful uprooting and washing in water before blotting on tissue paper and drying at 70°C for 2 days. Three biological replicates containing 20 plants each were collected per treatment. Dried whole plant tissues were then ground to a fine powder and 200 mg of dry weight per replicate were used for subsequent analyses. Elemental analysis was carried out by inductively coupled plasma (ICP) atomic absorption with a Varian Vista Pro ICP Optical Emission Spectrometer (OES). Samples were digested in nitric/perchloric acid at a ratio of 5:1. The above procedure was repeated for corresponding rhizosphere soil that was collected by shaking the associated soil off carefully uprooted plants (**Figure S1**). Soil was then sieved through a 2 mm sieve to remove root residues (if any) before performing acid digestion. Total C and N concentration for plant tissues were separately determined by combustion using an automated dry combustion instrument LECO CNS 2000 (LECO, St. Joseph, MI, USA) at 1100°C. In addition, soil samples were analyzed using the DTPA (diethylenetriaminepentaacetic acid)-extraction method (Lindsay and Norvell, 1978) to determine bioavailable copper, iron, zinc, and manganese.

RESULTS

WHOLE SOIL MICROBIAL COMMUNITIES PROMOTE ARABIDOPSIS SHOOT GROWTH

To investigate the effects of whole soil microbial communities on *Arabidopsis* growth, plants were cultivated for 2 weeks in either sterile soil (also here referred to as "microbe-free soil"), or non-sterile soil (soil-containing microbes). For ideal comparisons, all soil was initially sterilized by γ-irradiation. These were then either inoculated with microbial extract from compost soil (non-sterile soil) or filter-sterilized extract from compost soil (microbe-free control). The addition of sterile soil extract was considered necessary to rule out any differences caused by the transfer of nutrients. In addition, the effect of the soil sterilization method used was evaluated by comparing plants grown in γ-irradiated as well as in autoclaved soils. Irrespective of the soil sterilization method used, plants cultivated in the presence of microbes displayed more vigorous growth than plants grown under sterile conditions. These

plants had approximately twice the shoot weight ($P < 0.05$) and visibly larger leaf areas, while root biomass was not markedly different between treatments ($P > 0.05$; **Figures 1A,B**). Leaves of plants grown in sterile soil were also smaller, more poorly developed and fragile with signs of leaf curling, elongated leaf axes and crispy, brittle, but not dry, leaf structure (**Figures 1**, **S1**). Plants grown in sterile soil also often displayed a poorly developed caudal stem with developing inflorescences, while plants grown in the presence of microbes showed no signs of early flowering. Cotyledons of *Arabidopsis* plants in the absence of soil microbes were frequently yellow or dead (**Figure S1**), suggesting signs of early senescence in these plants. The above phenotypic differences were confirmed by independent additional experiments with three biological replicates (50 plants each) using autoclaved soil.

CHANGES IN NUTRITIONAL STATUS INDUCED BY MICROBES

A multi-element analysis of plant tissues was performed to investigate whether the enhanced growth in non-sterile soils resulted from a higher availability/acquisition of nutrients provided by microbes. No significant difference in carbon-nitrogen (C/N) ratio was observed in tissues from plants cultivated in sterile compared with non-sterile conditions (**Figure S2**). This implies that plant growth promotion was not associated with a higher N acquisition. However, a two-fold increase in Fe and Mn concentrations was found in plant tissues harvested from soils containing microbes compared to sterile soils (**Figure 2**). No significant differences in concentrations were found for other nutrients measured, such as calcium, potassium, magnesium, sodium, phosphate, sulphur, aluminium and boron (**Figure 2**).

A multi-element analysis (ICP-OES) was also carried out for bulk and rhizosphere soils to determine the nutrient status of the root-associated soil at the time of the harvest (28-day-old plants). Fe concentrations were significantly higher

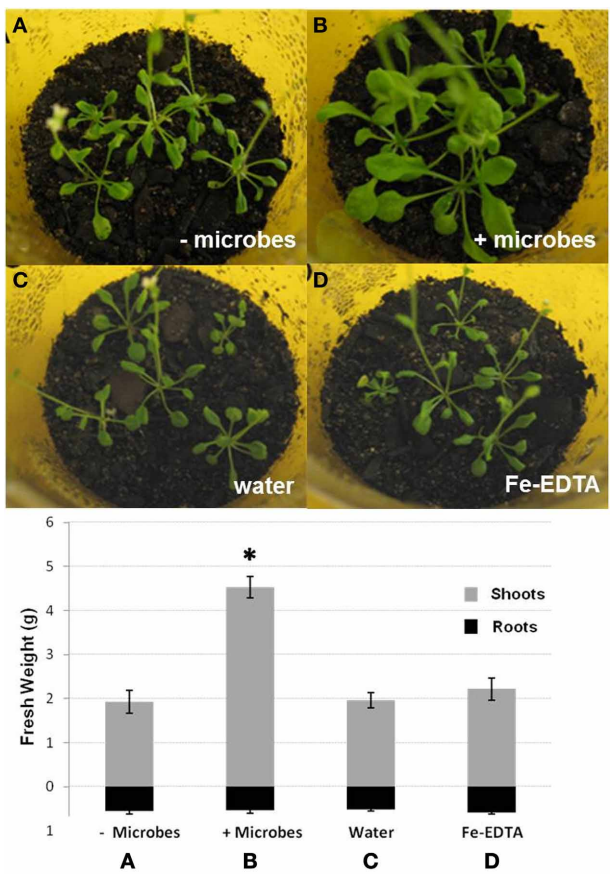


FIGURE 1 | Photographs of shoots and fresh weights of shoots and roots of *Arabidopsis* plants grown in sterile soil that was inoculated either with filter-sterilized soil extract (A; − microbes), non-sterile soil extract (B; + microbes), water (C) or Fe-EDTA (D). Bars represent mean values ±SE from three biological replicates (50 pooled plants/replicate). The asterisk indicates a statistically significant difference ($P < 0.05$).

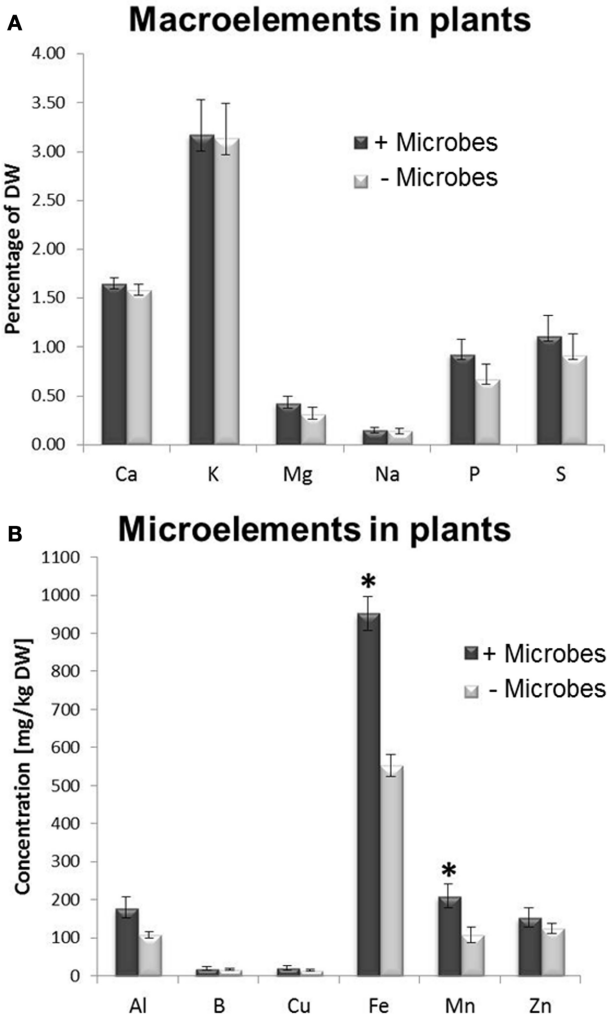


FIGURE 2 | Macro and micronutrient element analysis in *Arabidopsis* plants grown in the presence of soil microbes (non-sterile) or in microbe-free soils (sterile treatment). (A) Macronutrients, **(B)** Micronutrients. Bars represent mean values per dry weight (DW) ± SE from three biological replicates (20 pooled plants/replicate). The asterisk indicates a statistically significant difference ($P < 0.05$).

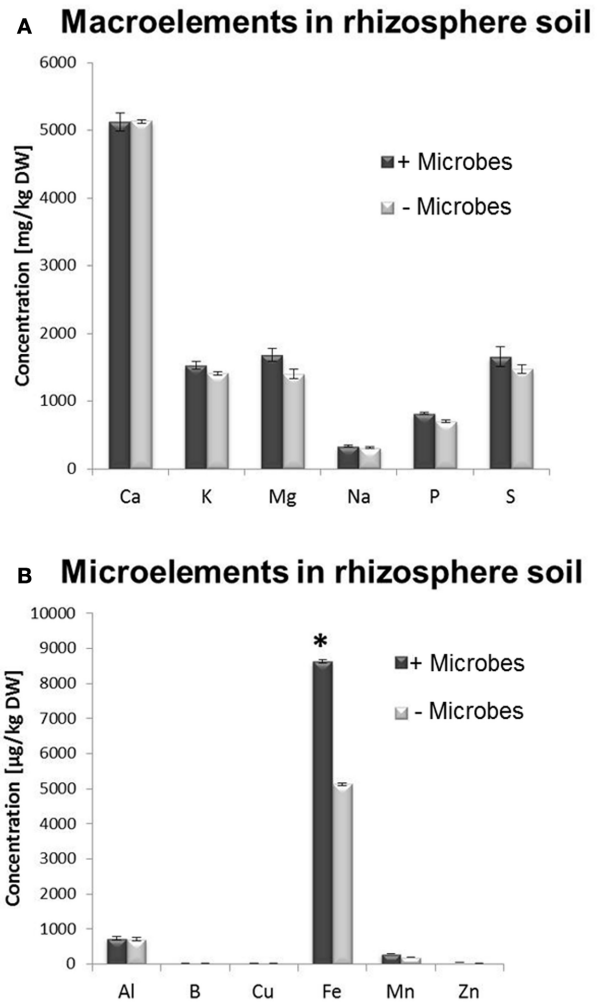


FIGURE 3 | ICP-OES analysis of total element concentration in sieved rhizosphere soil from *Arabidopsis* plants grown in the presence or absence of soil microbes. (A) Macronutrients, **(B)** Micronutrients. Bars present mean values in mg/kg of soil dry weight (DW) ±SE from 3 independent replicates (100 g soil pooled from 10 pots/replicate). The asterisk indicates a statistically significant difference ($P < 0.05$).

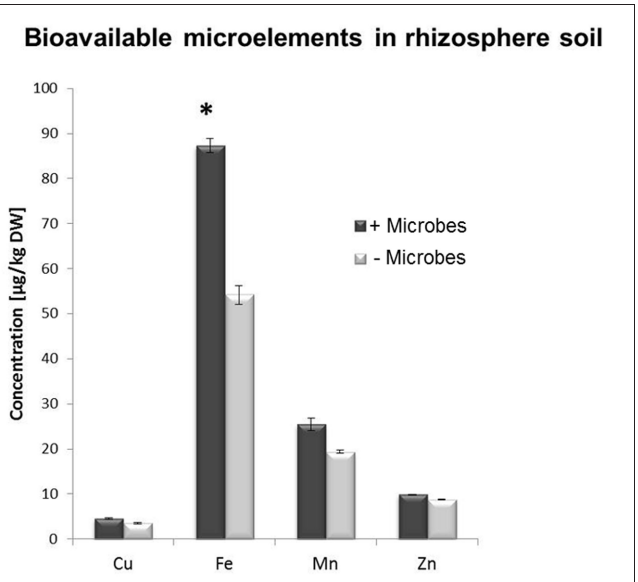


FIGURE 4 | DTPA extraction-based analysis of bioavailable trace element concentrations in rhizosphere soil from *Arabidopsis* plants grown in the presence or absence of soil microbes. Bars present mean values in µg/kg of soil dry weight (DW) ±SE from 3 independent replicates (pooled rhizosphere soil from 50 plants/replicate). The asterisk indicates a statistically significant difference ($P < 0.05$).

in non-sterile compared to sterile rhizosphere soils (**Figure 3**), while amounts of other elements were relatively similar and no significant differences could be observed for bulk soil distant from roots (**Figure S3**). A different method for micronutrient analysis (DTPA-extraction method) was also performed to measure bioavailable trace elements in rhizosphere soils. These confirmed that a higher concentration of Fe was available to plants in rhizosphere microbe-containing soils compared to microbe-free soils (**Figure 4**).

Fe-EDTA was added into sterile soils to investigate whether differences in *Arabidopsis* growth in non-sterile and microbe-free soils could be attributed solely to a higher availability of Fe. Water or filter-sterilized soil extract was used as controls. No significant differences in plant growth could be observed between additions of Fe-EDTA, sterile soil extract, or water (**Figures 1C,D**). These results indicate that a lower availability of Fe was not the only factor causing decreased shoot growth in microbe-free soils compared to non-sterile soils.

GENE EXPRESSION ANALYSIS OF *ARABIDOPSIS* PLANTS GROWN IN THE PRESENCE OR ABSENCE OF SOIL MICROBES

Gene expression profiling was conducted on selected marker genes to identify processes involved in the interactions between *Arabidopsis* roots and soil microbial communities and to better understand the observed differences between plants grown in the presence or absence of soil microbes. The selection of genes for qRT-PCR was based on putative processes identified in the results of phenotypical analyses described previously, such as increased plant growth and enhanced iron incorporation. A number of marker genes associated to iron acquisition and metal homeostasis were chosen, such as *IRT1*, *FRO2*, *OPT3, MYB72,* and At3g07720. Although no differences in C/N ratio were observed in plant tissues (**Figure S2**), a gene involved in N acquisition (*NIA1*) was included as N is one of the major macronutrients required in plant nutrition. Given that beneficial microbes have been reported to alleviate stress derived from biotic and abiotic sources (de Zelicourt et al., 2013), genes that are representative of several stress-related responses were also selected, including pathogen defense responses (*PR1*, *PDF1.2, LECTIN1, LECTIN2, WRKY70, WRKY25, MYC2, ERF104*), oxidative stress responses (*CAT1*, *PER50, ERF6, ZAT10, OPR2*), abiotic stress (*WRKY25*, *MYB15*) and senescence (*SEN1*). A full list of selected marker genes, their locus names and qRT-PCR primers is shown in **Table S1**.

NIA1, which is required for nitrate assimilation (Scheible et al., 2004), was downregulated in roots grown in the presence of microbes (**Figure 5A**). Genes directly involved in Fe acquisition (*IRT1*, *FRO2*) were also downregulated in roots of *Arabidopsis* roots grown in soil containing microbes (**Figure 5A**). Furthermore, genes involved in upstream signaling/regulation of Fe acquisition and metal homeostasis (*MYB72, OPT3*, *At3g07720*) were also downregulated (**Figure 5A**). qRT-PCR analysis of *IRT1* and *MYB72* using autoclaved instead of γ-irradiated soil gave similar results (**Figure S4**). This confirms that *Arabidopsis* plants were responding to the lower Fe availability in sterile soils compared to plants grown in the presence of microbes.

Genes involved in oxidative stress and redox homeostasis were generally lower expressed in roots when plants were grown in soil containing microbes. These include catalase (*CAT1*)- and peroxidase (*PER50*)-encoding genes that are required for ROS detoxification (Miller et al., 2008; **Figure 5A**). Furthermore, genes involved in upstream redox signaling (*WRKY25, ERF6; OPR2;* Zheng et al., 2007; Jiang and Deyholos, 2009; Wang et al., 2013) were also downregulated (**Figure 5A**). Similarly, *SEN1*, a gene involved in senescence, but also other pathways, including

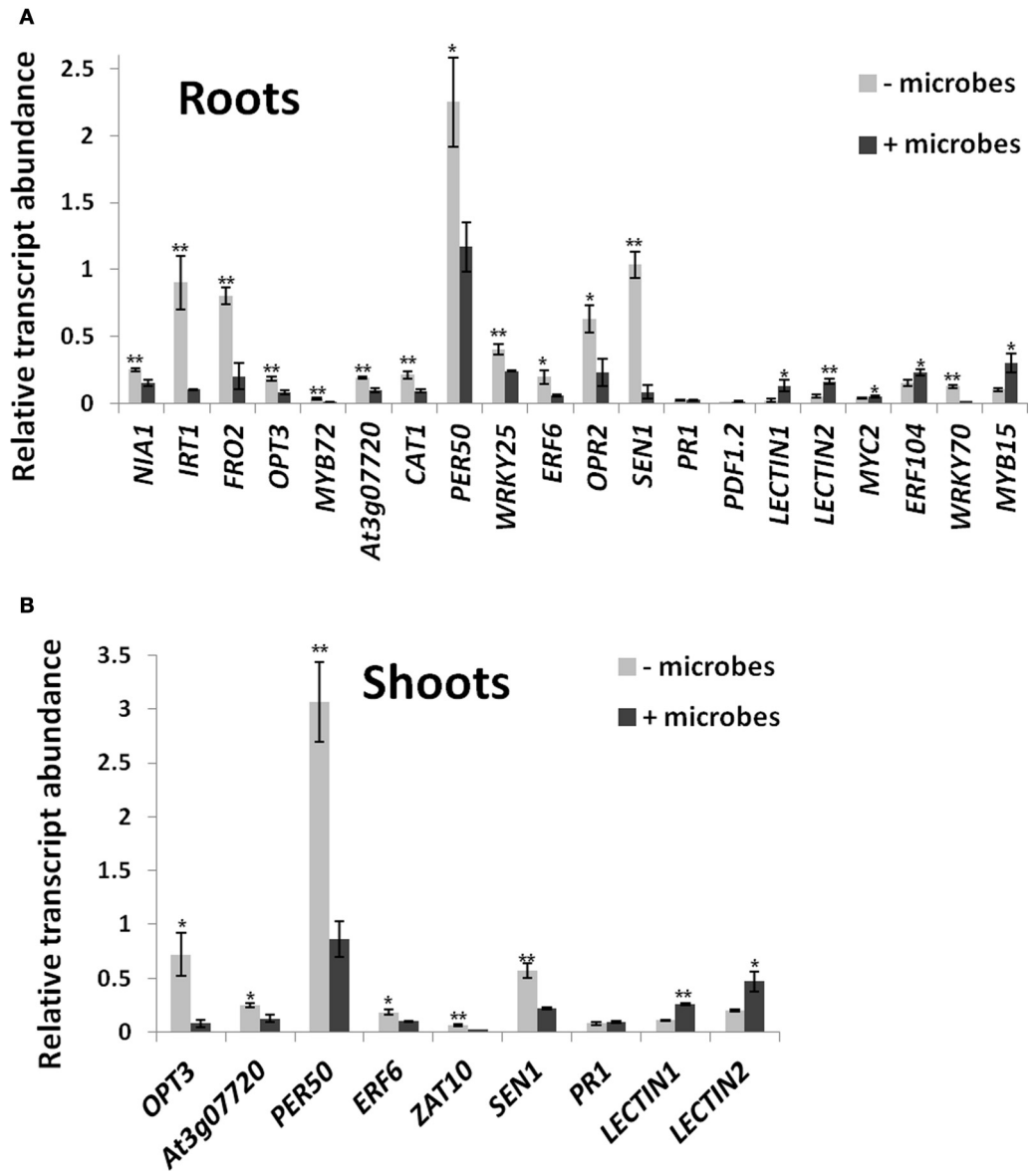


FIGURE 5 | Differential expression of marker genes in roots (A) and shoots (B) of *Arabidopsis* grown in soil in the presence or absence of whole microbial communities. Transcript abundances are shown relative to *ACTIN* genes measured by qRT-PCR from three independent biological replicates. Each replicate contained pooled samples from 50 plants. Bars represent mean ± SE. The asterisk indicates a statistically significant difference ($P < 0.05$), two asterisks ($P < 0.01$). See **Table S1** for full gene locus names.

oxidative stress and SA-mediated plant defense (Schenk et al., 2005) was downregulated in roots in the presence of microbes (**Figure 5A**). Interestingly, the classical defense marker genes for the SA and JA defense pathways, *PR1* and *PDF1.2* (Thomma et al., 2000; Metraux, 2002) were not differentially expressed in roots, but their expression levels were also very low (<2% of *ACTIN* transcript levels; **Figure 5A**). JA signaling, a pathway also known to involve beneficial interactions with microbes for ISR (Van der Ent et al., 2009; Zamioudis and Pieterse, 2012), was upregulated in roots as shown by the induction of JA-regulated *LECTIN* genes (At3g15356 and At3g16530; Jung et al., 2007; **Figure 5A**) as well as the pathway's key regulatory gene *MYC2* (Anderson et al., 2004; Kazan and Manners, 2013), a gene that is also required for ISR triggered by beneficial soil microbes (Kazan and Manners, 2013). In addition, the JA/ethylene regulatory gene *ERF104* (Bethke et al., 2009) was upregulated, while *WRKY70*, a negative regulator of the JA pathway and a positive regulator of the SA pathway (Li et al., 2004), was downregulated in roots exposed to soil microbes (**Figure 5A**). The abiotic stress regulators *WRKY25* (SA-inducible; Zheng et al., 2007) and *MYB15* [abscisic acid (ABA)-inducible; Ding et al., 2009; Chinnusamy et al., 2010; **Figure 5A**] were also repressed.

Some of the physiological processes occurring in roots in the presence of soil microbes were also found in shoots. This includes downregulation of genes involved in Fe and metal homeostasis (*OPT3; At3g07720*), oxidative stress (*CAT1, PER50; ERF6; ZAT10*) and senescence (*SEN1*) **Figure 5B**). As in roots, *PR1* was not differentially expressed, while JA-responsive lectin-encoding genes At3g15356 and At3g16530 were induced in shoots in the presence of soil microbes (**Figure 5B**).

To identify other physiological processes associated with the presence of whole soil microbial communities that may lead to the increased shoot growth; a microarray analysis was carried on shoots and roots of *Arabidopsis* plants cultivated under the above conditions using γ-irradiated soil (**Table S2**). A GO enrichment analysis was performed in lists of up or downregulated genes to provide an overview of potential biological functions associated in soil microbe-root interactions (**Tables 1**, **S3**). In roots, GO terms that were enriched in the upregulated gene list, included response to stimulus, response to abiotic stimulus, response to oxidative stress, response to light stimulus, C fixation and plant-type cell wall organization or biogenesis (**Table S3**). In the downregulated gene list in roots, GO terms associated with iron transport and homeostasis were enriched (**Table S3**), which corroborates the results of the qRT-PCR (**Figure 5A**). In shoots, several GO terms were overrepresented in the upregulated gene list, such as those related to photosynthesis and responses to chemical and biotic stimulus, including response to other organisms as bacteria and fungi (chitin), stress related responses, JA signaling and ISR (**Table 1**). Most of these GO terms reveal responses involved in biotic interactions, which were apparently more pronounced in shoots compared to roots in terms of gene expression. Indeed, the overall phenotypical outcome of introducing a compost soil-derived community of microbes was an enhanced shoot growth, as opposed to no changes found in root length and biomass (**Figure 1**). Interestingly, several genes involved in defense responses were also up-regulated in shoots, as evidenced by the GO term enrichment analysis (**Table 1**). The enrichment of GO terms related to photosynthesis both in shoots and roots suggests that soil microbe-mediated plant growth promotion also coincided with increased photosynthetic activity. Most of the genes that contributed to the overrepresentation of the GO term "response to biotic stimulus" are also involved in defense responses, specifically jasmonate signaling (*CYP71A12*), SA signaling (*CRK4, AT5G02490, ATMPK3, WRKY70*), and oxidative stress (*At5G64120, RBOHD, At2G37130, ATMPK3*).

DISCUSSION

PLANT NUTRITION IN THE PRESENCE OF RHIZOSPHERE MICROORGANISMS

The present study shows that plants grown in the presence of whole soil microbial communities exhibited enhanced shoot growth when compared to plants cultivated on sterile soil. This is consistent with related studies that reported that plants inoculated with individual beneficial microorganisms displayed an increase of fresh weight compared to axenically grown plants (Persello-Cartieaux et al., 2001; Ryu et al., 2005). However, so far it remained unclear whether this growth promoting effect can also be achieved by a complex assemblage of soil microbial populations. The increased shoot growth may be partially attributed to improved plant nutrition (**Figures 2B**, **3B**, and **4**). Global gene expression profiling using microarray analyses of shoots and roots also showed that many genes were down-regulated in the presence of soil microorganisms which may have translated into metabolic cost savings for these plants. Although the presence of soil microbes did not affect plant C/N ratio (**Figure S2**), the downregulation of *NIA1*, which is involved in nitrate assimilation (Scheible et al., 2004), indicates that microbes compete for the nitrate available in the soil, as reported previously (Song et al., 2007; Xu et al., 2011). Instead, plants may utilize other N sources available to them, including organic forms. Indeed, there are several ways that rhizosphere bacteria, many of which also fix atmospheric N_2, contribute to N uptake in plants, including organic forms such as amino acids, oligopeptides, DNA, as well as whole proteins (Paungfoo-Lonhienne et al., 2008, 2010a). In addition, whole bacteria and yeast cells have been shown to be taken up and consumed by plant roots (Paungfoo-Lonhienne et al., 2010b), although it is currently uncertain how significant this process is to N acquisition.

Tissues as well as rhizosphere soils collected from plants grown in the presence of microbes showed higher Fe content (**Figures 2**, **3**, **4**). In addition, genes involved in Fe acquisition (e.g., the high affinity iron transporter IRT1; Colangelo and Guerinot, 2004) and metal homeostasis in these plants were down-regulated (**Figure 5A**). There are two strategies that plants utilize to acquire Fe in conditions of deprivation. Strategy I is employed by dicots and non-graminaceous monocots and it relies on reductases and proton secretion to increase the availability of insoluble inorganic iron by means of lowering the redox conditions and rhizosphere acidification. Conversely, Strategy II applies to graminaceous monocots that release chelating organic molecules known as siderophores to scavenge iron from the soil solution. Siderophores

Table 1 | Identification of Gene Ontology (GO) functional categories that are enriched in transcript populations in shoots ($n = 3$) in the presence of whole soil microbial communities.

GO term	*P*-value	Sample frequency	Background frequency	Gene names
UPREGULATED				
GO:0050896 response to stimulus	1.06E-06	66/243 (27.2%)	3815/29970 (12.7%)	FHL, AT1G72920, VTC2, ACS6, AT5G64120, AT4G34810, LHCA1, CRK4, CYP71A12, RBOHD, WRKY38, AT5G38420, SUR1, WRKY46, CYP83B1, PRXR1, CYP83A1, AT2G04795, AT5G38410, PMSR2, ERF2, MYB51, AT4G09420, WRKY54, ATL2, AT3G26590, AT5G51470, AT1G78410, PIL1, CYP707A2, ATBBD1, ERF11, AT1G72910, AT1G02820, TCH2, AT1G20620, SHI, AT5G38344, APX1, JAZ6, NIP6;1, AT5G02490, AT3G02840, PUB22, AT1G74670, ERF104, SZF1, PUB23, ECS1, AT2G37130, NAXT1, AT1G70000, TIP2, AT5G51190, ERF4, MGD2, AT1G32920, STO, AT1G20823, AT4G24350, ATRLP26, EBF1, CYP71B2, ATMPK3, WRKY70, AT4G30370
GO:0009607 response to biotic stimulus	1.83E-02	18/243 (7.4%)	703/29970 (2.3%)	VTC2, AT5G64120, CRK4, CYP71A12, RBOHD, WRKY38, CYP83B1, ERF2, MYB51, ATBBD1, AT5G02490, AT3G02840, ERF104, AT2G37130, TIP2, ERF4, ATMPK3, WRKY70
GO:0042221 response to chemical stimulus	4.50E-03	36/243 (14.8%)	1984/29970 (6.6%)	VTC2, ACS6, AT4G34810, CRK4, WRKY38, WRKY46, PRXR1, AT2G04795, PMSR2, ERF2, MYB51, ATL2, AT3G26590, AT5G51470, AT1G78410, ERF11, TCH2, AT1G20620, SHI, APX1, JAZ6, NIP6;1, AT5G02490, AT3G02840, PUB22, AT1G74670, SZF1, PUB23, AT1G70000, AT5G51190, ERF4, AT1G20823, EBF1, ATMPK3, WRKY70,AT4G30370
GO:0009743 response to carbohydrate stimulus	1.72E-05	13/243 (5.3%)	209/29970 (0.7%)	WRKY46, ERF2, ATL2, PUB22, AT1G74670, SZF1, PUB23, AT5G51190, ERF4, AT1G20823, ATMPK3, WRKY70, AT4G30370
GO:0051707 response to other organism	7.87E-03	18/243 (7.4%)	660/29970 (2.2%)	VTC2, AT5G64120, CRK4, CYP71A12, RBOHD, WRKY38, CYP83B1, ERF2, MYB51, ATBBD1, AT5G02490, AT3G02840, ERF104, AT2G37130, TIP2, ERF4, ATMPK3, WRKY70
GO:0009617 response to bacterium	4.76E-03	12/243 (4.9%)	291/29970 (1.0%)	VTC2, CRK4, CYP71A12, WRKY38, CYP83B1, ERF2, MYB51, AT5G02490, TIP2, ERF4, ATMPK3, WRKY70
GO:0010200 response to chitin	5.37E-07	12/243 (4.9%)	127/29970 (0.4%)	WRKY46, ERF2, ATL2, PUB22, SZF1, PUB23, AT5G51190, ERF4, AT1G20823, ATMPK3, WRKY70, AT4G30370
GO:0006952 defense response	1.37E-05	25/243 (10.3%)	815/29970 (2.7%)	AT1G72920, VTC2, AT5G64120, CRK4, RBOHD, WRKY38, CYP83B1, ERF2, MYB51, AT4G09420, WRKY54, ATL2, ATBBD1, AT1G72910, TCH2, AT5G38344, PUB22, ERF104, PUB23, ECS1, AT2G37130, TIP2, ERF4, AtRLP26, WRKY70
GO:0006950 response to stress	1.02E-04	42/243 (17.3%)	2161/29970 (7.2%)	AT1G72920, VTC2, ACS6, AT5G64120, CRK4, RBOHD, WRKY38, CYP83B1, PRXR1, AT2G04795, PMSR2, ERF2, MYB51, AT4G09420, WRKY54, ATL2, AT1G78410, ATBBD1, AT1G72910, AT1G02820, TCH2, AT1G20620, AT5G38344, APX1, JAZ6, AT5G02490, PUB22, ERF104, PUB23, ECS1, AT2G37130, AT1G70000, TIP2, ERF4, MGD2, AT1G32920, STO, AT4G24350, AtRLP26, CYP71B2, ATMPK3, WRKY70

(Continued)

Table 1 | Continued

GO term	*P*-value	Sample frequency	Background frequency	Gene names
GO:0010033 response to organic substance	3.97E-03	26/243 (10.7%)	1181/29970 (3.9%)	VTC2, ACS6, AT4G34810, CRK4, WRKY38, WRKY46, ERF2, MYB51, ATL2, AT5G51470, ERF11, TCH2, SHI, JAZ6, PUB22, AT1G74670, SZF1, PUB23, AT1G70000, AT5G51190, ERF4, AT1G20823, EBF1, ATMPK3, WRKY70, AT4G30370
GO:0045730 respiratory burst	4.81E-03	3/243 (1.2%)	5/29970 (0.0%)	AT5G64120, PUB22, PUB23
GO:0015979 photosynthesis	8.47E-03	9/243 (3.7%)	166/29970 (0.6%)	LHCA1, LHCA2, LHB1B2, LHCA3, AT5G28450, LHCA4, PSAF, CAB1, PSI-P
GO:0042435 indole derivative biosynthetic process	1.62E-02	5/243 (2.1%)	40/29970 (0.1%)	SUR1, CYP83B1, MYB51, NIT1, ATMPK3
GO:0042430 indole and derivative metabolic process	3.25E-02	5/243 (2.1%)	46/29970 (0.2%)	SUR1, CYP83B1, MYB51, NIT1, ATMPK3
GO:0009753 response to jasmonic acid stimulus	4.76E-02	8/243 (3.3%)	160/29970 (0.5%)	VTC2, ACS6, ERF2, MYB51, JAZ6, AT1G70000, ERF4, WRKY70
GO:0009682 induced systemic resistance	4.96E-03	4/243 (1.6%)	15/29970 (0.1%)	CYP83B1, ERF2, ERF4, WRKY70
GO:0009864 induced systemic resistance, jasmonic acid mediated signaling pathway	1.66E-02	3/243 (1.2%)	7/29970 (0.0%)	ERF2, ERF4, WRKY70
GO:0006790 sulfur metabolic process	2.29E-02	9/243 (3.7%)	188/29970 (0.6%)	APR1, ACS6, SUR1, CYSD2, CYP83B1, APS3, CYP83A1, MYB51, ATMPK3
DOWNREGULATED				
GO:0050896 response to stimulus	1.20E-02	19/55 (34.5%)	3815/29970 (12.7%)	GT72B1, VTC2, RAP2.4, AT1G76190, CP12-2, HSF, A4A, AT1G70000, ATMRP7, CBL5, CRY1, AT2G31730, AT2G40460, STO, AT5G41750, AT3G23600, EBF1, PRXR1, RING1, YSL1
GO:0042221 response to chemical stimulus	4.36E-03	14/55 (25.5%)	1984/29970 (6.6%)	GT72B1, VTC2, RAP2.4, CP12-2, HSF, A4A, AT1G70000, CBL5, AT2G31730, EBF1, PRXR1, RING1, YSL1
GO:0009651 response to salt stress	3.33E-02	6/55 (10.9%)	386/29970 (1.3%)	GT72B1, RAP2.4, AT1G70000, CBL5, STO, AT3G23600

are also able to release iron from complexes contained in humic and fulvic acids present in the organic matter, as well as to mobilize Fe from minerals in the solid phase. Such molecules can be produced by nearly all cultured microbial isolates (Crowley, 2006) and especially microbe-derived siderophores have been reported to confer resistance to hydrogen peroxide (Dellagi et al., 1998; Oide et al., 2006). This may partly explain why plants in the absence of microbes displayed higher expression levels for genes involved in oxidative stress/redox homeostasis (*CAT1, PER50*; **Figure 5A**). In plants that adopt strategy I for iron acquisition; such as *Arabidopsis*, the plasma membrane-bound Fe^{3+} chelate reductase FRO2 catalyzes the reduction of Fe^{3+} at the cell surface (Robinson et al., 1999), which is then taken up by IRT1, an iron regulated transporter of the ZIP family (Varotto et al., 2002; Vert et al., 2002). Consistent with our findings, *IRT1, FRO2*, *MYB72* and *At3g07720* were previously found to be upregulated during Fe deficiency and are directly regulated by FIT, a basic helix–loop–helix (bHLH) transcription factor required for root iron uptake (Colangelo and Guerinot, 2004; Sivitz et al., 2012). MYB72 has a demonstrated role in iron uptake regulation (Sivitz et al., 2012), but interestingly is also activated by beneficial soil microbes (Van der Ent et al., 2008). *At3g07720*

encodes a Kelch repeat protein and is regulated by Fe deficiency (Sivitz et al., 2012) but has putative biochemical functions including SA biosynthesis. OPT3 is also involved in metal ion homeostasis (Stacey et al., 2008). Upregulation of these genes in plants grown in the absence of microbes (**Figure 5**) is consistent with the finding that these plants did not have the same amount of bioavailable Fe than plants grown in the presence of microbes. This effect was observed irrespective of the soil sterilization method used (**Figure S4**). Although γ-irradiation is known for posing the least disturbance to physical and chemical properties in soils in comparison to autoclaving (Alef and Nannipleri, 1995; Berns et al., 2008), significant differential expression for *IRT1* and *MYB72* were maintained irrespective of the sterilization method used (**Figure S4**). Plant growth can be indirectly affected by siderophore-producing bacteria as they exhibit improved rhizosphere competence in Fe-deficient soils (Babalola, 2010). However, the addition of Fe-EDTA to the sterile soil did not trigger significantly increased plant growth compared to plants grown in microbe-free soil (**Figures 1C,D**). This indicates that iron supply was not the dominant factor associated to the enhanced plant growth in non-sterile conditions. Interestingly, Mn was also less abundant in *Arabidopsis* plants in the absence of microbes (**Figures 3**, **4**). Indeed, both elements, Fe and Mn, use the same ITR1- and FRO2-mediated transport mechanisms for uptake in *Arabidopsis* plants (Colangelo and Guerinot, 2004; Sivitz et al., 2012). It is worth considering that *Arabidopsis* plants have been shown to incorporate Fe chelated to the microbial siderophore pyoverdine more efficiently than Fe chelated to EDTA (Vansuyt et al., 2007). Therefore, it appears possible that incorporation of Fe chelated to microbial siderophores may have contributed to the increased shoot growth of *Arabidopsis* as well as to the higher Fe concentration in plant tissues and rhizosphere soil.

OXIDATIVE STRESS, REDOX HOMEOSTASIS AND SENESCENCE

Beneficial plant–rhizobacteria interactions have been shown to alleviate plant abiotic stress conditions associated with oxidative stress (Dimkpa et al., 2009). Plants inoculated with known beneficial microbes generally show lower activities of antioxidant enzymes, such as catalases and peroxidases as compared to uninoculated plants (Bianco and Defez, 2009; Sandhya et al., 2010). In addition to these enzymes, several regulatory genes for oxidate stress signaling have been characterized in *Arabidopsis*, for example *ERF6*, *WRKY25,* and *ZAT10* (Wang et al., 2013; Zheng et al., 2007; Mittler et al., 2006). The lower expression of *CAT1, PER50, WRKY25, ERF6,* and *OPR2* in roots (**Figure 5A**) and of *CAT1, PER50*, *ERF6,* and *ZAT10* in shoots (**Figure 5B**) of plants grown in microbe-containing soil suggests that beneficial microbes may be present in the whole soil microbial communities used in this study. A marker gene involved in senescence (*SEN1*) that is highly expressed in cotyledons (Schenk et al., 2005) was also downregulated in leaves in the presence of microbes (**Figure 5B**). It should be noted that *SEN1* is also involved in other pathways, including oxidative stress and SA-mediated plant defense (Oh et al., 1996; Schenk et al., 2005). Taken together, this may explain why *Arabidopsis* plants in the absence of microbes showed signs of abiotic stress and senescence, especially in the cotyledons (**Figure S1**).

PHOTOSYNTHESIS

Microarray data analysis of *Arabidopsis* shoots and roots showed that genes required for photosynthesis (e.g., *RBCS* and *CAB*) were up-regulated in plants grown with soil microbes (**Table S2**). In addition, GO terms related to photosynthesis were enriched in roots and shoots, including response to light stimulus (GO:0009416), C fixation (GO:0015977), and photosynthesis (GO:0015979) (**Tables 1**, **S3**). This finding is consistent with the study by Zhang et al. (2008) who showed that the PGPR *B. subtilis* GB03 augments the photosynthetic capacity of *Arabidopsis* plants by decreasing glucose sensing and ABA levels. Growth promotion and increased photosynthesis have also been reported for *Phaseolus vulgaris* and rice when inoculated with phosphorus solubilizing bacteria and *Sinorhizobium meliloti* 1021, respectively (Collavino et al., 2010). A higher photosynthetic efficiency was also conferred by endophytic bacteria to sugar beet (Shi et al., 2010). Common to the above studies is that they report an increase in growth and photosynthetic capacity of plants inoculated with individual microbial strains. The present study indicates that increased leaf growth and photosynthesis could still be observed when plants were exposed to whole soil microbial communities, possibly a result of the synergistic activities of a number of PGPR.

It is well documented that increased photosynthesis can also lead to higher oxidative stress in leaves (Hideg and Schreiber, 2007) and an increase of the GO term "Respiratory burst" could be observed in microarray data from plant leaves grown in the presence of microbes (**Table 1**). This includes some genes that encode proteins associated with oxidative stress in leaves, for example NADPH oxidase (*RBOHD*), MAPK3, and peroxidases 21 and 71 (**Table S2**; **Figure 5B**). Interestingly, this is in contrast to genes encoding other peroxidases (peroxidase 42, 50), catalase 1, ERF6, ZAT10 and SEN1 that were down-regulated in leaves in the presence of microbes (**Figure 5B**; **Table S2**). While there is no simple explanation for this observation, this adds to the growing body of evidence showing that redox homeostasis and ROS production are associated with many different processes in the plant where different gene family members also play different roles and are often involved in many other functions (Miller et al., 2008).

PLANT DEFENSE AND BENEFICIAL INTERACTIONS

The GO term enrichment analysis revealed the major biological processes involved in roots and shoots when exposed to compost soil-derived whole microbial communities (**Tables 1**, **S3**). JA and ET signaling were upregulated in the presence of microbes especially in shoots, which was evidenced by the genes involved in these processes that contributed to the enrichment of the GO terms "response to biotic stimulus" (e.g., *CYP71A12, CYP83B1, ERF104*) and "response to stress" (e.g., *WRKY38, ERF2, WRKY54, ATL2, JAZ6, At1G32920*). The fact that such responses occurred mainly in shoots indicates that they are systemic rather than local, given that most of the interactions in this study were more likely to be occurring underground (Van Wees et al.,

2008; Van der Ent et al., 2009). Although jasmonate and ethylene signaling have been associated to ISR and the recognition of specific strains of beneficial microbes (Matilla et al., 2009; Alizadeh et al., 2013; Chowdappa et al., 2013), it appears that these signaling pathways also play a major role in recognizing microbes at the community level. This suggests that these interactions are more frequent than previously thought. Certain microbe-derived molecules are recognized by plants as non-self through receptors and elicit the MAMPs-triggered immunity (MTI, Zamioudis and Pieterse, 2012). MAMPs include flagellin, lipopolysaccharides (LPS), peptidoglycans, and elongation factor Tu (Pel and Pieterse, 2012). MTI responses such as production of ROS are elicited by non-symbiotic microbes at first (Zamioudis and Pieterse, 2012). As the observed outcome of the system that we were investigating represents a combination of a multitude of interactions with different microbes, the induction of several genes involved in oxidative stress in shoots may be a net result of these initial encounters that continuously occur during the plant's lifecycle with microbes in the environment. However, these microbes are also believed to actively suppress this initial defense response by utilizing effector molecules and hormone-like compounds (Zamioudis and Pieterse, 2012). Effectors include low molecular weight molecules and LPS. Another strategy used by some bacteria to avoid recognition by the plant host is to reversibly switch between phenotypic stages (Pel and Pieterse, 2012; Zamioudis and Pieterse, 2012). This process is called phase variation (Van der Woude, 2011). Thereafter, usually just a mild systemic immune response is elicited and this prepares the host for future pathogen attacks which is then combated more promptly and intensively, also referred to as priming and that is also effective against insect attacks (Conrath et al., 2006; Pieterse and Dicke, 2007). Interestingly, genes involved in indole glucosinolate biosynthesis, which is believed to play a role in plant responses against insect attack (Agerbirk et al., 2008), were upregulated in shoots (*MYB51*, *CYP83B1*, **Table 1**). JA signaling has also been associated with a reduction in ROS (Ton et al., 2002; Pieterse et al., 2009), mainly for two scenarios: (1) to prevent harm towards beneficial microbes and (2) to prevent cell death when attacked by a necrotrophic pathogen. JA signaling is also generally regarded to be antagonistic to SA and ABA signaling, two pathways that also involve ROS production for protection against biotrophic pathogens and abiotic stress, respectively (Anderson et al., 2004; Kazan and Manners, 2013). A recent 16 S rRNA pyrotag sequencing study on *Arabidopsis*-acclimated rhizosphere soil suggests that plants under normal conditions attract growth-promoting bacteria, while during conditions of JA-mediated plant defense, soil bacteria with antimicrobial and insecticidal attributes were enriched (Carvalhais et al., 2013). Further experimentation should focus on the effect of different types of whole soil microbial communities on plant growth promotion and ISR, a promising area of research that may lead to increased crop yields and effective biocontrol of pathogens and pests.

MULTIFUNCTIONALITY DURING MULTIPLE INTERACTIONS

Many genes that were up or downregulated in the presence of microbes have multiple functions. This was apparent in this study in particular for genes involved in signaling, including those that encode transcription factors (e.g., *MYB72, WRKY25*) and genes required for redox homeostasis (e.g., the peroxidase-encoding gene family and *ERF6*). Gene expression profiling, including the genome-wide microarray data (**Table S2**) can serve as a platform to provide additional leads on functionality. However, ultimately, the genetic approach (use of mutants, up or downregulation of genes or gene families) in combination with physiological analyses should be used to determine plant function during multiple plant-microbe interactions. GO term analysis may provide an overview of some of the processes occurring in plants as it takes into account multiple known roles of genes (**Table 1** and **S3**). For example, another functional GO term found to be overrepresented in the list of genes which were induced in roots in the presence of microbes was "Plant cell wall organization or biogenesis" (**Table S3**). *E. coli* and *Saccharomyces cerevisiae* are able to be taken up by root cells and serve as nutrient sources to *Arabidopsis* and tomato plants (Paungfoo-Lonhienne et al., 2010b). This process is accompanied by extensive modifications in root cell wall, including cell wall outgrowth, and enhanced expression of genes involved in cell wall modification (Paungfoo-Lonhienne et al., 2010b). Here, plants were exposed to a wider range of microbes, therefore it is possible that *Arabidopsis* roots have sensed microbes and regulated cell wall modification genes to uptake microbial cells into the roots and use them as a source of nutrients. However, mechanisms involved in this process and whether there is a preferential uptake of certain microbes as opposed to others still needs to be further investigated. Finally, it should be mentioned that, although *Arabidopsis* plants used in this study were checked for the presence of culturable microorganisms, the possibility cannot be excluded that plants may still have contained endophytic organisms (Bulgarelli et al., 2012).

To our knowledge this is the first study to use microbe-free soil to compare some of the main processes involved in interactions between plants and whole microbial communities. Iron acquisition, JA signaling, photosynthesis, redox homeostasis, and plant cell wall organization appear to be the driving mechanisms affected by *Arabidopsis* and rhizosphere microbial communities interactions. Although most previous studies have focused on individual plant-microbe interactions, multi-species analyses such as simultaneous plant and microbial metatranscriptomics coupled to metagenomics (Berendsen et al., 2012; Carvalhais et al., 2012; Delmont et al., 2012; Schenk et al., 2012) may be required to further increase our understanding of the intricate networks underlying plant-microbe interactions in their diverse environments.

ACKNOWLEDGMENTS

We are grateful to the Australian Research Council (DP1094749) for financial support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant-Microbe_Interaction/10.3389/fpls.2013.00235/abstract

Table S1 | List of genes and primers used for qRT-PCR analyses.

Table S2 | Genome-wide microarray data analysis of *Arabidopsis* plants grown in the absence or presence of whole soil microbial communities. The complete data sets are shown for root and shoot tissues, as well as lists of differentially expressed genes (up and downregulated in the presence of microbes) that passed all selection criteria for data analysis (see Materials and Methods for details).

Table S3 | Identification of Gene Ontology (GO) functional categories that are enriched in transcript populations in roots in the presence of whole soil microbial communities.

Figure S1 | Sampling of plant shoots, roots and rhizosphere soil. First, plants were carefully uprooted, soil attached to roots (rhizosphere soil) was shaken off and sieved. Plants were then carefully washed to remove excess soil and briefly blotted onto tissue paper. Shoots and roots were sampled separately. All samples were immediately snap-frozen in liquid N_2 before storage at −80°C. The photograph shows a typical plant grown in the absence of microbes. The red arrow indicates senescence at the cotyledons.

Figure S2 | Carbon-nitrogen (C/N) ratios in whole plant tissues from *Arabidopsis* plants cultivated in the presence or absence of microbes.

Figure S3 | ICP-OES analysis of total element concentration in sieved bulk soil from *Arabidopsis* plants grown in the presence or absence of soil microbes. (A) Macronutrients, **(B)** Micronutrients. Bars represent mean values in mg/kg soil dry weight (DW) ±SD from 3 independent replicates (100 g soil pooled from 10 vessels/replicate).

Figure S4 | Comparison of *IRT1* and *MYB72* expression in *Arabidopsis* plants grown in the presence or absence of soil microorganisms using either autoclaved or γ-irradiated soil as the growth substrate. qRT-PCR results from 3 biological replicates and SDs are shown.

REFERENCES

Agerbirk, N., De Vos, M., Kim, J. H., and Jander, G. (2008). Indole glucosinolate breakdown and its biological effects. *Phytochem. Rev.* 8, 101–120. doi: 10.1007/s11101-008-9098-0

Alef, K., and Nannipleri, P. (1995). *Methods in Applied Soil Microbiology and Biochemistry.* San Diego, CA: Academic Press.

Alizadeh, H., Behboudi, K., Ahmadzadeh, M., Javan-Nikkhah, M., Zamioudis, C., Pieterse, C. M. J., et al. (2013). Induced systemic resistance in cucumber and *Arabidopsis thaliana* by the combination of *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14. *Biol. Control* 65, 14–23. doi: 10.1016/j.biocontrol.2013.01.009

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16, 3460–3479. doi: 10.1105/tpc.104.025833

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnol. Lett.* 32, 1559–1570. doi: 10.1007/s10529-010-0347-0

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berns, A. E., Philipp, H., Narres, H. D., Burauel, P., Vereecken, H., and Tappe, W. (2008). Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. *Eur. J. Soil Sci.* 59, 540–550. doi: 10.1111/j.1365-2389.2008.01016.x

Bethke, G., Unthan, T., Uhrig, J. F., Poschl, Y., Gust, A. A., Scheel, D., et al. (2009). Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in *Arabidopsis thaliana* via ethylene signaling. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8067–8072. doi: 10.1073/pnas.0810206106

Bianco, C., and Defez, R. (2009). *Medicago truncatula* improves salt tolerance when nodulated by an indole-3-acetic acid-overproducing *Sinorhizobium meliloti* strain. *J. Exp. Bot.* 60, 3097–30107. doi: 10.1093/jxb/erp140

Bolstad, B. M., Irizarry, R. A., Åstrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19, 185–193. doi: 10.1093/bioinformatics/19.2.185

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Burkett-Cadena, M., Kokalis-Burelle, N., Lawrence, K. S., van Santen, E., and Kloepper, J. W. (2008). Suppressiveness of root-knot nematodes mediated by rhizobacteria. *Biol. Control* 47, 55–59. doi: 10.1016/j.biocontrol.2008.07.008

Cartieaux, F., Contesto, C., Gallou, A., Desbrosses, G., Kopka, J., Taconnat, L., et al. (2008). Simultaneous interaction of *Arabidopsis thaliana* with *Bradyrhizobium* sp strain ORS278 and *Pseudomonas syringae* pv. tomato DC3000 leads to complex transcriptome changes. *Mol. Plant Microbe Interact.* 21, 244–259. doi: 10.1094/MPMI-21-2-0244

Cartieaux, F., Thibaud, M. C., Zimmerli, L., Lessard, P., Sarrobert, C., David, P., et al. (2003). Transcriptome analysis of *Arabidopsis* colonized by a plant-growth promoting rhizobacterium reveals a general effect on disease resistance. *Plant J.* 36, 177–188. doi: 10.1046/j.1365-313X.2003.01867.x

Carvalhais, L. C., Dennis, P. G., Badri, D. V., Tyson, G. W., Vivanco, J. M., and Schenk, P. M. (2013). Activation of the jasmonic acid plant defense pathway alters the composition of rhizosphere bacterial communities. *PLoS ONE* 8:e56457. doi: 10.1371/journal.pone.0056457

Carvalhais, L. C., Dennis, P. G., Fedoseyenko, D., Hajirezaei, M. R., Boriss, R., and von Wirén, N. (2011). Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *J. Plant Nutr. Soil Sci.* 174, 3–11. doi: 10.1002/jpln.201000085

Carvalhais, L. C., Dennis, P. G., Tyson, G. W., and Schenk, P. M. (2012). Application of metatranscriptomics to soil environments. *J. Microbiol. Methods* 91, 246–251. doi: 10.1016/j.mimet.2012.08.011

Chen, X. H., Koumoutsi, A., Scholz, R., and Borriss, R. (2009). More than anticipated - production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. *J. Mol. Microbiol. Biotechnol.* 16, 14–24. doi: 10.1159/000142891

Chinnusamy, V., Zhu, J. K., and Sunkar, R. (2010). Gene regulation during cold stress acclimation in plants. *Methods Mol. Biol.* 639, 39–55. doi: 10.1007/978-1-60761-702-0_3

Chowdappa, P., Kumar, S. P. M., Lakshmi, M. J., and Upreti, K. K. (2013). Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. *Biol. Control* 65, 109–117. doi: 10.1016/j.biocontrol.2012.11.009

Colangelo, E. P., and Guerinot, M. L. (2004). The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell* 16, 3400–3412. doi: 10.1105/tpc.104.024315

Collavino, M. M., Sansberro, P. A., Mroginski, L. A., and Aguilar, O. M. (2010). Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biol. Fertil. Soils* 46, 727–738. doi: 10.1007/s00374-010-0480-x

Conrath, U., Beckers, G. J. M., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant Microbe Ineract.* 19, 1062–1071. doi: 10.1094/MPMI-19-1062

Crowley, D. E. (2006). "Microbial siderophores in the plant rhizosphere," in *Iron Nutrition in Plants and Rhizospheric Microorganisms,* eds L. L. Barton and J. Abadia (Dorecht: Springer), 169–198. doi: 10.1007/1-4020-4743-6_8

de Zelicourt, A., Al-Yousif, M., and Hirt, H. (2013). Rhizosphere microbes as essential partners for plant stress tolerance. *Mol. Plant* 6, 242–245. doi: 10.1093/mp/sst028

Dellagi, A., Brisset, M. N., Paulin, J. P., and Expert, D. (1998). Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. *Mol. Plant Microbe Ineract.* 11, 734–742. doi: 10.1094/MPMI.1998.11.8.734

Delmont, T. O., Prestat, E., Keegan, K. P., Faubladier, M., Robe, P., Clark, I. M., et al. (2012). Structure, fluctuation and magnitude of a natural grassland soil metagenome. *ISME J.* 6, 1677–1687. doi: 10.1038/ismej.2011.197

Dennis, P. G., Miller, A. J., and Hirsch, P. R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities. *FEMS Microbiol. Ecol.* 72, 313–327. doi: 10.1111/j.1574-6941.2010.00860.x

Dey, R., Pal, K. K., Bhatt, D. M., and Chauhan, S. M. (2004). Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microbiol. Res.* 159, 371–394. doi: 10.1016/j.micres.2004.08.004

Dimkpa, C., Weinand, T., and Asch, F. (2009). Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant Cell Environ.* 32, 1682–1694. doi: 10.1111/j.1365-3040.2009.02028.x

Ding, Z. H., Li, S. M., An, K. L., Liu, X. J., Qin, H. M., and Wang, D. (2009). Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. *J. Genet. Genomics.* 36, 17–29. doi: 10.1016/S1673-8527(09)60003-5

Fan, B., Carvalhais, L. C., Becker, A., Fedoseyenko, D., von Wirén, N., and Borriss, R. (2012). Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. *BMC Microbiol.* 12:116. doi: 10.1186/1471-2180-12-116

Feddermann, N., Finlay, R., Boller, T., and Elfstrand, M. (2010). Functional diversity in arbuscular mycorrhiza - the role of gene expression, phosphorous nutrition and symbiotic efficiency. *Fungal Ecol.* 3, 1–8. doi: 10.1016/j.funeco.2009.07.003

Franche, C., Lindstrom, K., and Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant Soil* 321, 35–59. doi: 10.1007/s11104-008-9833-8

Garg, N., and Chandel, S. (2010). Arbuscular mycorrhizal networks: process and functions. A review. *Agron. Sustain Dev.* 30, 581–599. doi: 10.1051/agro/2009054

Glick, B. R., Todorovic, B., Czarny, J., Cheng, Z. Y., Duan, J., and McConkey, B. (2007). Promotion of plant growth by bacterial ACC deaminase. *Crit. Rev. Plant Sci.* 26, 227–242. doi: 10.1080/07352680701572966

Grant, M. R., Kazan, K., and Manners, J. M. (2013). Exploiting pathogens' tricks of the trade for engineering of plant disease resistance: challenges and opportunities. *Microbiol. Biotechnol.* 6, 212–222. doi: 10.1111/1751-7915.12017

Gulati, A., Sharma, N., Vyas, P., Sood, S., Rahi, P., Pathania, V., et al. (2010). Organic acid production and plant growth promotion as a function of phosphate solubilization by *Acinetobacter rhizosphaerae* strain BIHB 723 isolated from the cold deserts of the trans-Himalayas. *Arch. Microbiol.* 192, 975–983. doi: 10.1007/s00203-010-0615-3

Gupta, M., Bisht, S., Singh, B., Gulati, A., and Tewari, R. (2011). Enhanced biomass and steviol glycosides in *Stevia rebaudiana* treated with phosphate-solubilizing bacteria and rock phosphate. *Plant Growth Regul.* 65, 449–457. doi: 10.1007/s10725-011-9615-9

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60, 579–598. doi: 10.1007/s13213-010-0117-1

Hideg, E., and Schreiber, U. (2007). Parallel assessment of ROS formation and photosynthesis in leaves by fluorescence imaging. *Photosynth Res.* 92, 103–108. doi: 10.1007/s11120-007-9146-4

Idris, E. E., Iglesias, D. J., Talon, M., and Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Mol. Plant Microbe Ineract.* 20, 619–626. doi: 10.1094/MPMI-20-6-0619

Jan, A. T., Azam, M., Ali, A., and Haq, Q. M. R. (2011). Novel approaches of beneficial Pseudomonas in mitigation of plant diseases - an appraisal. *J. Plant Interact.* 6, 195–205. doi: 10.1080/17429145.2010.541944

Jiang, Y., and Deyholos, M. K. (2009). Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. *Plant Mol. Biol.* 69, 91–105. doi: 10.1007/s11103-008-9408-3

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Jung, C., Lyou, S. H., Yeu, S., Kim, M. A., Rhee, S., Kim, M., et al. (2007). Microarray-based screening of jasmonate-responsive genes in *Arabidopsis thaliana*. *Plant Cell Rep.* 26, 1053–1063. doi: 10.1007/s00299-007-0311-1

Kazan, K., and Manners, J. M. (2013). MYC2: the master in action. *Mol. Plant* 6, 686–703. doi: 10.1093/mp/sss128

Kellenberger, E. (2001). Exploring the unknown: the silent revolution of microbiology. *EMBO Rep.* 2, 5–7. doi: 10.1093/embo-reports/kve014

Li, J., Brader, G., and Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. *Plant Cell* 16, 319–331. doi: 10.1105/tpc.016980

Lindsay, W. L., and Norvell, W. A. (1978). Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Sci. Soc. Am. J.* 42, 421–428. doi: 10.2136/sssaj1978.03615995004200030009x

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core of *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Masson-Boivin, C., Giraud, E., Perret, X., and Batut, J. (2009). Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes. *Trends Microbiol.* 17, 458–466. doi: 10.1016/j.tim.2009.07.004

Matilla, M. A., Ramos, J. L., Bakker, P. A. H. M., Doornbos, R., Badri, D. V., Vivanco, J. M., et al. (2009). Pseudomonas putida KT2440 causes induced systemic resistance and changes in *Arabidopsis* root exudation. *Environ. Microbiol. Rep.* 2, 381–388. doi: 10.1111/j.1758-2229.2009.00091.x

Medeiros, F. H. V., Souza, R. M., Medeiros, F. C. L., Zhang, H. M., Wheeler, T., Payton, P., et al. (2011). Transcriptional profiling in cotton associated with Bacillus subtilis (UFLA285) induced biotic-stress tolerance. *Plant Soil* 347, 327–337. doi: 10.1007/s11104-011-0852-5

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 322, 1097–1100. doi: 10.1126/science.1203980

Metraux, J. P. (2002). Recent breakthroughs in the study of salicylic acid biosynthesis. *Trends Plant Sci.* 7, 332–334. doi: 10.1016/S1360-1385(02)02313-0

Meziane, H., Van der Sluis, I., Van Loon, L. C., Hofte, M., and Bakker, P. A. H. M. (2005). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Mol. Plant Pathol.* 6, 177–185. doi: 10.1111/j.1364-3703.2005.00276.x

Miller, G., Shulaev, V., and Mittler, R. (2008). Reactive oxygen signaling and abiotic stress. *Physiol. Plantarum* 133, 481–489. doi: 10.1111/j.1399-3054.2008.01090.x

Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., et al. (2010). Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. *Plant Cell* 22, 973–990. doi: 10.1105/tpc.109.069658

Mittler, R., Kim, Y., Song, L., Coutu, J., Coutu, A., Ciftci-Yilmaz, S., et al. (2006). Gain-and loss-of-function mutations in ZAT10 enhance the tolerance of plants to abiotic stress. *FEBS Lett.* 580, 6537–6542. doi: 10.1016/j.febslet.2006.11.002

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., et al. (2011). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe Ineract.* 24, 533–542. doi: 10.1094/MPMI-09-10-0213

Oh, S. A., Lee, S. Y., Chung, I. K., Lee, C. H., and Nam, H. G. (1996). A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. *Plant Mol. Biol.* 30, 739–754. doi: 10.1007/BF00019008

Oide, S., Moeder, W., Krasnoff, S., Gibson, D., Haas, H., Yoshioka, K., et al. (2006). NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. *Plant Cell* 18, 2836–2853. doi: 10.1105/tpc.106.045633

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., et al. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as

elicitors of induced systemic resistance in plants. *Environ. Microbiol.* 9, 1084–1090. doi: 10.1111/j.1462-2920.2006.01202.x

Ongena, M., Jourdan, E., Schafer, M., Kech, C., Budzikiewicz, H., Luxen, A., et al. (2005). Isolation of an n-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. *Mol. Plant Microbe Ineract.* 18, 562–569. doi: 10.1094/MPMI-18-0562

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6, 763–775. doi: 10.1038/nrmicro1987

Paungfoo-Lonhienne, C., Lonhienne, T. G. A., Rentsch, D., Robinson, N., Christie, M., Webb, R. I., et al. (2008). Plants can use protein as a nitrogen source without assistance from other organisms. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4524–4529. doi: 10.1073/pnas.0712078105

Paungfoo-Lonhienne, C., Lonhienne, T. G., Mudge, S. R., Schenk, P. M., Christie, M., Carroll, B. J., et al. (2010a). DNA is taken up by root hairs and pollen, and stimulates root and pollen tube growth. *Plant Physiol.* 153, 799–805. doi: 10.1104/pp.110.154963

Paungfoo-Lonhienne, C., Rentsch, D., Robatzek, S., Webb, R. I., Sagulenko, E., Nasholm, T., et al. (2010b). Turning the table: plants consume microbes as a source of nutrients. *PLoS ONE* 7:e11915. doi: 10.1371/journal.pone.0011915

Pel, M. J. C., and Pieterse, C. M. J. (2012). Microbial recognition and evasion of host immunity. *J. Exp. Bot.* 64, 1237–1248. doi: 10.1093/jxb/ers262

Perez-Garcia, A., Romero, D., and de Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Curr. Opin. Biotech.* 22, 187–193. doi: 10.1016/j.copbio.2010.12.003

Persello-Cartieaux, F., David, P., Sarrobert, C., Thibaud, M. C., Achouak, W., Robaglia, C., et al. (2001). Utilization of mutants to analyze the interaction between *Arabidopsis thaliana* and its naturally root-associated *Pseudomonas*. *Planta.* 212, 190–198. doi: 10.1007/s004250000384

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29, 2002–2007. doi: 10.1093/nar/29.9.e45

Phi, Q. T., Park, Y. M., Seul, K. J., Ryu, C. M., Park, S. H., Kim, J. G., et al. (2010). Assessment of root-associated *Paenibacillus polymyxa* groups on growth promotion and induced systemic resistance in pepper. *J. Microbiol. Biotechnol.* 20, 1605–1613.

Pieterse, C. M. J., Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends Plant Sci.* 12, 564–569. doi: 10.1016/j.tplants.2007.09.004

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Ran, L. X., Li, Z. N., Wu, G. J., van Loon, L. C., and Bakker, P. A. H. M. (2005). Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. *Eur. J. Plant Pathol.* 113, 59–70. doi: 10.1007/s10658-005-0623-3

Raposeiras, R., Marriel, I. E., Muzzi, M. R. S., Paiva, E., Filho, I. A. P., Carvalhais, L. C., et al. (2006). Rhizobium strains competitiveness on bean nodulation in Cerrado soils. *Pesqui. Agropecu. Bra.* 41, 439–447. doi: 10.1590/S0100-204X2006000300010

Richardson, A. E., Barea, J. M., McNeill, A. M., and Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305–339. doi: 10.1007/s11104-009-9895-2

Robinson, N. J., Procter, C. M., Connolly, E. L., Guerinot, M. L. (1999). A ferricchelate reductase for iron uptake from soils. *Nature* 397, 694–697. doi: 10.1038/17800

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Pare, P. W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* 134, 1017–1026. doi: 10.1104/pp.103.026583

Ryu, C. M., Hu, C. H., Locy, R. D., and Kloepper, J. W. (2005). Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. *Plant Soil* 268, 285–292. doi: 10.1007/s11104-004-0301-9

Ryu, C. M., Murphy, J. F., Reddy, M. S., and Kloepper, J. W. (2007). A two-strain mixture of rhizobacteria elicits induction of systemic resistance against *Pseudomonas syringae* and Cucumber mosaic virus coupled to promotion of plant growth on *Arabidopsis thaliana*. *J. Microbiol. Biotechnol.* 17, 280–286.

Sandhya, V., Ali, S.k. Z., Grover, M., Reddy, G., and Venkateswarlu, B. (2010). Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. *Plant Growth Regul.* 62, 21–30. doi: 10.1007/s10725-010-9479-4

Scheible, W. R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., et al. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol.* 136, 2483–2499. doi: 10.1104/pp.104.047019

Schenk, P. M., Carvalhais, L. C., and Kazan, K. (2012). Unraveling plant–microbe interactions: can multi-species transcriptomics help. *Trends Biotechnol.* 30, 177–184. doi: 10.1016/j.tibtech.2011.11.002

Schenk, P. M., Kazan, K., Rusu, A. G., Manners, J. M., and Maclean, D. J. (2005). The SEN1 gene of *Arabidopsis* is regulated by signals that link plant defense responses and senescence. *Plant Physiol. Biochem.* 43, 997–1005. doi: 10.1016/j.plaphy.2005.09.002

Scholz, R., Molohon, K. J., Nachtigall, J., Vater, J., Markley, A. L., Sussmuth, R. D., et al. (2011). Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* 193, 215–224. doi: 10.1128/JB.00784-10

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., et al. (2006). Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29, 909–918. doi: 10.1111/j.1365-3040.2005.01471.x

Scotti, M. R., Sa, N., Marriel, I., Carvalhais, L. C., Matias, S. R., Correa, E. J., et al. (2007). "Effect of plant species and mycorrhizal inoculation on soil phosphate-solubilizing microorganisms in semi-arid Brazil: growth promotion effect of rhizospheric phosphate-solubilizing microorganisms on *Eucalyptus camaldulensis*," in *First International Meeting on Microbial Phosphate Solubilization* ed E. Velazquez (Dordrecht: Kluwer Academic Publishers), 167–172.

Shi, Y. W., Lou, K., and Li, C. (2010). Growth and photosynthetic efficiency promotion of sugar beet (*Beta vulgaris* L.) by endophytic bacteria. *Photosynth. Res.* 105, 5–13. doi: 10.1007/s11120-010-9547-7

Siddikee, M. A., Chauhan, P. S., Anandham, R., Han, G. H., and Sa, T. (2010). Isolation, characterization, and use for plant growth promotion under salt stress, of acc deaminase-producing halotolerant bacteria derived from coastal soil. *J. Microbiol. Biotechnol.* 20, 1577–1584. doi: 10.4014/jmb.1007.07011

Sivitz, A. B., Hermand, V., Curie, C., and Vert, G. (2012). *Arabidopsis* bHLH100 and bHLH101 control iron homeostasis via a FIT-independent pathway. *PLoS ONE.* 7:e44843. doi: 10.1371/journal.pone.0044843

Song, M. H., Xu, X. L., Hu, Q. W., Tian, Y. Q., Hua, O. Y., Zhou, C. P. (2007). Interactions of plant species mediated plant competition for inorganic nitrogen with soil microorganisms in an alpine meadow. *Plant Soil* 297, 127–137. doi: 10.1007/s11104-007-9326-1

Spaepen, S., and Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol.* 3:a001438. doi: 10.1101/cshperspect.a001438

Stacey, M. G., Patel, A., McClain, W. E., Mathieu, M., Remley, M., Rogers, E. E., et al. (2008). The *Arabidopsis* AtOPT3 protein functions in metal homeostasis and movement of iron to developing seeds. *Plant Physiol.* 146, 589–601. doi: 10.1104/pp.107.108183

Thomma, B. P. H. J., Eggermont, K., Broekaert, W. F., and Cammue, B. P. A. (2000). Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. *Plant Physiol. Biochem.* 38, 421–427. doi: 10.1016/S0981-9428(00)00756-7

Ton, J., Van Pent, J. A., Van Loon, L. C., and Pieterse, C. M. (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene dependent induced resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.* 15, 27–34. doi: 10.1094/MPMI.2002.15.1.27

Torres, M. A. (2010). ROS in biotic interactions. *Physiol. Plantarum* 138, 414–429. doi: 10.1111/j.1399-3054.2009.01326.x

Van der Ent, S., Van Wees, S. C. M., and Pieterse, C. M. J. (2009). Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* 70, 1581–1588. doi: 10.1016/j.phytochem.2009.06.009

Van der Ent, S., Verhagen, B. W., Van Doorn, R., Bakker, D., Verlaan, M. G., Pel, M. C. J., et al. (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiol.* 146, 1293–1304. doi: 10.1104/pp.107.113829

Van der Woude, M. W. (2011). Phase variation: how to create and coordinate population diversity. *Curr. Opin. Microbiol.* 14, 205–211. doi: 10.1016/j.mib.2011.01.002

Van Wees, S. C. M., Van der Ent, S., Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* 11, 443–448. doi: 10.1016/j.pbi.2008.05.005

Vansuyt, G., Robin, A., Briat, J. F., Curie, C., and Lemanceau, P. (2007). Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 20, 441–447. doi: 10.1094/MPMI-20-4-0441

Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F., and Leister, D. (2002). The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. *Plant J.* 31, 589–599. doi: 10.1046/j.1365-313X.2002.01381.x

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., et al. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* 14, 1223–1233. doi: 10.1105/tpc.001388

Wang, P., Du, Y., Zhao, X., Miao, Y., and Song, C. P. (2013). The MPK6-ERF6-ROS-responsive cis-acting element7/GCC box complex modulates oxidative gene transcription and the oxidative response in *Arabidopsis*. *Plant Physiol.* 161, 1392–1408. doi: 10.1104/pp.112.210724

Wang, S. A., Wu, H. J., Qiao, J. Q., Ma, L. L., Liu, J., Xia, Y. F., et al. (2009). Molecular mechanism of plant growth promotion and induced systemic resistance to tobacco mosaic virus by *Bacillus* spp. *J. Microbiol. Biotechn.* 19, 1250–1258. doi: 10.4014/jmb.0901.008

Wang, Y. Q., Ohara, Y., Nakayashiki, H., Tosa, Y., and Mayama, S. (2005). Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. *Mol. Plant Microbe Interact.* 18, 385–396. doi: 10.1094/MPMI-18-0385

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi: 10.1146/annurev.phyto.40.030402.110010

Xu, X. L., Ouyang, H., Richter, A., Wanek, W., Cao, G. M., and Kuzyakov, Y. (2011). Spatio-temporal variations determine plant-microbe competition for inorganic nitrogen in an alpine meadow. *J. Ecol.* 99, 563–571.

Yang, L., Wang, Y. J., Song, J., Zhao, W., He, X. L., Chen, J., et al. (2011). Promotion of plant growth and *in situ* degradation of phenol by an engineered *Pseudomonas fluorescens* strain in different contaminated environments. *Soil Biol. Biochem.* 43, 915–922. doi: 10.1016/j.soilbio.2011.01.001

Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R., and Berg, G. (2010). Strain-specific colonization pattern of Rhizoctonia antagonists in the root system of sugar beet. *FEMS Microbiol. Ecol.* 74, 124–135. doi: 10.1111/j.1574-6941.2010.00930.x

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Zhang, H. M., Xie, X. T., Kim, M. S., Kornyeyev, D. A., Holaday, S., and Pare, P. W. (2008). Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. *Plant J.* 56, 264–273. doi: 10.1111/j.1365-313X.2008.03593.x

Zheng, Z. Y., Mosher, S. L., Fan, B. F., Klessig, D. F., and Chen, Z. X. (2007). Functional analysis of *Arabidopsis* WRKY25 transcription factor in plant defense against *Pseudomonas syringae*. *BMC Plant Biol.* 7:2. doi: 10.1186/1471-2229-7-2

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 March 2013; accepted: 14 June 2013; published online: 04 July 2013.

Citation: Carvalhais LC, Muzzi F, Tan C-H, Hsien-Choo J and Schenk PM (2013) Plant growth in Arabidopsis is assisted by compost soil-derived microbial communities. Front. Plant Sci. 4:235. doi: 10.3389/fpls.2013.00235

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Carvalhais, Muzzi, Tan, Hsien-Choo and Schenk. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 18 June 2013
doi: 10.3389/fpls.2013.00204

Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis

Caroline Gutjahr*[1] *and* ***Uta Paszkowski*[2]***

[1] *Institute of Genetics, University of Munich, Martinsried, Germany*
[2] *Department of Plant Sciences, University of Cambridge, Cambridge, UK*

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Didier Reinhardt, University of Fribourg, Switzerland
Ainhoa Martinez-Medina, Utrecht University, Netherlands

****Correspondence:***
Caroline Gutjahr, Institute of Genetics, University of Munich, Biocenter Martinsried, 82152 Martinsried, Germany
e-mail: caroline.gutjahr@lmu.de;
Uta Paszkowski, Department of Plant Sciences, Downing Street, Cambridge CB2 3EA, UK
e-mail: up220@cam.ac.uk

In nature, the root systems of most plants develop intimate symbioses with glomeromycotan fungi that assist in the acquisition of mineral nutrients and water through uptake from the soil and direct delivery into the root cortex. Root systems are endowed with a strong, environment-responsive architectural plasticity that also manifests itself during the establishment of arbuscular mycorrhizal (AM) symbioses, predominantly in lateral root proliferation. In this review, we collect evidence for the idea that AM-induced root system remodeling is regulated at several levels: by AM fungal signaling molecules and by changes in plant nutrient status and distribution within the root system.

Keywords: arbuscular mycorrhiza, root system architecture, lateral root, plant nutrition, symbiosis, Glomeromycota

INTRODUCTION

When plants made the transition from freshwater to terrestrial environments more than 400 million years ago, fundamental morphological changes were needed for the acquisition of mineral nutrients from the soil instead of from the aqueous substratum. Preceding the development of complex root systems the alliance with symbiotic fungi such as the Glomeromycetes and Mucoromycotina is believed to have greatly assisted this transition (Humphreys et al., 2010; Bidartondo et al., 2012; Field et al., 2012, and citations therein). The aseptate hyphal network of the glomeromycotan fungi functions as a mineral nutrient-transfer pipeline from the soil-exploring extraradical mycelium to the intracellularly colonized plant cell. Extensively branched tree-like fungal haustoria, the arbuscules, form within living plant cells and are the site of mineral nutrient delivery. It is widely accepted that these hyphal conduits have served mineral nutrient uptake by ancestral rootless gametophytes and continue to do so on today's complexly rooted sporophytes. Liverworts constitute the earliest diverging plant lineage known (for recent review, see Jones and Dolan, 2012), that supports the development of arbuscular mycorrhizal (AM) symbioses with Glomeromycetes. The fungus enters via the rhizoid, develops arbuscules within the green thallus parenchyma (Russell and Bulman, 2005; Ligrone et al., 2007; Hata et al., 2010) and confers nutritional benefit to the plant host (Humphreys et al., 2010). In higher plants, arbuscules develop in root cortex cells where they deliver inorganic phosphate to the plant (Javot et al., 2007a; Yang et al., 2012). The extant ability of AM fungal species to equivalently colonize thallus parenchyma and root cortex suggests the genetic repertoire of AM fungi to ascertain a seamless adaption from ancient to newly invented organs and the two participating plant cell types to represent sufficiently similar niches for colonization. Within the highly patterned "modern" root system, arbuscular colonization is restricted to cortex cells.

Root systems consist of individual modules with different function: the shoot-born dicotyledon tap roots and monocotyledon crown roots (CRs) are mainly involved in anchorage and support whereas lateral roots mediate nutrient uptake (McCully and Canny, 1988). Root system architecture displays a high developmental plasticity in response to environmental stimuli such as nutrient and humidity levels or temperature (Lopez-Bucio et al., 2003; Osmont et al., 2007; Hodge, 2009). Importantly, among other biota AM fungi influence root system architecture, most prominently, by enhancing lateral root formation. In this review, we summarize current knowledge on the selective colonization of root types by AM fungi and its impact on root architectural changes, which we propose is regulated at multiple levels.

NON-RANDOM AM COLONIZATION OF ROOT SYSTEMS

In both di- and monocotyledon root systems AM colonization is not evenly distributed since AM fungi preferentially colonize lateral roots and rather neglect dicotyledon primary roots or monocotyledon CRs (**Figure 1**; Hooker et al., 1992; Gutjahr et al., 2009a). Intuitively, this might be due to a higher sturdiness and lignin content in shoot-born roots with anchoring function that are more challenging to penetrate than the young expanding, and therefore softer tissue of growing lateral roots (Hepper, 1985; Amijee et al., 1993). Consistently, rigid CRs of rice are mainly colonized in patches close to lateral roots or emerging lateral root primordia (Gutjahr et al., 2009a). However, longer periods of plant co-cultivation with AM fungi increase the percentage

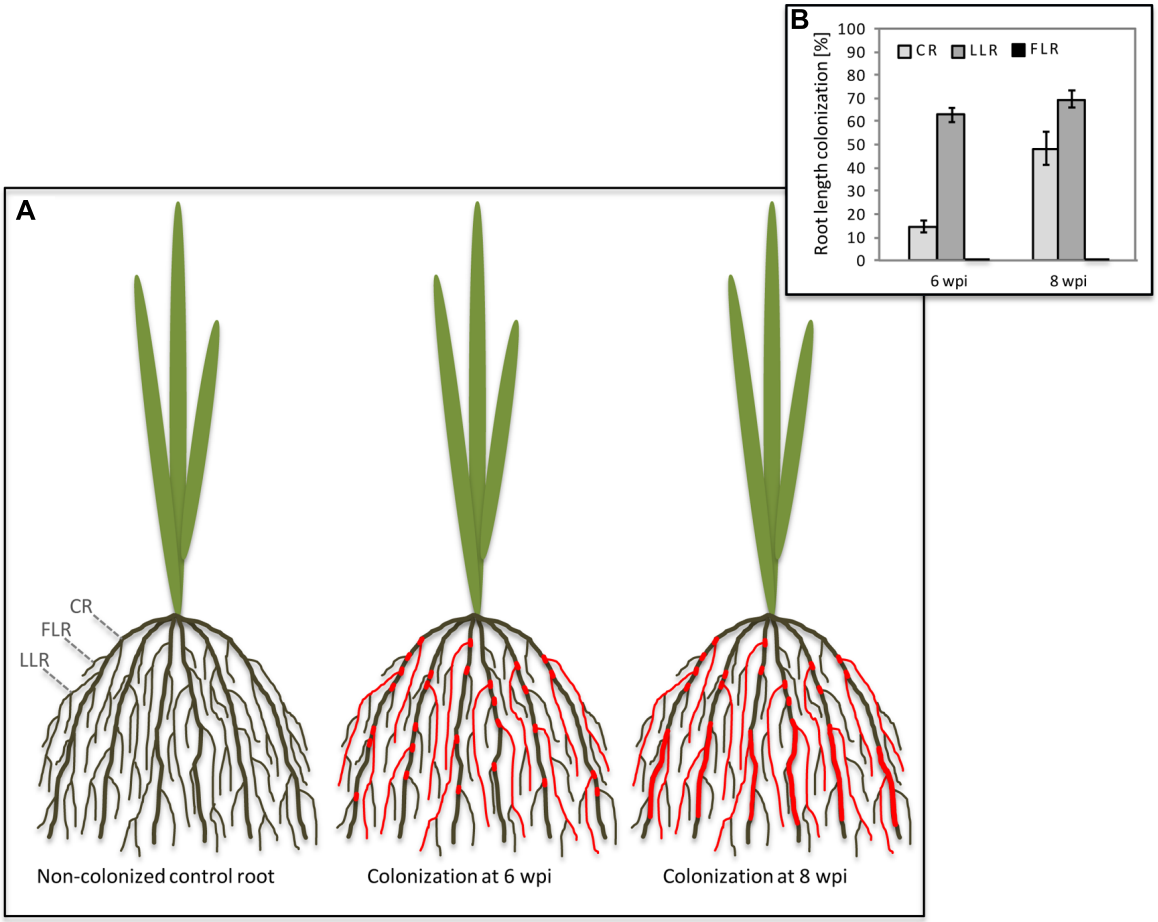


FIGURE 1 | Colonization of rice crown roots by *Glomus intraradices* upon prolonged co-cultivation. (A) Schematic illustration of the rice root system with crown roots (CRs), large lateral roots (LLRs) and fine lateral roots (FLRs). Colonization of the different root types is indicated by red color. At 6 weeks post-inoculation (wpi), CR show fungal colonization at the site of lateral root emergence; at 8 wpi, the CRs are normally colonized. **(B)** Percent root length colonization of rice CR, LLR, and FLR was determined at 6 and 8 wpi with *Glomus intraradices*. Mean values and SE of four biological replicates are shown.

of CR length colonized (**Figure 1**). It has been shown in maize that phosphate starvation stress leads to an increased transcription of genes involved in secondary cell wall biosynthesis (Calderon-Vazquez et al., 2008). Phosphate supply through AM fungi reduces starvation and might thus contribute to a decrease in secondary cell wall biosynthesis in CRs, thereby possibly facilitating further colonization when symbiotic phosphate transfer had conferred the effect. Also the colonization of the liverwort *Conocephalum conicum* leads to the disappearance of thallus cell wall autofluorescence at infected sites, indicating a localized decrease in cell wall phenolics (Ligrone et al., 2007). Similar to rice, also in soybean colonization was described to be particularly evident at points of lateral roots emergence. Corresponding spatial expression patterns of soluble acid invertase and sucrose synthase genes suggested an enhanced carbohydrate supply to the emerging and elongating laterals to account for this localized fungal root invasion (Blee and Anderson, 2002). Interestingly, lateral roots exhibit an increased responsiveness to AM fungal signaling molecules as evidenced by activation of a p*ENOD11*-GUS transgene in *Medicago* hairy roots (Kosuta et al., 2003). Thus they might induce the symbiotic program more swiftly and promote colonization more readily than other root types.

An unequal distribution of AM colonization is particularly evident in rice root systems, that are equipped with two types of lateral roots, the strongly colonized large lateral roots (LLRs) and the fine lateral roots (FLRs), which lack cortex tissue (Rebouillat et al., 2009), and are therefore not able to host arbuscules (Gutjahr et al., 2009a). While absence of arbuscules from FLRs was predictable, the absence of fungal hyphopodium differentiation is surprising and implies that FLRs are not recognized by the fungus (**Figure 1**; Gutjahr et al., 2009a), possibly due to differences in either their surface composition or exudation of diffusible signals. Cutin monomers have recently been shown to induce hyphopodium formation on *Medicago truncatula* roots (Wang et al., 2012). Although not yet confirmed for rice, it is an attractive possibility that FLRs release insufficient amounts of cutin or related compounds. The chemical composition of the rhizodermal surface of any plant species is not well described but

there is evidence from *Arabidopsis* that it differs among root zones (Kosma et al., 2012). This is exemplified by rhizoplane bacteria, that accumulate in species-specific patterns on the *Arabidopsis* root surface (Bulgarelli et al., 2012; Lundberg et al., 2012). These patterns are likely at least in part evoked by localized chemical surface composition or differential exudation patterns. Strigolactones are constitutively exuded from higher plant roots and rhizoids of bryophytic gametophytes (Akiyama et al., 2005; Delaux et al., 2012). They induce the metabolic activity of AM fungi and provide a directional cue to guide the fungus to colonizable tissue (Parniske, 2005; Besserer et al., 2006). PDR1 (pleiotropic drug resistance protein 1), a strigolactone ATP-binding cassette (ABC)-exporter in Petunia is expressed in hypodermal passage cells of lateral roots only (Kretzschmar et al., 2012). This might explain – at least for dicotyledons – why AM fungi are firstly attracted to lateral roots. It remains an intriguing open question whether an orthologous strigolactone transporter is expressed in outer cell layers of rice FLRs.

LATERAL ROOT INDUCTION BY AM FUNGI IS REGULATED AT MULTIPLE LEVELS

Numerous studies report root system changes in response to arbuscular mycorrhiza leading to an increased root branching and root system volume (reviewed in Hodge et al., 2009; Sukumar et al., 2013) but also reductions in root branching and length were detected (Hetrick, 1991). The basis of the observed differences is not clear but could be related to the studied plant species or the varying growth conditions. Diverging AM induced root system changes across different maize or soybean cultivars, grown under the same condition, suggested that at least part of the response is subject to genetic variation (Zhu et al., 2005; Wang et al., 2011). Although not systematically investigated an influence of the fungal genotype on the type and extend of root system remodeling can also be expected (Veresoglou et al., 2012). Yano et al. (1996) reported the induction of lateral root formation to be a highly localized response. AM inoculation of only one half of a split-root system of peanut and pigeon pea resulted in a higher number of lateral roots in the inoculated as compared to the non-inoculated half. However, systemic inhibitory or stimulatory effects on lateral root proliferation were not examined. The power of AM colonization over lateral root development was demonstrated in knock-down *Lotus japonicus* hairy root cultures of the putative transcription factor gene meristem and arbuscular mycorrhiza induced *(LjMAMI)* (Volpe et al., 2013). Here, colonization by AM fungi rescues the reduced lateral root growth phenotype and restores wild-type root system morphology. However, the most dramatic influence of AM colonization on root system architecture was found in the maize mutant *lateral rootless1 (lrt1)* that lacks embryonic lateral roots (Hochholdinger and Feix, 1998). Inoculation with AM fungi-induced bushy lateral roots even at elevated phosphate levels (Paszkowski and Boller, 2002). Taken together these data indicate that AM fungi trigger a signaling pathway that bypasses the default lateral root developmental control exerted by MAMI and/or LRT1.

Root system architectural changes in response to AM colonization are regulated on at least two levels as evidenced by their induction prior to or after establishment of AM colonization (Berta et al., 1990, 1995; Maillet et al., 2011; Mukherjee and Ane, 2011).

ROOT SYSTEM CHANGES IN RESPONSE TO PRE-SYMBIOTIC SIGNALING

In the legume *M. truncatula*, germinating AM fungal spores that were separated from the root by a semipermeable membrane induced lateral root formation, indicating that diffusible signals released by these spores activate the lateral root developmental program (Olah et al., 2005). This is in agreement with the observation that the recently identified lipochitooligosaccharide Myc factors (Myc-LCOs) also induce lateral root formation in *M. truncatula* (**Figure 2**; Maillet et al., 2011). Intra-radical colonization of angiosperm roots is dependent on a signal transduction pathway, which includes Ca^{2+}-oscillations as a second messenger and is also required for nodulation and accommodation of rhizobia and therefore named the common SYM pathway (for a recent review, see Singh and Parniske, 2012; Gutjahr and Parniske, 2013; Venkateshwaran et al., 2013). Lateral root induction by the presence of AM fungi was dependent only on *DMI1* (*POLLUX*) and *DMI2* (*SYMRK*), two genes that act upstream of Ca^{2+}-spiking as part of the common SYM pathway (Olah et al., 2005). By contrast, Myc-LCO-mediated lateral root induction, additionally required the third common SYM gene *DMI3* (*CCamK*), that acts downstream of Ca^{2+}-spiking (Maillet et al., 2011) and is also required for rhizobial Nod factor-mediated lateral root induction (Olah et al., 2005). This raises the question whether germinating spore exudates (GSEs) also contain diffusible signaling molecules other than Myc-LCOs that do not require DMI3, but signal through alternative components downstream of DMI1 and DMI2 to induce lateral root formation in legumes. Lateral root development might be sustained by enhanced carbon accumulation that has been described in GSE-stimulated *Lotus japonicus* roots to be dependent on CASTOR, another SYM pathway component upstream of Ca^{2+}-spiking (Gutjahr et al., 2009b).

Remarkably, the monocot rice does not require the common SYM genes *CASTOR*, *DMI1* (*POLLUX*), and *DMI3* (*CCAMK*) for lateral root induction by GSEs (Gutjahr et al., 2009a; Mukherjee and Ane, 2011). It is intriguing whether this is due to a fundamental genetic difference between monocotyledons and dicotyledons or whether legumes, due to their specific genetic layout, that grants the development of nodules, have incorporated the common SYM pathway into a regulatory network, that directs development of all root accessory organs. Congruent with the latter hypothesis, the *Lotus japonicus* mutant *hypernodulation aberrant root formation 1* (*har1*), that hypernodulates and is hypercolonized by AM fungi, constitutively forms supernumerary lateral roots (Solaiman et al., 2000; Wopereis et al., 2000; Nishimura et al., 2002).

Lateral root formation is regulated by auxin in conjunction with other phytohormone signaling pathways (Nibau et al., 2008). Impairment of pre-symbiotic lateral root induction in hairy root culture of the auxin-resistant *diageotropica* tomato mutant suggests that Myc factor-dependent lateral root induction is similarly channeled into the auxin-controlled developmental outcome (Hanlon and Coenen, 2010). Ectomycorrhizal fungi such as *Laccaria bicolor* and *Tuber melanosporum* trigger the production of lateral roots prior to colonization through the stimulation of

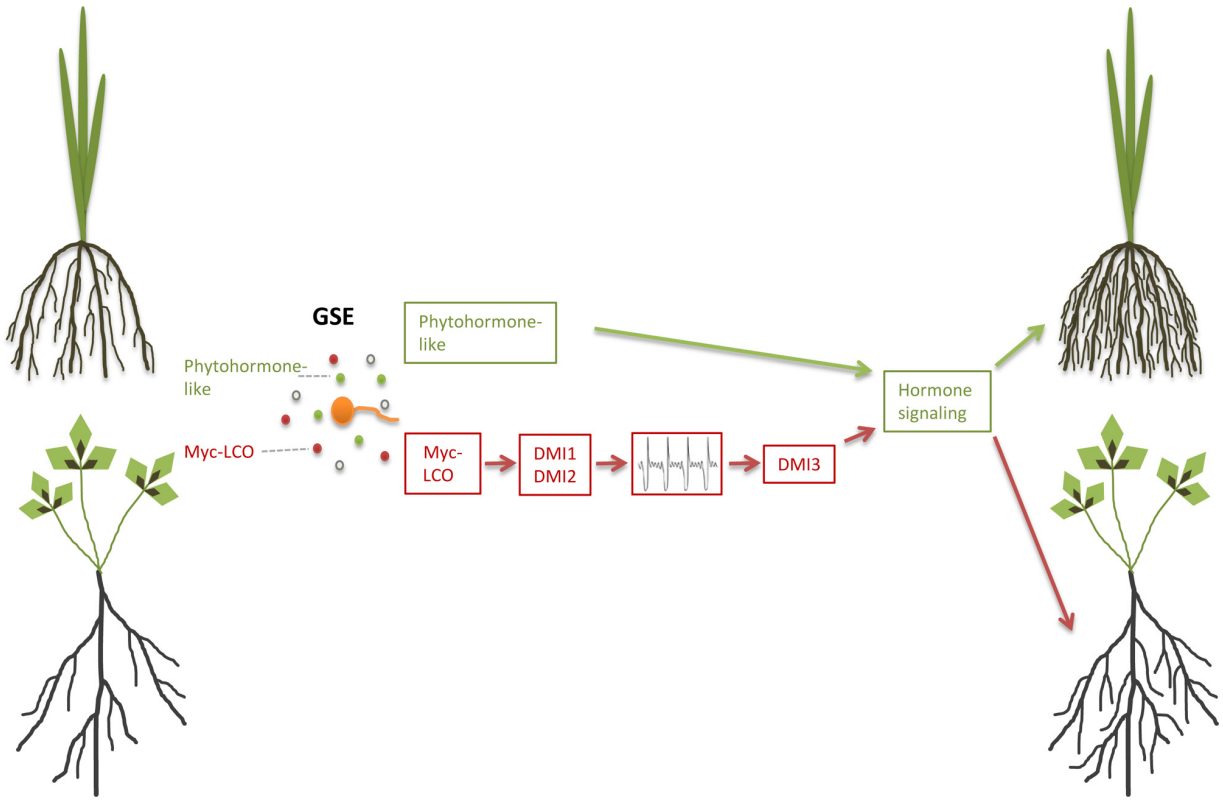


FIGURE 2 | Pre-symbiotic induction of lateral root formation in arbuscular mycorrhiza. Germinating spore exudates (GSE) contain Myc-LCOs and possibly phytohormone-like compounds. Perception of Myc-LCOs leads to lateral root induction in *Medicago truncatula*, which requires the common symbiosis signaling components DMI1, DMI2, and DMI3 (brown pathway). The green pathway hypothesizes phytohormone-like signaling to operate either downstream or independent of common symbiosis signaling in *M. truncatula* and rice, respectively.

auxin signaling, likely due to their production and release of auxin and ethylene or other volatile compounds (Rupp et al., 1989; Karabaghli-Degron et al., 1998; Ivanchenko et al., 2008; Felten et al., 2009, Felten et al., 2010; Splivallo et al., 2009; Sukumar et al., 2013). Likewise it is possible that also AM fungi produce plant hormones such as auxin and ethylene or other volatile compounds in addition to Myc-LCOs (**Figure 2**), and this might for example explain SYM pathway-independent lateral root induction in rice, while in nodulating legumes common SYM-mediated lateral root induction might be epistatic to auxin signaling.

ROOT SYSTEM CHANGES IN RESPONSE TO INTRA-RADICAL COLONIZATION

Arbuscular mycorrhizal colonization preceding alterations in root system architecture has also been observed, e.g., in *Allium porrum* and *Prunus cerasifera* (Berta et al., 1990, 1995). Enhancement of lateral root formation after colonization has been related to nutritional effects. AM fungi deliver phosphate and nitrogen directly into the root cortex where the minerals are taken up by specific plant ion transporters localized in the peri-arbuscular membrane, a plant-derived membrane domain that surrounds the arbuscule branches (Harrison et al., 2002; Javot et al., 2007b; Kobae and Hata, 2010; Yang et al., 2012). The patchy distribution of AM colonization must lead to transient local increases of phosphate and/or nitrogen concentrations in the root, which may serve as a hallmark of symbiosis (**Figure 3**; Fitter, 2006). Plants can perceive localized differences in nutrient distribution also within the surrounding environment and respond with lateral root proliferation into phosphate or nitrogen-rich soil pockets (**Figure 3**; Drew, 1975; Linkohr et al., 2002). A nitrate transporter NRT1.1 has been identified in *Arabidopsis thaliana*, which acts as a nitrate transporter and sensor and triggers lateral root elongation into nitrate rich soil pockets (Remans et al., 2006). Besides nitrate it also facilitates auxin transport away from the lateral root meristem at low nitrogen conditions, leading to reduced lateral root outgrowth and elongation. In a patch of high nitrate concentration auxin transport by NRT1.1 is inhibited and auxin accumulates in lateral root tips leading to increased lateral root growth (Krouk et al., 2010). Thus NRT1.1 directly influences root system architecture via an orchestration of nitrate transport, -sensing as well as auxin transport. It will be highly interesting to determine if related mechanisms are at play in the regulation of root system architecture by mycorrhizal nutrient uptake. Mutants perturbed in mycorrhizal nutrient acquisition, e.g., defective in mycorrhiza-specific phosphate transporters such as *Medicago* PT4 or rice PT11 (Javot et al., 2007a; Yang et al., 2012), will provide a first means to study the impact of AM-mediate phosphate uptake on lateral root proliferation.

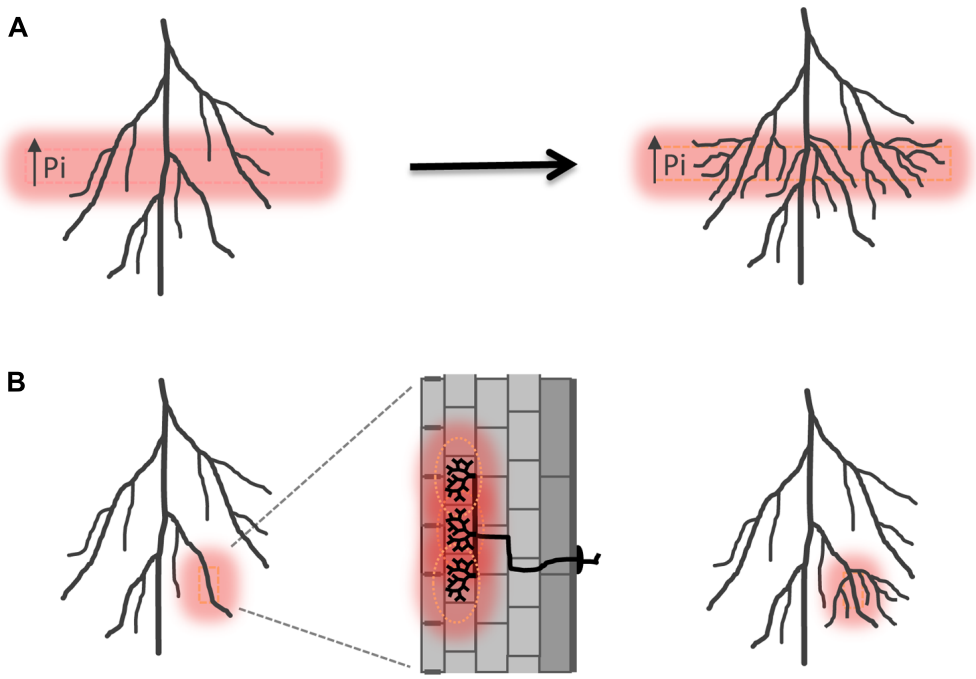


FIGURE 3 | Induction of lateral root formation in response to locally high phosphate. (A) Schematic illustration of lateral root induction in high phosphate fertilized layers of the rhizosphere according to Drew (1975). **(B)** Hypothetical induction of lateral roots as a consequence of sensing a locally high concentration of phosphate within the tissue resulting from symbiotic phosphate uptake. Pi, inorganic phosphate.

In mycorrhizal roots, the symbiotic phosphate (possibly also nitrogen) uptake pathway dominates and involves suppression of the transporter genes involved in epidermal direct uptake (Smith et al., 2003; Smith and Smith, 2011; Yang and Paszkowski, 2011). It is a currently unexplored but attractive possibility that some transport proteins belonging to the direct epidermal nutrient uptake pathway are involved in nutrient sensing similar to NRT1.1 (Krouk et al., 2010). Downregulation of their expression during the switch from the direct to the mycorrhizal nutrient uptake pathway, might inhibit sensing of the nutrient status of the surrounding soil medium, and thus alter the root system architecture response to the local soil environment thereby enhancing the influence of mycorrhizal nutrient delivery on root system architecture.

Lateral root formation can be triggered by carbon supply in the growth medium, suggesting its dependence on sufficient carbon (Jain et al., 2007; MacGregor et al., 2008). There is evidence that in the AM symbiosis fungus-delivered phosphate is traded for plant-derived carbon (Kiers et al., 2011). However, the balance of this trade can depend on the plant–fungus species combination and competition among plants that are connected via the common hyphal network (Walder et al., 2012). As long as the carbon-cost imposed by the fungus is lower than the amount of sugar transported into a given colonized part of the root system, this redirection to colonized parts of the root system could perhaps provide a mechanism by which mycorrhiza-mediated mineral nutrient uptake promotes lateral root formation (Fitter, 2006; Yang and Paszkowski, 2011). A second mechanism for liberating carbon resources might be the putative reduction of secondary cell wall biosynthesis upon phosphate starvation release (Calderon-Vazquez et al., 2008). AM colonization has been reported to induce changes in the amount of phytohormones such as cytokinins, jasmonic acid (JA), certain auxins, abscisic acid (ABA), ethylene, salicylic acid (SA), strigolactones in roots (reviewed in Hause et al., 2007; Foo et al., 2013). These phytohormones are also involved in the regulation of root system architecture (Nibau et al., 2008; Fukaki and Tasaka, 2009; Koltai, 2011). It is currently unknown in how far the changes in phytohormone levels are related to AM-induced changes in root nutrient status evoked by mineral nutrient supply via the fungus or by an increase in root carbon sink strength. Nevertheless changes in phytohormone levels might contribute to root system remodeling in response to AM colonization either independently or as part of a nutrient signaling network.

CONCLUSIONS AND PERSPECTIVES

Plant productivity strongly depends on an appropriately adapted root system architecture for the uptake of nutrients and water under adverse soil conditions. Thus modulation of the root system architecture in response to environmental conditions is considered an important target for genetic crop improvement (de Dorlodot et al., 2007). AM fungi represent an inherent component of natural and agricultural ecosystems and influence root system architecture prior and post-colonization. It is therefore of high interest to enhance knowledge about the molecular mechanisms that underpin these morphological modulations and to elucidate the cross-talk between the two regulatory "étappes" of root system remodeling.

REFERENCES

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435, 824–827. doi: 10.1038/nature03608

Amijee, F., Stribley, D., and Lane, P. (1993). The susceptibility of roots to infection by an arbuscular mycorrhizal fungus in relation to age and phosphorus supply. *New Phytol.* 125, 581–586. doi: 10.1111/j.1469-8137.1993.tb03906.x

Berta, G., Fusconi, A., Trotta, A., and Scannerini, S. (1990). Morphogenetic modifications induced by the mycorrhizal fungus *Glomus* strain E3 in the root system of *Allium porrum*. *New Phytol.* 114, 207–215. doi: 10.1111/j.1469-8137.1990.tb00392.x

Berta, G., Trotta, A., Fusconi, A., Hooker, J., Munro, M., Atkinson, D., et al. (1995). Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. *Tree Physiol.* 15, 281–293. doi: 10.1093/treephys/15.5.281

Besserer, A., Puech-Pagés, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., et al. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PLoS Biol.* 4:e226. doi: 10.1371/journal.pbio.0040226

Bidartondo, M., Read, D., Trappe, J., Merckx, V., Ligrone, R., and Duckett, J. (2012). The dawn of symbiosis between plants and fungi. *Biol. Lett.* 7, 574–577. doi: 10.1098/rsbl.2010.1203

Blee, K., and Anderson, A. (2002). Transcripts for genes encoding soluble acid invertase and sucrose synthase accumulate in root tip and cortical cells containing mycorrhizal arbuscules. *Plant Mol. Biol.* 50, 197–211. doi: 10.1023/A:1016038010393

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren Van Thermaat, E., Ahmadinejad, N., Assenza, F., et al. (2012). Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

Calderon-Vazquez, C., Ibarra-Laclette, E., Caballero-Perez, J., and Herrera-Estrella, L. (2008). Transcript profiling of *Zea mays* roots reveals gene responses to phosphate deficiency at the plant- and species-specific levels. *J. Exp. Bot.* 59, 2479–2497. doi: 10.1093/jxb/ern115

de Dorlodot, S., Forster, B., Pagès, L., Price, A., Tuberosa, R., and Draye, X. (2007). Root system architecture: opportunities and constraints for genetic improvement of crops. *Trends Plant Sci.* 12, 474–481. doi: 10.1016/j.tplants.2007.08.012

Delaux, P.-M., Xie, X., Timme, R. E., Puech-Pages, V., Dunand, C., Lecompte, E., et al. (2012). Origin of strigolactones in the green lineage. *New Phytol.* 195, 857–871. doi: 10.1111/j.1469-8137.2012.04209.x

Drew, M. (1975). Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytol.* 75, 479–490. doi: 10.1111/j.1469-8137.1975.tb01409.x

Felten, J., Kohler, A., Morin, E., Bhalerao, R. P., Palme, K., Martin, F., et al. (2009). The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and *Arabidopsis* through auxin transport and signaling. *Plant Physiol.* 151, 1991–2005. doi: 10.1104/pp.109.147231

Felten, J., Legué, V., and Ditengou, F. A. (2010). Lateral root stimulation in the early interaction between *Arabidopsis thaliana* and the ectomycorrhizal fungus *Laccaria bicolor*: is fungal auxin the trigger? *Plant Signal. Behav.* 5, 864–867. doi: 10.1104/pp.109.147231.

Field, K. J., Cameron, D. D., Leake, J. R., Tille, S., Bidartondo, M. I., and Beerling, D. J. (2012). Contrasting arbuscular mycorrhizal responses of vascular and non-vascular plants to a simulated Palaeozoic CO(2) decline. *Nat. Commun.* 3, 835. doi: 10.1038/ncomms1831

Fitter, A. H. (2006). What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. *New Phytol.* 172, 3–6. doi: 10.1111/j.1469-8137.2006.01861.x

Foo, E., Ross, J. J., Jones, T. W., and Reid, J. B. (2013). Plant hormones in arbuscular mycorrhizal symbiosis: an emerging role for gibberellins. *Ann. Bot.* 111, 769–779. doi: 10.1093/aob/mct041

Fukaki, H., and Tasaka, M. (2009). Hormone interactions during lateral root formation. *Plant Mol. Biol.* 69, 437–449. doi: 10.1007/s11103-008-9417-2

Gutjahr, C., Casieri, L., and Paszkowski, U. (2009a). *Glomus intraradices* induces changes in root system architecture of rice independently of common symbiosis signaling. *New Phytol.* 182, 829–837. doi: 10.1111/j.1469-8137.2009.02839.x

Gutjahr, C., Novero, M., Guether, M., Montanari, O., Udvardi, M., and Bonfante, P. (2009b). Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots. *New Phytol.* 183, 53–61. doi: 10.1111/j.1469-8137.2009.02871.x

Gutjahr, C., and Parniske, M. (2013). Cell and developmental biology of arbuscular mycorrhiza symbiosis. *Annu. Rev. Cell Dev. Biol.* 29. doi: 10.1146/annurev-cellbio-101512-122413

Hanlon, M. T., and Coenen, C. (2010). Genetic evidence for auxin involvement in arbuscular mycorrhiza initiation. *New Phytol.* 189, 701–709. doi: 10.1111/j.1469-8137.2010.03567.x

Harrison, M., Dewbre, G., and Liu, J. (2002). A phosphate transporter of *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* 14, 2413–2429. doi: 10.1105/tpc.004861

Hata, S., Kobae, Y., and Banba, M. (2010). Interactions between plants and arbuscular mycorrhizal fungi. *Int. Rev. Cell Mol. Biol.* 281, 1–48. doi: 10.1016/S1937-6448(10)81001-9

Hause, B., Mrosk, C., Isayenkov, S., and Strack, D. (2007). Jasmonates in arbuscular mycorrhizal interactions. *Phytochemistry* 68, 101–110. doi: 10.1016/j.phytochem.2006.09.025

Hepper, C. (1985). Influence of age of roots on the pattern of vesicular-arbuscular mycorrhizal infection of leek and clover. *New Phytol.* 101, 685–693. doi: 10.1111/j.1469-8137.1985.tb02874.x

Hetrick, B. (1991). Mycorrhizas and root architecture. *Experientia* 47, 355–362. doi: 10.1007/BF01972077

Hochholdinger, F., and Feix, G. (1998). Early post-embryonic root formation is specifically affected in the maize mutant *lrt1*. *Plant J.* 16, 247–255. doi: 10.1046/j.1365-313x.1998.00280.x

Hodge, A. (2009). Root decisions. *Plant Cell Environ.* 32, 628–640. doi: 10.1111/j.1365-3040.2008.01891.x

Hodge, A., Berta, G., Doussan, C., Merchan, F., and Crespi, M. (2009). Plant root growth, architecture and function. *Plant Soil* 321, 153–187. doi: 10.1007/s11104-009-9929-9

Hooker, J., Munro, M., and Atkinson, D. (1992). Vesicular-arbuscular mycorrhizal fungi induced alterations in poplar root system morphology. *Plant Soil* 145, 207–214. doi: 10.1007/BF00010349

Humphreys, C. P., Franks, P. J., Rees, M., Bidartondo, M. I., Leake, J. R., and Beerling, D. J. (2010). Mutualistic mycorrhiza-like symbiosis in the most ancient group of land plants. *Nat. Commun.* 1, 103. doi: 10.1038/ncomms1105

Ivanchenko, M., Muday, G., and Dubrovsky, J. (2008). Ethylene-auxin interactions regulate lateral root initiation and emergence in *Arabidopsis thaliana*. *Plant J.* 55, 335–347. doi: 10.1111/j.1365-313X.2008.03528.x

Jain, A., Poling, M. D., Karthikeyan, A. S., Blakeslee, J. J., Peer, W. A., Titapiwatanakun, B., et al. (2007). Differential effects of sucrose and auxin on localized phosphate deficiency-induced modulation of different traits of root system architecture in *Arabidopsis*. *Plant Physiol.* 144, 232–247. doi: 10.1104/pp.106.092130

Javot, H., Varma Penmetsa, R., Terzaghi, N., Cook, D. R., and Harrison, M. J. (2007a). A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1720–1725. doi: 10.1073/pnas.0608136104

Javot, H., Pumplin, N., and Harrison, M. (2007b). Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. *Plant Cell Environ.* 30, 310–322. doi: 10.1111/j.1365-3040.2006.01617.x

Jones, V. A., and Dolan, L. (2012). The evolution of root hairs and rhizoids. *Ann. Bot.* 110, 205–212. doi: 10.1093/aob/mcs136

Karabaghli-Degron, C., Sotta, B., Bonnet, M., Gay, G., and Le Tacon, F. (1998). The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) inhibits the stimulation of in vitro lateral root formation and the colonization of the tap-root cortex of Norway spruce (*Picea abies*) seedlings by the ectomycorrhizal fungus *Laccaria bicolor*. *New Phytol.* 140, 723–733. doi: 10.1046/j.1469-8137.1998.00307.x

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333, 880–882. doi: 10.1126/science.1208473

Kobae, Y., and Hata, S. (2010). Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. *Plant Cell Physiol.* 51, 341–353. doi: 10.1093/pcp/pcq013

Koltai, H. (2011). Strigolactones are regulators of root development. *New Phytol.* 190,

545–549. doi: 10.1111/j.1469-8137.2011.03678.x

Kosma, D., Molina, I., Ohlrogge, J., and Pollard, M. (2012). Identification of an *Arabidopsis* fatty alcohol:caffeoyl-coenzyme A acyltransferase required for the synthesis of alkyl hydroxycinnamates in root waxes. *Plant Physiol.* 160, 237–248. doi: 10.1104/pp.112.201822

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Dénarié, J., Barker, D., et al. (2003). A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. *Plant Physiol.* 131, 952–962. doi: 10.1104/pp.011882.

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J. B., et al. (2012). A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. *Nature* 483, 341–344. doi: 10.1038/nature10873

Krouk, G., Lacombe, B., Bielach, A., Perrine-Walker, F., Malinska, K., Mounier, E., et al. (2010). Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Dev. Cell* 18, 927–937. doi: 10.1016/j.devcel.2010.05.008

Ligrone, R., Carafa, A., Lumini, E., Bianciotto, V., Bonfante, P., and Duckett, J. (2007). Glomeromycotan associations in liverworts: a molecular, cellular and taxonomic analysis. *Am. J. Bot.* 94, 1756–1777. doi: 10.3732/ajb.94.11.1756

Linkohr, B., Williamson, L., Fitter, A., and Leyser, H. (2002). Nitrate and phosphate availability and distribution have different effects on root system architecture of *Arabidopsis*. *Plant J.* 29, 751–760. doi: 10.1046/j.1365-313X.2002.01251.x

Lopez-Bucio, J., Cruz-Ramirez, A., and Herrera-Estrella, L. (2003). The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* 6, 280–287. doi: 10.1016/S1369-5266(03)00035-9

Lundberg, D., Lebeis, S., Herrera Paredes, S., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

MacGregor, D. R., Deak, K. I., Ingram, P. A., and Malamy, J. E. (2008). Root system architecture in *Arabidopsis* grown in culture is regulated by sucrose uptake in the aerial tissues. *Plant Cell* 20, 2643–2660. doi: 10.1105/tpc.107.055475

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., et al. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58–63. doi: 10.1038/nature09622

McCully, M., and Canny, M. (1988). Pathways and processes of water and nutrient uptake in roots. *Plant Soil* 111, 159–170. doi: 10.1007/BF02139932

Mukherjee, K., and Ane, J. (2011). Germinating spore exudates from arbuscular mycorrhizal fungi: molecular and developmental responses in plants and their regulation by ethylene. *Mol. Plant Microbe Interact.* 24, 260–270. doi: 10.1094/MPMI-06-10-0146

Nibau, C., Gibbs, D., and Coates, J. (2008). Branching out in new directions: the control of root architecture by lateral root formation. *New Phytol.* 179, 595–614. doi: 10.1111/j.1469-8137.2008.02472.x

Nishimura, R., Hayashi, M., Wu, G., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., et al. (2002). HAR1 mediates systemic regulation of symbiotic organ development. *Nature* 420, 426–429. doi: 10.1038/nature01231

Olah, B., Brière, C., Bécard, G., Dénarié, J., and Gough, C. (2005). Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. *Plant J.* 44, 195–207. doi: 10.1111/j.1365-313X.2005.02522.x

Osmont, K., Sibout, R., and Hardtke, C. (2007). Hidden branches: developments in root system architecture. *Annu. Rev. Plant Biol.* 58, 93–113. doi: 10.1146/annurev.arplant.58.032806.104006

Parniske, M. (2005). Cue for the branching connection. *Nature* 435, 450–451. doi: 10.1038/435750a

Paszkowski, U., and Boller, T. (2002). The growth defect of *lrt1*, a maize mutant lacking lateral roots, can be complemented by symbiotic fungi or high phosphate nutrition. *Planta* 214, 584–590. doi: 10.1007/s004250100642

Rebouillat, J., Dievart, A., Verdeil, J., Escoute, J., Giese, G., Breitler, J., et al. (2009). Molecular genetics of rice root development. *Rice* 2, 15–34. doi: 10.1007/s12284-008-9016-5

Remans, T., Nacry, P., Pervent, M., Filleur, S., Diatloff, E., Mounier, E., et al. (2006). The *Arabidopsis* NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate rich patches. *Proc. Natl. Acad. Sci.* 103, 19206–19211. doi: 10.1073/pnas.0605275103

Rupp, L., Devries, H., and Mudge, K. (1989). Effect of aminocyclopropane carboxylic acid and aminoethoxyvinylglycine on ethylene production by ectomycorrhizal fungi. *Can. J. Bot.* 67, 483–485. doi: 10.1139/b89-068

Russell, J., and Bulman, S. (2005). The liverwort *Marchantia foliacea* forms a specialized symbiosis with arbuscular mycorrhizal fungi in the genus *Glomus*. *New Phytol.* 165, 567–579. doi: 10.1111/j.1469-8137.2004.01251.x

Singh, S., and Parniske, M. (2012). Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. *Curr. Opin. Plant Biol.* 15, 444–453. doi: 10.1016/j.pbi.2012.04.002

Smith, S. E., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Annu. Rev. Plant Biol.* 62, 227–250. doi: 10.1146/annurev-arplant-042110-103846

Smith, S. E., Smith, F. A., and Jakobsen, I. (2003). Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiol.* 133, 16–20. doi: 10.1104/pp.103.024380

Solaiman, M., Senoo, K., Kawaguchi, M., Imaizumi-Anraku, H., Akao, S., Tanaka, A., et al. (2000). Characterization of mycorrhizas formed by *Glomus* sp. on roots of hypernodulating mutants of *Lotus japonicus*. *J. Plant Res.* 113, 443–448. doi: 10.1007/PL00013953

Splivallo, R., Fischer, U., Göbel, C., Feussner, I., and Karlovsky, P. (2009). Truffles regulate plant root morphogenesis via the production of auxin and ethylene. *Plant Physiol.* 150, 2018–2029. doi: 10.1104/pp.109.141325

Sukumar, P., Legué, V., Vayssièees, A., Martin, F., Tuskan, G. A., and Kalluri, U. C. (2013). Involvement of auxin pathways in modulating root architecture during beneficial plant–microorganism interactions. *Plant Cell Environ.* 36, 909–919. doi: 10.1111/pce.12036

Venkateshwaran, M., Volkening, J. D., Sussman, M. R., and Ané, J.-M. (2013). Symbiosis and the social network of higher plants. *Curr. Opin. Plant Biol.* 16, 118–127. doi: 10.1016/j.pbi.2012.11.007

Veresoglou, S. D., Menexes, G., and Rillig, M. C. (2012). Do arbuscular mycorrhizal fungi affect the allometric partition of host plant biomass to shoots and roots? A meta-analysis of studies from 1990 to 2010. *Mycorrhiza* 22, 227–235. doi: 10.1007/s00572-011-0398-7

Volpe, V., Dell'Aglio, E., Giovannetti, M., Ruberti, C., Costa, A., Genre, A., et al. (2013). An AM-induced, MYB-family gene of *Lotus japonicus* (LjMAMI) affects root growth in an AM-independent manner. *Plant J.* 73, 442–455. doi: 10.1111/tpj.12045

Walder, F., Niemann, H., Natarajan, M., Lehmann, M. F., Boller, T., and Wiemken, A. (2012). Mycorrhizal networks: common goods of plants shared under unequal terms of trade. *Plant Physiol.* 159, 789–797. doi: 10.1104/pp.112.195727

Wang, E., Schornack, S., Marsh, J., Gobbato, E., Schwessinger, B., Eastmond, P., et al. (2012). A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Curr. Biol.* 22, 2242–2246. doi: 10.1016/j.cub.2012.09.043

Wang, X., Pan, Q., Chen, F., Yan, X., and Liao, H. (2011). Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. *Mycorrhiza* 21, 173–181. doi: 10.1007/s00572-010-0319-1

Wopereis, J., Pajuelo, E., Dazzo, F., Jiang, Q., Gresshoff, P., De Brujin, F., et al. (2000). Short root mutant of *Lotus japonicus* with a dramatically altered symbiotic phenotype. *Plant J.* 23, 97–114. doi: 10.1046/j.1365-313x.2000.00799.x

Yang, S.-Y., Grønlund, M., Jakobsen, I., Grotemeyer, M. S., Rentsch, D., Miyao, A., et al. (2012). Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the PHOSPHATE TRANSPORTER1 gene family. *Plant Cell* 24, 4236–4251. doi: 10.1105/tpc.112.104901

Yang, S.-Y., and Paszkowski, U. (2011). Phosphate import at the arbuscule: just a nutrient? *Mol. Plant Microbe Interact.* 24, 1296–1299. doi: 10.1094/MPMI-06-11-0151

Yano, K., Yamauchi, A., and Kono, Y. (1996). Localized alteration in lateral root development in roots colonized by an arbuscular mycorrhizal fungus. *Mycorrhiza* 6, 409–415. doi: 10.1007/s005720050140

Zhu, J., Kaeppler, S., and Lynch, J. (2005). Topsoil foraging and phosphorous acquisition efficiency in maize (*Zea mays*). *Funct. Plant Biol.* 32, 749–762. doi: 10.1071/FP05005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 March 2013; paper pending published: 20 March 2013; accepted: 31 May 2013; published online: 18 June 2013.

Citation: Gutjahr C and Paszkowski U (2013) Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. Front. Plant Sci. ***4****:204. doi: 10.3389/fpls.2013.00204*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Gutjahr and Paszkowski. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 11 July 2013
doi: 10.3389/fpls.2013.00228

Effector candidates in the secretome of *Piriformospora indica*, a ubiquitous plant-associated fungus

Maryam Rafiqi[1]*, Lukas Jelonek[2], Ndifor F. Akum[1], Feng Zhang[1] and Karl-Heinz Kogel[1]*

[1] Institute of Phytopathology and Applied Zoology, Research Centre for BioSystems, Land use, and Nutrition (IFZ), Justus Liebig University, Giessen, Germany
[2] CeBiTec, Bielefeld University, Bielefeld, Germany

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Weixing Shan, Northwest A&F University, China
Corné M. J. Pieterse, Utrecht University, Netherlands

***Correspondence:**
Maryam Rafiqi and Karl-Heinz Kogel, Institute of Phytopathology and Applied Zoology, Research Centre for BioSystems, Land use, and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26-32, Giessen, Germany
e-mail: maryam.rafiqi@agrar.uni-giessen.de; karl-Heinz.Kogel@agrar.uni-giessen.de

One of the emerging systems in plant–microbe interaction is the study of proteins, referred to as effectors, secreted by microbes in order to modulate host cells function and structure and to promote microbial growth on plant tissue. Current knowledge on fungal effectors derives mainly from biotrophic and hemibiotrophic plant fungal pathogens that have a limited host range. Here, we focus on effectors of *Piriformospora indica*, a soil borne endophyte forming intimate associations with roots of a wide range of plant species. Complete genome sequencing provides an opportunity to investigate the role of effectors during the interaction of this mutualistic fungus with plants. We describe *in silico* analyses to predict effectors of *P. indica* and we explore effector features considered here to mine a high priority protein list for functional analysis.

Keywords: fungal effector biology, small secreted proteins, biotrophy, symbiosis, endophyte

INTRODUCTION

Plant roots interact constantly with rhizosphere-resident microorganisms. These interactions, which can be either pathogenic or mutualistic, influence plant growth, immunity, and tolerance to abiotic stress (Richardson et al., 2009; Zamioudis and Pieterse, 2012). Beneficial symbioses that supply plants with growth limiting nutrients, such as nitrogen and phosphorus, are of a particular interest to agriculture because they minimize crops requirement for fertilizers. *Piriformospora indica* is a ubiquitous soil borne fungus that associates with roots of a wide range of plant species, including important crops, such as barley and wheat, medicinal plants as well as the model plants *Arabidopsis* and tobacco (Verma et al., 1998; Varma et al., 1999; Rai et al., 2001; Peskan-Berghofer et al., 2004). *P. indica* was initially investigated for its beneficial effects on plant's growth and resistance to pathogenic infections. Earlier reports have shown that fungal culture filtrates as well as infestation by *P. indica* spores promote shoots growth and increase root branching of plants grown on sterile nutrient-rich media (Barazani et al., 2005; Waller et al., 2005; Deshmukh and Kogel, 2007; Harrach et al., 2007; Serfling et al., 2007), suggesting possible induction of long distance hormonal signals rather than nutrient supply by the fungus. Indeed many microorganisms produce phytohormones or their analogs that induce plants growth and modify root structures (Grunewald et al., 2009). However, recent studies report that while *P. indica* indeed produces auxin during association with *Arabidopsis* and barley roots, fungal auxin production was not found to be required for triggering plant's growth (Vadassery et al., 2008; Hilbert et al., 2012; Nongbri et al., 2012). More studies are needed to specify the role of hormonal signals mediating the interaction between *P. indica* and plants. While accumulated evidence supports a mutualistic association between plants and *P. indica*, and suggests the use of this fungus as a biocontrol agent, the exact molecular process underlying the antagonistic effect of *P. indica* on pathogenic infections is unknown.

Piriformospora indica is a facultative saprophyte that grows on dead plant material and colonizes living root cells, mostly biotrophically, though a switch to a late cell death-associated stage has been described (Deshmukh et al., 2006; Qiang et al., 2012). This late growth stage is symptomless and poorly characterized. Whether this transition in the lifestyle affects mutualistic interactions with plants is as yet unknown. In general, biotrophic fungi have a narrow host range. *P. indica* forms associations with roots of a large range of plant species. Although it is still unclear if these interactions are mutualistic or more parasitic, an intriguing question is what are the cellular and molecular mechanisms developed by this fungus to ensure biotrophic growth and to undermine host defense strategies in different plant species? One scenario is that *P. indica* deploys an effector repertoire targeting conserved cellular processes in many plant species.

Key feature of the virulence of many biotrophic and hemibiotrophic fungal pathogens is the ability to deliver virulence proteins called effectors into their host cells. These effector proteins manipulate the host immunity, physiology, and metabolism, in favor of fungal growth and disease development. Some secreted fungal effectors exert their action extracellularly, in the plant apoplastic space. Many others have their molecular targets inside the plant cell, in the cytoplasm, the nucleus or other host subcellular compartments (Rafiqi et al., 2012). During biotrophic growth on barley root cells, *P. indica* intercellular hyphae extend differentiated branched hyphal structures into infected cells of root tissue (**Figure 1**). These structures are morphologically analogous

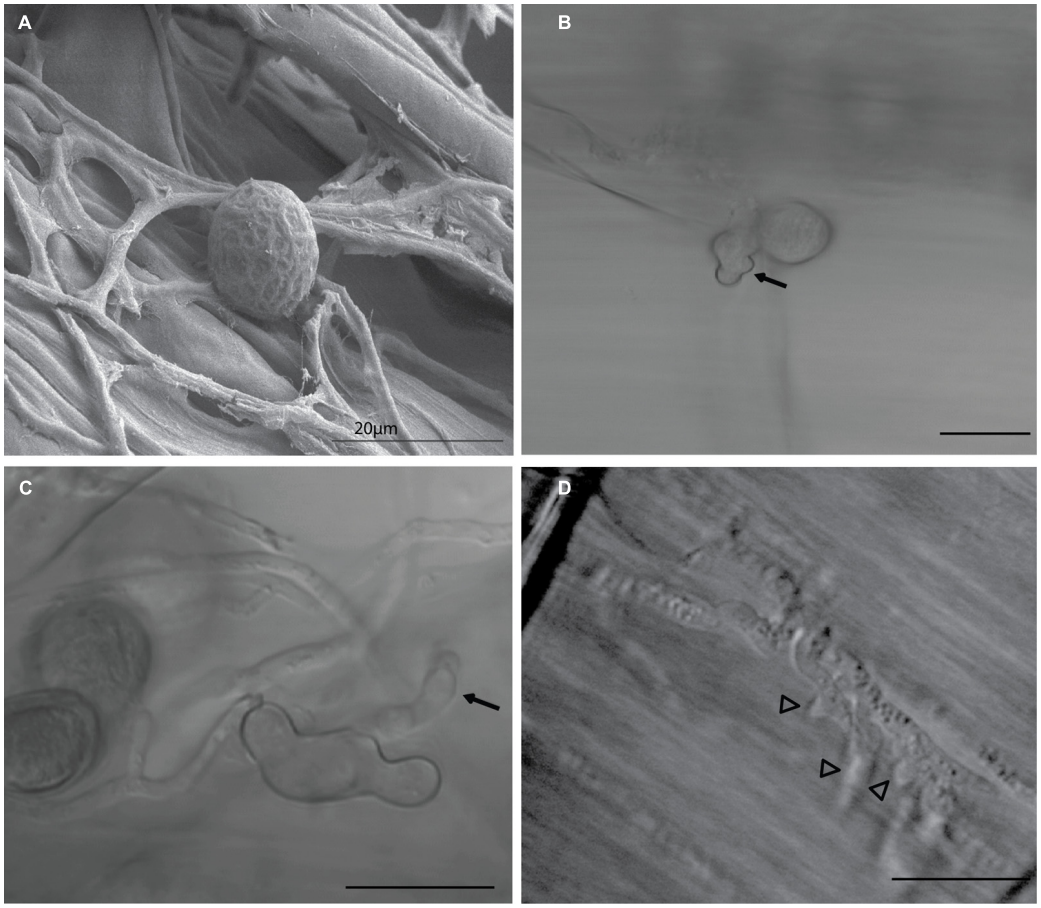


FIGURE 1 | *Piriformospora indica* biotrophic hyphal structures. During biotrophic growth on barley root cells, *P. indica* spores attach to the root surface, as seen **(A)** germinate and extend intercellular hyphae (arrows) on root tissue within 10 h **(B,C)** Differentiated swollen hyphal structures (arrowheads) are extended into colonized living cells of root tissue **(D)** These structures are morphologically analogous and may share similar functions to haustoria and arbuscules formed by pathogenic and mycorrhizal fungi, respectively, suggesting possible roles in acquisition of nutrients and secretion of effectors into host tissue. Image **(A)** was taken using scanning electron microscope (SEM), Images **(B–D)** were taken using a light microscope. Bars =20 μm.

and may share similar functions to the haustoria and arbuscules formed by pathogenic and mycorrhizal fungi, respectively. *P. indica* biotrophic hyphal structures penetrate the cell wall and invaginate the plasma membrane of infected barley root cells, suggesting possible roles in acquisition of nutrients and secretion of effectors in host tissue, similar to haustoria and arbuscules (Voegele and Mendgen, 2003; Catanzariti et al., 2006; O'Connell and Panstruga, 2006; Corradi and Bonfante, 2012). In this review, we use the whole genome sequence of *P. indica* (Zuccaro et al., 2011) to generate a refined list of effector candidates in the secretome of this endophytic fungus.

IDENTIFYING EFFECTOR CANDIDATES OF *P. indica*

Recent work on predicting effector candidates from fungal genomes has relied on selecting fungal genes up-regulated during *in planta* growth and coding for predicted small secreted proteins (SSPs) with a size cut-off of 300 amino acids (aa) that do not code for known functions (Martin et al., 2008; Hacquard et al., 2012; Zuccaro et al., 2011). However, more recent research has shown that fungal and oomycete effectors can exceed the size of 300 aa (Rafiqi et al., 2010; van Damme et al., 2012), and that despite being under high selective pressure, some effectors can still carry recognizable Pfam domains, which would help predict their biological function. Examples of these effectors are CRN8 of *Phytophthora infestans* and AvrM of *Melampsora lini*. CRN8 is 600 aa in size and carries a serine/threonine RD kinase domain that has been shown to function in the plant nucleus. AvrM is a 343 aa avirulence protein that is intercepted by the tonoplast-resident flax resistance protein M (Catanzariti et al., 2006; Takemoto et al., 2012; van Damme et al., 2012). Similarly, Ecp6 of *Cladosporium fulvum* and Slp1 of *Magnaporthe oryzae* carry LysM domains, (de Jonge et al., 2010; Mentlak et al., 2012). Thus, for identification of *P. indica* effector protein candidates, we established an *in silico* pipeline that does not take in account protein size and that includes Pfam domain-containing proteins (**Figure 2**).

Using SignalP (Petersen et al., 2011), 976 genes were predicted to code for proteins with signal peptide. Sequence similarity search was run using BlastP. Secreted proteins with predicted apoplastic

functions, such as cell wall hydrolysis, were excluded from this set based on their function and not on their size, and proteins with Pfam domains suggesting possible intracellular functions were retained. This resulted in a reduced set of 543 secreted proteins that are considered effector candidates (**Figure 2**). The majority, 389 proteins, are with unknown functions, a feature that characterize many predicted fungal effectors. 154 proteins carry predicted Pfam domains, of which 64 are predicted to have protease activity and 23 carry the carbohydrate-binding protein domain LysM. Effector protein families with LysM domains are expanded in many fungal species and are predicted to contribute to fungal virulence through binding to chitin oligosaccharides, and subsequently preventing their hydrolysis by plant chitinases (de Jonge and Thomma, 2009; Gan et al., 2012; Mentlak et al., 2012) and/or their recognition by membrane-anchored pattern recognition receptors (PRRs) such as *Arabidopsis* chitin elicitor receptor kinase (AtCERK1) that binds chitin directly through its extracellular LysM-containing domain (Liu et al., 2012).

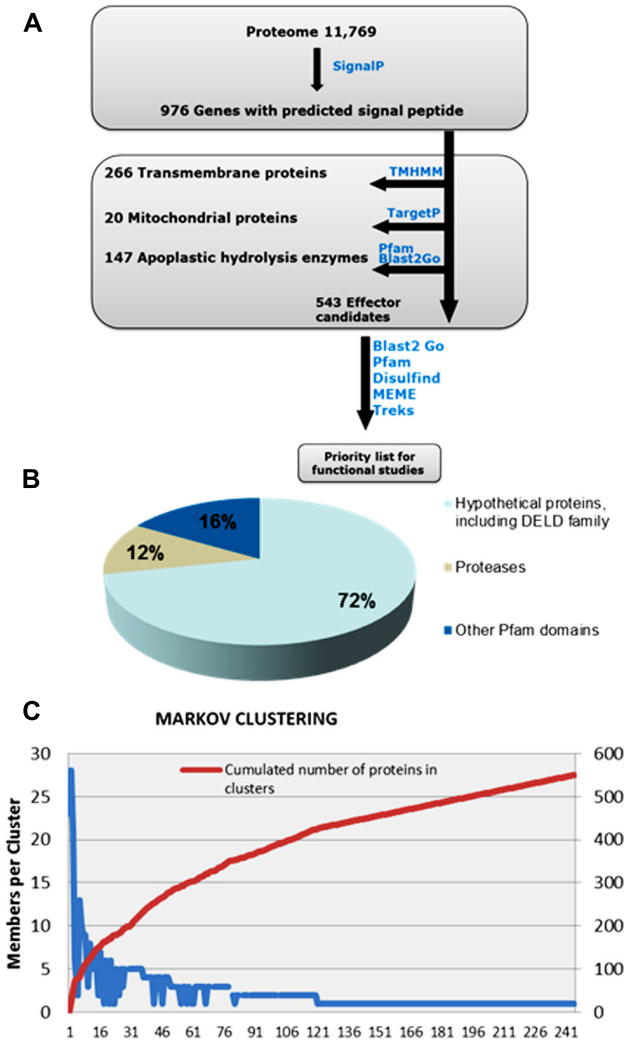


FIGURE 2 | Overview of the computational pipeline used to mine the list of effector candidates in the secretome of *P. indica*. (A) *P. indica* secretome, consisting of 972 proteins, was predicted using SignalP. Proteins containing transmembrane domains and proteins with mitochondrial signals were removed using TMHMM and TargetP, respectively. Apoplastic hydrolysis enzymes, such as chitinases and glucanases, were removed based on their function and not on their size, using Pfam and Blast2Go. The remaining total number of 543 proteins are considered effector candidates. Notably, 72% of effector candidates are novel sequences of unknown function **(B)** MCL analysis **(C)** has resulted in a high number of singletons and has shown no evidence for gene clustering.

Piriformospora indica EFFECTOR CANDIDATES WITH NO Pfam DOMAIN ARE ENRICHED FOR CYSTEINE RESIDUES AND INTERNAL REPEAT-RICH SEQUENCES BUT SHOW NO EVIDENCE FOR CLUSTERING

132 of the 389 SSPs lacking Pfam domains are enriched for cysteine residues, of which 65 are predicted by Disulfind algorithm (Ceroni et al., 2006) to have three or more disulphide bonds. 14 SSPs showed similarity to predicted proteins in the secretome of *Laccaria bicolor*. Using T-REKS program (Jorda and Kajava, 2009), 110 SSPs lacking Pfam domains were found to contain internal repeat-rich sequences. Search for conserved motifs (RxLR, [L/I]xAR, [R/K]CxxCx12H, [Y/F/W]xC, YxSL[R/K], and G[I/F/Y][A/L/S/T]R) showed no evidence for the presence of conserved motifs identified in SSPs of other fungal and oomycete species. Some of these motifs were present in one or a few sequences. However, because of their low frequency and their short sequences when compared to the more complex SSPs sequences, we consider their presence to occur by random chance. Using the Markov-Cluster-Algorithm (MCL; http://micans.org/mcl/) and MCL-Tribe (Enright et al., 2002), 215 SSPs could be clustered into tribes with five or more proteins (**Figure 2**). The remaining 328 sequences were split into 212 smaller clusters, including 138 singletons, and showed no evidence for gene clustering. Among SSPs rich in small repeats, 25 effector candidates carry the conserved C-terminal motif RSIDELD (Zuccaro et al., 2011). The function of this motif is as yet unknown. One new DELD gene (deposited to NCBI GenBank under the accession number KC342232.1) that was missing in the *P. indica* genome database, likely due to the presence of repetitive sequences, was amplified by PCR, indicating that DELD protein family might be more expanded than *ab initio* deduced from the assembled genome. Homologs of DELD proteins are also conserved in the closely related sebacinalean fungus *Piriformospora williamsii* (Rafiqi, unpublished). Proteins of this family have related sequences enriched for alanine and histidine residues and may have expanded from a single ancestral sequence. With the exception of DELD proteins and 14 other SSPs showing similarity to predicted secreted proteins of *L. bicolor*, the majority of *P. indica* SSPs are novel sequences showing no significant homology to known sequences in other organisms, which is in accord with previous studies highlighting the evolutionary diverse nature of fungal effectors (Saunders et al., 2012).

FAMILIES OF EFFECTOR CANDIDATES WITH PREDICTED INTRACELLULAR FUNCTIONS

Among Pfam-containing effector candidates, 35 indicate intracellular regulatory functions, suggesting that they perform these functions after translocation into plant root cells. Examples of these predicted intracellular effectors are translation activators,

RNA-binding proteins, RING fingers and F-box-containing proteins that are involved in protein ubiquitination. In addition, 14 SSPs with no Pfam domains carry predicted nuclear localization signals (NLSs). *In planta* expression of three green fluorescent protein (GFP)-tagged NLS-harboring proteins lacking the signal peptide resulted in nuclear localization of GFP fusion proteins, confirming the functionality of the NLS in plant cells and presenting indirect evidence for the intracellular function of these effector candidates (Rafiqi, Unpublished). Effectors with predicted intracellular functions constitute a high priority list for further analysis of the biological role as well as the translocation mechanism of fungal effectors in plant cells. Preliminary yeast two hybrid screen results indicate interaction of one of *P. indica* effector candidates with CSN5a and CSN5b components of the COP9 signalosome in *Arabidopsis* and tobacco, and with a member of *Arabidopsis* stress-associated protein family (AtSAP) that act as E3 ligase (Boernke and Rafiqi, unpublished). CSN5 is an evolutionary conserved protein complex comprised of eight subunits, named CSN1-8, where CSN5 is the only catalytic subunit described so far. CSN5 is an isopeptidase that interferes with the ubiquitin-proteasome pathway and plays critical developmental roles in plants (Wei et al., 2008). Targeting both CSN5 and AtSAP gives molecular insights into how *P. indica* could manipulate protein ubiquitination in different plant species by targeting conserved molecular processes in plants.

Unlike pathogenic cytoplasmic effectors, which can be revealed through a screen for avirulence functions in resistant plants, mutualistic cytoplasmic effectors are more challenging to identify. In a recent study, Kloppholz et al. (2011) have used yeast secreted protein trap system to identify a cytoplasmic effector, SP7, of the arbuscular mycorrhiza, *Glomus intraradices*. SP7 that target the plant nucleus is thought to promote symbiotic biotrophy through interaction with the plant transcription factor ERF19 that inhibit host defenses during mycorrhization. Similarly, another cytoplasmic effector, MiSSP7, that enters the plant nucleus and alters host gene expression was identified in the genome sequence of the ectomycorrhiza *L. bicolor* (Plett et al., 2011). Cell death suppression is likely to be a redundant function in the effector repertoire of mutualistic fungi.

Besides their biological function, how *P. indica* cytoplasmic effectors enter host cells is an important question to address. Translocation of fungal effectors is a topic of great debate. Evidence has been presented that translocation of some oomycete and fungal effectors, including two mutualistic effectors MiSSP7 of *L. bicolor* and SP7 of *G. intraradices*, can be pathogen-independent (Kale et al., 2010; Rafiqi et al., 2010; Kloppholz et al., 2011; Plett et al., 2011). However, the question of how fungal effector proteins reach the cytoplasm of plant cells is still widely debated.

PERSPECTIVES

As more and more evidence comes in to support the biological role of fungal effectors in manipulating plant immunity in favor of fungal virulence, selecting biologically significant proteins among hundreds of predicted effector candidates revealed by genome sequencing, and establishing a priority list for functional analysis remain critical. Isolation of *P. indica* biotrophic hyphal structures and construction of complementary DNA (cDNA) library of genes that are differentially expressed in these structures are necessary to identify effectors deployed at different stages of fungal morphogenesis. Available transcriptome sampled from colonized roots masks the expression pattern of *in planta* induced genes due to abundant extracellular and saprophytic mycelia, and to the low ratio of fungal to plant biomass in the early stages of root colonization. An important question is how *P. indica* evades recognition by the plant surveillance system, and whether it switches from restricted mutualistic to proliferative parasitic or pathogenic growth. Investigating the biological activity of effector proteins may provide mechanistic insights into how *P. indica* colonizes plants, at the molecular level.

ACKNOWLEDGMENTS

This work was supported by the German research funding organization "Deutsche Forschungsgemeinschaft" (DFG, Research unit 666). We thank Alexander Goesmann of CeBiTec, University of Bielefeld for provision of valuable bioinformatics support.

REFERENCES

Barazani, O., Benderoth, M., Groten, K., Kuhlemeier, C., and Baldwin, I. T. (2005). *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. *Oecologia* 146, 234–243. doi: 10.1007/s00442-005-0193-2

Catanzariti, A. M., Dodds, P. N., Lawrence, G. J., Ayliffe, M. A., and Ellis, J. G. (2006). Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell* 18, 243–256. doi: 10.1105/tpc.105.035980

Ceroni, A., Passerini, A., Vullo, A., and Frasconi, P. (2006). DISULFIND: a disulfide bonding state and cysteine connectivity prediction server. *Nucleic Acids Res.* 34, W177–W181. doi: 10.1093/nar/gkl266

Corradi, N., and Bonfante, P. (2012). The arbuscular mycorrhizal symbiosis: origin and evolution of a beneficial plant infection. *PLoS Pathog.* 8:e1002600. doi: 10.1371/journal.ppat.1002600

de Jonge, R., and Thomma, B. P. (2009). Fungal LysM effectors: extinguishers of host immunity? *Trends Microbiol.* 17, 151–157. doi: 10.1016/j.tim.2009.01.002

de Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., et al. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329, 953–955. doi: 10.1126/science.1190859

Deshmukh, S., Hueckelhoven, R., Schaefer, P., Imani, J., Sharma, M., Weiss, M., et al. (2006). The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc. Natl. Acad. Sci. U.S.A.* 103, 18450–18457. doi: 10.1073/pnas.0605697103

Deshmukh, S. D., and Kogel, K. H. (2007). *Piriformospora indica* protects barley from root rot caused by *Fusarium graminearum*. *J. Plant Dis. Prot.*114, 263–268.

Enright, A. J., Van Dongen, S., and Ouzounis, C. A. (2002). An efficient algorithm for large-scale detection of protein families. *Nucleic Acids Res.* 30, 1575–1584. doi: 10.1093/nar/30.7.1575

Gan, P., Ikeda, K., Irieda, H., Narusaka, M., O'Connell, R. J., Narusaka, Y., et al. (2012). Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. *New phytol.* 197, 1236–1249. doi: 10.1111/nph.12085

Grunewald, W., van Noorden, G., Van Isterdael, G., Beeckman, T., Gheysen, G., and Mathesius, U. (2009). Manipulation of auxin transport in plant roots during *Rhizobium* symbiosis and nematode parasitism. *Plant Cell* 21, 2553–2562. doi: 10.1105/tpc.109.069617

Hacquard, S., Joly, D. L., Lin, Y. C., Tisserant, E., Feau, N., Delaruelle, C., et al. (2012). A comprehensive

analysis of genes encoding small secreted proteins identifies candidate effectors in Melampsora laricipopulina (poplar leaf rust). *Mol. Plant Microbe. Interact.* 25, 279–293. doi: 10.1094/MPMI-09-11-0238

Harrach, B. D., Fodor, J., Barna, B., and Skoczowski, A. (2007). Salt tolerance induced in barley by *Piriformospora indica*. *Acta Physiol. Plant.* 29, S18–S19. doi: 10.1111/j.1469-8137.2008.02583.x

Hilbert, M., Voll, L. M., Ding, Y., Hofmann, J., Sharma, M., and Zuccaro, A. (2012). Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots. *New Phytol.* 196, 520–534. doi: 10.1111/j.1469-8137.2012.04275.x

Jorda, J., and Kajava, A. V. (2009). T-REKS: identification of Tandem REpeats in sequences with a K-meanS based algorithm. *Bioinformatics* 25, 2632–2638. doi: 10.1093/bioinformatics/btp482

Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., et al. (2010). External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell* 142, 284–295. doi: 10.1016/j.cell.2010.06.008

Kloppholz, S., Kuhn, H., and Requena, N. (2011). A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. *Curr. Biol.* 21, 1204–1209. doi: 10.1016/j.cub.2011.06.044

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., et al. (2012). Chitin-induced dimerization activates a plant immune receptor. *Science* 336, 1160–1164. doi: 10.1126/science.1218867

Martin, F., Aerts, A., Ahren, D., Brun, A., Danchin, E. G., Duchaussoy, F., et al. (2008). The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature* 452, 88–92. doi: 10.1038/nature06556

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., et al. (2012). Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* 24, 322–335. doi: 10.1105/tpc.111.092957

Nongbri, P. L., Johnson, J. M., Sherameti, I., Glawischnig, E., Halkier, B. A., and Oelmuler, R. (2012). Indole-3-acetaldoxime-derived compounds restrict root colonization in the beneficial interaction between *Arabidopsis* roots and the endophyte *Piriformospora indica*. *Mol. Plant Microbe Interact.* 25, 1186–1197. doi: 10.1094/MPMI-03-12-0071-R

O'Connell, R. J., and Panstruga, R. (2006). Tete a tete inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. *New phytol.* 171, 699–718. doi: 10.1111/j.1469-8137.2006.01829.x

Peskan-Berghofer, T., Shahollari, B., Giong, P. H., Hehl, S., Markert, C., Blanke, V., et al. (2004). Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represents a novel system to study beneficial plant-microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. *Physiol. Plant.* 122, 465–477. doi: 10.1111/j.1399-3054.2004.00424.x

Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* 8, 785–786. doi: 10.1038/nmeth.1701

Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legue, V., Brun, A., et al. (2011). A secreted effector protein of *Laccaria bicolor* is required for symbiosis development. *Curr. Biol.* 21, 1197–1203. doi: 10.1016/j.cub.2011.05.033

Qiang, X., Zechmann, B., Reitz, M. U., Kogel, K. H., and Schafer, P. (2012). The mutualistic fungus *Piriformospora indica* colonizes *Arabidopsis* roots by inducing an endoplasmic reticulum stress-triggered caspase-dependent cell death. *Plant Cell* 24, 794–809. doi: 10.1105/tpc.111.093260

Rafiqi, M., Ellis, J. G., Ludowici, V. A., Hardham, A. R., and Dodds, P. N. (2012). Challenges and progress towards understanding the role of effectors in plant-fungal interactions. *Curr. Opin. Plant Biol.* 15, 477–482. doi: 10.1016/j.pbi.2012.05.003

Rafiqi, M., Gan, P. H., Ravensdale, M., Lawrence, G. J., Ellis, J. G., Jones, D. A., et al. (2010). Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. *Plant Cell* 22, 2017–2032. doi: 10.1105/tpc.109.072983

Rai, M., Acharya, D., Singh, A., and Varma, A. (2001). Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. *Mycorrhiza* 11, 123–128. doi: 10.1007/s005720100115

Richardson, A., Barea, J.-M., McNeill, A., and Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305–339. doi: 10.1007/s11104-009-9895-2

Saunders, D. G., Win, J., Cano, L. M., Szabo, L. J., Kamoun, S., and Raffaele, S. (2012). Using hierarchical clustering of secreted protein families to classify and rank candidate effectors of rust fungi. *PLoS ONE* 7:e29847. doi: 10.1371/journal.pone.0029847

Serfling, A., Wirsel, S. G. R., Lind, V., and Deising, H. B. (2007). Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. *Phytopathology* 97, 523–531. doi: 10.1094/PHYTO-97-4-0523

Takemoto, D., Rafiqi, M., Hurley, U., Lawrence, G. J., Bernoux, M., Hardham, A. R., et al. (2012). N-terminal motifs in some plant disease resistance proteins function in membrane attachment and contribute to disease resistance. *Mol. Plant Microbe. Interact.* 25, 379–392.

Vadassery, J., Ritter, C., Venus, Y., Camehl, I., Varma, A., Shahollari, B., et al. (2008). The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. *Mol. Plant Microbe Interact.* 21, 1371–1383. doi: 10.1094/MPMI-21-10-1371

van Damme, M., Bozkurt, T. O., Cakir, C., Schornack, S., Sklenar, J., Jones, A. M. et al.(2012). The irish potato famine pathogen phytophthora infestans translocates the CRN8 kinase into host plant cells. *PLoS Pathog*, 8:e1002875. doi: 10.1371/journal.ppat.1002875

Varma, A., Savita, V., Sudha, Sahay, N., Butehorn, B., and Franken, P. (1999). *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl. Environ. Microbiol.* 65, 2741–2744.

Verma, S., Varma, A., Rexer, K. H., Hassel, A., Kost, G., Sarbhoy, A., et al. (1998). *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia* 90, 896–903. doi: 10.2307/3761331

Voegele, R. T., and Mendgen, K. (2003). Rust haustoria: nutrient uptake and beyond. *New Phytol.* 159, 93–100. doi: 10.1046/j.1469-8137.2003.00761.x

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., et al. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13386–13391. doi: 10.1073/pnas.0504423102

Wei, N., Serino, G., and Deng, X. W. (2008). The COP9 signalosome: more than a protease. *Trends Biochem. Sci.* 33, 592–600. doi: 10.1016/j.tibs.2008.09.004

Zamioudis, C., and Pieterse, C. M. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Zuccaro, A., Lahrmann, U., Guldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., et al. (2011). Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont *Piriformospora indica*. *PLoS Pathog.* 7:e1002290. doi: 10.1371/journal.ppat.1002290

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 March 2013; accepted: 10 June 2013; published online: 11 July 2013.

*Citation: Rafiqi M, Jelonek L, Akum NF, Zhang F and Kogel K-H (2013) Effector candidates in the secretome of Piriformospora indica, a ubiquitous plant-associated fungus. Front. Plant Sci. **4**:228. doi: 10.3389/fpls.2013.00228*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Rafiqi, Jelonek, Akum, Zhang and Kogel. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 24 June 2013
doi: 10.3389/fpls.2013.00206

Deciphering the hormonal signaling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato

Ainhoa Martínez-Medina[1]*, Iván Fernández[1], María J. Sánchez-Guzmán[2], Sabine C. Jung[1], Jose A. Pascual[3] and María J. Pozo[1]*

[1] *Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain*
[2] *Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, Málaga, Spain*
[3] *Department of Soil and Water Conservation and Organic Waste Management, Centro de Edafología y Biología Aplicada del Segura, Consejo Superior de Investigaciones Científicas, Murcia, Spain*

Edited by:
Erik Poelman, Wageningen University, Netherlands

Reviewed by:
Cesar Rodriguez-Saona, Rutgers University, USA
Christelle Aurélie Maud Robert, Max Planck Institute, Germany

****Correspondence:***
Ainhoa Martínez-Medina, Plant-Microbe Interactions, Utrecht University, H.R. Kruyt Building, Padualaan 8, W303, 3584 CH Utrecht, Netherlands
e-mail: a.martinezmedina@uu.nl;
María J. Pozo, Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Profesor Albareda 1, 18008 Granada, Spain
e-mail: mjpozo@eez.csic.es

Root colonization by selected *Trichoderma* isolates can activate in the plant a systemic defense response that is effective against a broad-spectrum of plant pathogens. Diverse plant hormones play pivotal roles in the regulation of the defense signaling network that leads to the induction of systemic resistance triggered by beneficial organisms [induced systemic resistance (ISR)]. Among them, jasmonic acid (JA) and ethylene (ET) signaling pathways are generally essential for ISR. However, *Trichoderma* ISR (TISR) is believed to involve a wider variety of signaling routes, interconnected in a complex network of cross-communicating hormone pathways. Using tomato as a model, an integrative analysis of the main mechanisms involved in the systemic resistance induced by *Trichoderma harzianum* against the necrotrophic leaf pathogen *Botrytis cinerea* was performed. Root colonization by *T. harzianum* rendered the leaves more resistant to *B. cinerea* independently of major effects on plant nutrition. The analysis of disease development in shoots of tomato mutant lines impaired in the synthesis of the key defense-related hormones JA, ET, salicylic acid (SA), and abscisic acid (ABA), and the peptide prosystemin (PS) evidenced the requirement of intact JA, SA, and ABA signaling pathways for a functional TISR. Expression analysis of several hormone-related marker genes point to the role of priming for enhanced JA-dependent defense responses upon pathogen infection. Together, our results indicate that although TISR induced in tomato against necrotrophs is mainly based on boosted JA-dependent responses, the pathways regulated by the plant hormones SA- and ABA are also required for successful TISR development.

Keywords: *Botrytis* sp., induced systemic resistance, jasmonic acid, phytohormone, priming, signaling, tomato, *Trichoderma* sp.

INTRODUCTION

Root colonization by selected *Trichoderma* isolates has been reported to increase resistance to different types of pathogens in various plant species, both below and aboveground (reviewed in Harman et al., 2004). This biological control can be achieved by a direct effect of *Trichoderma* on plant pathogens (reviewed in Vinale et al., 2008); or indirectly through plant-mediated effects by improving the plant nutritional status (Shoresh and Harman, 2008) or through partial activation of the plant immune system (reviewed in Shoresh et al., 2010). Indeed, some competent *Trichoderma* strains can colonize plants roots without any damage to plant tissues but inducing changes in plant physiology and the plant defense system (Yedidia et al., 1999; Alfano et al., 2007; Chacón et al., 2007; Brotman et al., 2012; Mathys et al., 2012). As in other beneficial plant–microbe interactions, these changes could be associated with a regulatory strategy of the plant to limit microbial colonization of the "beneficial invader" (Zamioudis and Pieterse, 2012).

Although a clear understanding of the *Trichoderma*–plant recognition process is lacking, several elicitors that can activate plant basal immunity have been described in *Trichoderma* including the ethylene (ET)-inducing xylanase (Hanson and Howell, 2004); the proteinaceous non-enzymatic elicitor Sm1 (Djonovic et al., 2006, 2007); or the 18mer peptaibols (Viterbo et al., 2007). Only a limited number of pattern recognition receptors able to recognize some of these *Trichoderma*-related microbe-associated molecular patterns (MAMPs) have been characterized so far (Ron and Avni, 2004; Petutschnig et al., 2010). During the "asymptomatic" infection of the roots, the plant limits the endophytic colonization of *Trichoderma* through the activation of certain plant defense responses, including cell wall reinforcement and the accumulation of antimicrobial compounds and reactive oxygen species (Yedidia et al., 1999, 2000; Chacón et al., 2007; Contreras-Cornejo et al., 2011; Salas-Marina et al., 2011). After the successful limitation of fungus penetration to the first few layers of root cortical cells, the expression of some defense-related genes and the

antimicrobial activity return to pre-infection levels (Yedidia et al., 1999, 2003; Masunaka et al., 2011). It is likely that *Trichoderma* is able to "short-circuit" plant defense signaling, possibly through the secretion of still unknown fungal effectors, which suppress plant defense to remain accommodated by the plant as an avirulent symbiont. The interaction between the plant and *Trichoderma* should then be finely regulated, assuring benefits to both partners, with the plant receiving protection and more available nutrients and the fungus obtaining organic compounds and a niche for growth.

Trichoderma colonization triggers, therefore, a wide array of plant responses which may result in an enhanced defensive capacity of the plant (Bailey et al., 2006; Marra et al., 2006; Alfano et al., 2007; Morán-Diez et al., 2012). Often, the effects of *Trichoderma* on the plant defense system are not restricted to the root, but they also manifest in aboveground plant tissues (Martínez-Medina et al., 2010, 2011a; Salas-Marina et al., 2011; Mathys et al., 2012), rendering the plant more resistant to a broad-spectrum of plant pathogens. This systemic resistance is likely the result of the modulation of the plant defense network that may translate *Trichoderma*-induced early signaling events into a more efficient activation of defense responses. It is well known that the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and ET act as dominant primary signals in the regulation of local and systemic defense responses in plants (reviewed in Pieterse et al., 2009), and accordingly, they play a central role in the induced resistance phenomena. Generally, pathogen-induced systemic acquired resistance (SAR), is dependent on the SA-regulated signaling pathway (Durrant and Dong, 2004), while ISR by beneficial microorganisms usually relies on JA signaling (Pieterse et al., 1996; Van Loon et al., 1998; Pozo et al., 2008; Van Wees et al., 2008; Van der Ent et al., 2009). However, as more resistance-inducing agents are characterized, the implication of other signaling pathways in the induction of resistance becomes evident. Indeed is the cross-talk among different signaling pathways what provides the plant with a powerful capacity to finely regulate its immune response to specific invaders (Pieterse et al., 2009), and as induced resistance is usually an enhancement of basal defenses, the implication of multiple hormones in shaping ISR is likely. Induced resistance may result of the direct activation of defense mechanisms – including increased basal levels of defense-related hormones, or of the priming of the plant defensive capacity. In the latter, a more efficient activation of defense mechanisms occurs upon attack, and it may not be related to changes in hormone content but in the susceptibility of the tissues to these hormones (Conrath et al., 2006).

Expression studies on marker genes linked to the main defense signaling pathways suggested that *Trichoderma*-induced systemic resistance (TISR) might involve the direct activation of both SA- and JA-related pathways (Alfano et al., 2007; Salas-Marina et al., 2011; Mathys et al., 2012; Morán-Diez et al., 2012). Despite this possible direct activation of defenses, most examples points to a boosted activation of defenses upon attack by several pathogens (Segarra et al., 2009; Perazzolli et al., 2011; Brotman et al., 2012; Mathys et al., 2012). Nevertheless, the activation of a pathway does not proof its role in resistance. The requirement of a specific signaling pathway in TISR can only be addressed by phenotypic studies of disease development on mutant lines impaired in those pathways, however, only a limited number of studies in the model plant *Arabidopsis* have addressed this issue. The pioneer study by Korolev et al. (2008) using multiple *Arabidopsis* mutant lines showed that the induction of resistance by *Trichoderma harzianum* Rifai T39 against *Botrytis cinerea* requires JA, ET, and ABA signaling, while SA was not required. Using different *Trichoderma* strains and the same *Arabidopsis–B. cinerea* pathosystem other authors have confirmed the requirement of JA for TISR, while the need of an intact SA and ET signaling pathways is more controversial (Segarra et al., 2009; Mathys et al., 2012). In summary, in *Arabidopsis* JA has been consistently reported as essential for TISR against *B. cinerea* and other pathogens, but the requirement of SA and ET may depend on the *Trichoderma* strain (Korolev et al., 2008; Segarra et al., 2009; Mathys et al., 2012).

According to the reported data, it is likely that the induction of resistance against specific pathogens in different hosts may require different signaling pathways. Although induction of TISR in tomato has been demonstrated against bacterial and fungal pathogens (Alfano et al., 2007; Tucci et al., 2011), the signaling pathways involved are yet to be investigated. Here we aim to gain further insights in the role of the main defense signaling pathways that operate in TISR in tomato against the major fungal pathogen *B. cinerea* (Dean et al., 2012). First we try to uncouple the role of plant defense mechanisms from the possible contribution of nutritional aspects. Then we analyzed the signaling pathways required for efficient TISR establishment through the phenotypic analysis of disease on tomato signaling mutants. Finally, we explore the plant defense response triggered upon pathogen attack in induced plants by monitoring the expression of defense-related marker genes.

In summary, we present an integrative analysis of the main mechanisms implicated in the systemic resistance induced by *T. harzianum* T-78 in an agronomically important crop, tomato, against the gray mold causal agent *B. cinerea*. The hormonal related pathways implicated in TISR have been analyzed in order to provide insights into the signaling network regulating systemic resistance induced by *Trichoderma*in tomato.

MATERIALS AND METHODS

MICROBIAL STRAINS AND INOCULA PREPARATION

Trichoderma harzianum T-78 (CECT 20714, Spanish collection of type cultures) inoculum was prepared using a specific solid medium, obtained by mixing commercial oat, bentonite, and vermiculite according to Martínez-Medina et al. (2009). The necrotrophic fungus used in this study was *B. cinerea* CECT2100 (Spanish collection of type cultures) kindly provided by Dr. Flors (Universidad de Valencia). For spore production, *B. cinerea* was cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit) supplemented with tomato leaves at 40 mg ml^{-1} at 24°C (Vicedo et al., 2009). *B. cinerea* spores were collected from 15-day-old cultures and incubated in Gambor's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM sucrose and 10 mM KH_2PO_4 for 2 h in the dark with no shaking, according to Vicedo et al. (2009).

PLANT MATERIAL

Ten different tomato (*Solanum lycopersicum*) genotypes were used in our studies including the four wild-type cultivars Castlemart, Moneymaker, UC82B, and Betterboy and the following defense-related mutant lines: The JA-impaired mutant *def1* (Howe et al., 1996) in background Castlemart (provided by G. Howe, Michigan State University). The SA- and ABA-impaired lines *NahG* (Brading et al., 2000) and *sitiens* (Taylor et al., 1988) respectively, in background Moneymaker (provided by J. Jones, John Innes Centre and C. Hanhart, Wageningen University, respectively). The ET-impaired mutant *ACD* (Klee et al., 1991), in background UC82B (provided by H. Klee, University of Florida). The prosystemin antisense line *PS-* (Orozco-Cardenas et al., 1993) and the over-expressing line *PS+* (McGurl et al., 1994) both in background Betterboy (provided by C. Ryan and G. Pearce, Washington State University). Seeds were surface-sterilized in 4% sodium hypochlorite containing 0.02% (v/v) Tween-20, rinsed thoroughly with sterile water and germinated for 1 week in sterile vermiculite at 25°C in darkness.

EXPERIMENTAL DESIGN AND GROWTH CONDITION

Individual seedlings were transferred to 0.25 l pots with a sterile sand:soil (4:1) mixture containing the *Trichoderma* inoculum. *T. harzianum* inoculum was mixed through the soil to a final density of 1×10^6 conidia per g of soil before transplanting the tomato seedlings. The same amount of sand:soil mix but free from *T. harzianum* was added to control plants. For each treatment a total of six plants were used. Plants were randomly distributed and grown in a greenhouse at 24/16°C with a 16/8 h photoperiod and 70% humidity, and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966). After 5 weeks, plants were harvested and the roots and shoot fresh weights were determined. The fourth and fifth leaves of each plant were detached for inoculation with the pathogen, and the rest of the shoots reserved for nutritional analyses. Root samples of each individual plant were thoroughly rinsed and collected for microbiological analyses. Substrate attached to the root system was considered as rhizospheric substrate and reserved for microbiological analyses.

Botrytis cinerea BIOASSAY

The fourth and fifth leaves of each individual plant were detached from the plant with a blade and challenged with the pathogen by applying 5-µl droplets of a suspension of *B. cinerea* spores at 5×10^6 ml^{-1}, previously incubated in Gambor's B5 medium supplemented with sucrose (0.1 mM) and phosphate (0.1 mM) for 4 h (Vicedo et al., 2009). One leaflet of each detached leaf from control and *T. harzianum*-inoculated plants were collected and immediately frozen in liquid nitrogen and stored at −80°C until use in molecular analyses as uninfected controls (time 0). Two 5-µl droplets were applied on each of the remaining leaflets, one on each side of the midrib. Detached *Botrytis*-inoculated leaves were placed on wet paper within plastic trays covered with transparent film to maintain high relative humidity conditions, and kept at 15–20°C with a photoperiod of 16 h light. Fungal hyphae grew concentrically from the inoculation site, resulting in visible necrosis at 48 h after inoculation. Disease symptoms were scored 72 and 96 h post inoculation (hpi) by determining the average lesion diameter in 12 leaves per genotype and treatment.

PLANT NUTRIENT CONTENT ANALYSES

Nutrient content of shoots was measured at CEBAS-CSIC (Spain). Leaves were briefly rinsed with deionized water and oven-dried at 60°C for 72 h, and ground to a fine powder. The samples were digested by a microwave technique, using a Milestone Ethos I microwave digestion instrument, according to Martínez-Medina et al. (2011b). A standard aliquot (0.1 g) of dry, finely ground plant material was digested with concentrated nitric acid (HNO_3; 8 mL) and hydrogen peroxide (H_2O_2; 2 mL). Subsequently, plant content of nutrition elements, including phosphorus and potassium, were simultaneous analyzed using ICP (Iris intrepid II XD2 Thermo). Nitrogen content was determined using a Flash 1112 series EA carbon/nitrogen analyzer. Six biological replicates from six independent plants were measured for each treatment.

Trichoderma QUANTIFICATION IN THE RHIZOSPHERE

Serial dilutions of the sand:soil mixture samples in sterile, quarter-strength ringer solution were used for quantifying *T. harzianum* colony forming units (cfu), by a plate count technique using PDA amended with 50 mg L^{-1} rose bengal and 100 mg L^{-1} streptomycin sulfate, according to Martínez-Medina et al. (2011b). Plates were incubated at 28°C and cfu were counted after 5 days. Data were expressed per gram of dry soil.

ANALYSIS OF GENE EXPRESSION BY RT-qPCR

Total RNA from tomato leaves was extracted using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel), and stored at −80°C until use. Leaf tissue was collected from tomato leaves 96 h upon pathogen infection. The second leaflet of the leaves also was collected as uninfected control. The complementary DNA (cDNA) synthesis, the conditions of RT-qPCR (reverse transcription-quantitative polymerase chain reaction) experiments and the relative quantification of specific mRNA levels was performed according to López-Ráez et al. (2010) and using the gene-specific primers described in **Table 1**. Expression values were normalized using the housekeeping gene *SlEF*, which encodes for the tomato elongation factor-1α. The experiments were independently repeated and each reaction was performed in duplicate.

STATISTICAL ANALYSES

The statistical analyses were performed using SPSS software, version 20 (SPSS Inc., Chicago, IL, USA). The data on lesion diameter in different tomato genotypes were subjected to two-way analysis of variance (ANOVA). The statistical significance of the results was determined by performing Tukey's multiple-range test ($P < 0.05$). For data on plant nutritional content, pairwise comparisons were made for each genotype between *Trichoderma*-inoculated and control plants with Student's *t*-test ($P < 0.05$). Regarding *T. harzianum* quantification in soil, the non-inoculated treatments were excluded from the analyses since *T. harzianum* was not detected in any of the non-inoculated treatments, and pairwise

Table 1 | Primer sequences used in the gene expression analysis. The genes monitored are used as markers for the pathways indicated. Jasmonate (JA), salicylic acid (SA), abscisic acid (ABA), and ethylene (ET).

ID	Target Gen	Related pathway	Primer (5′–3′)
AF083253	Multicystatin[1] (*MC*)	JA inducible	GAGAATTTCAAGGAAGTTCAA
			GGCTTTATTTCACACAGAGATA
K03291	Proteinase inhibitor II[1] (*PI II*)	JA inducible	GAAAATCGTTAATTTATCCCAC
			ACATACAAACTTTCCATCTTTA
M84801	Prosystemin[2] (*PS*)	JA and ABA inducible	AATTTGTCTCCCGTTAGA
			AGCCAAAAGAAAGGAAGCAAT
M69247	Pathogenesis-related protein PR1a[3] (*PR1*)	SA inducible	GTGGGATCGGATTGATATCCT
			CCTAAGCCACGATACCATGAA
M83314	Phenylalanine ammonia lyase[2] (*PAL*)	SA biosynthesis	CGTTATGCTCTCCGAACATC
			GAAGTTGCCACCATGTAAGG
X51904	Desiccation protective protein[3] (*Le4*)	ABA inducible	ACTCAAGGCATGGGTACTGG
			CCTTCTTTCTCCTCCCACCT
NM001247876	β-1,3-glucanase[2] (*gluB*)	ET inducible	CCATCACAGGGTTCATTTAGG
			CCATCCACTCTCTGACACAACT
X14449	Elongation factor 1α [4] (*SlEF*)	Housekeeping	GATTGGTGGTATTGGAACTGTC
			AGCTTCGTGGTGCATCTC
XM_001560987.1	β-tubulin[5]	Quantification of *B. cinerea* tubulin mRNA levels	CCGTCATGTCCGGTGTTACCAC
			CGACCGTTACGGAAATCGGAA

[1] *Uppalapati et al. (2005);* [2] *This work;* [3] *López-Ráez et al. (2010);* [4] *Rotenberg et al. (2006);* [5] *Brouwer et al. (2003).*

comparisons were made between each impaired mutant and its corresponding wild-type with Student's *t*-test ($P < 0.05$). For gene expression analyses in the wild-type Moneymaker, pairwise comparisons were made for each gene between *Trichoderma*-inoculated and control plants with Student's *t*-test ($P < 0.05$). Pairwise comparisons with Student's *t*-test ($P < 0.05$) were also made for expression analysis between *Trichoderma*-inoculated and control plants for each gene in the genotypes *def1* and Moneymaker. All the experiments were repeated at least 2 times, with similar results.

RESULTS

Trichoderma harzianum INDUCES SYSTEMIC PROTECTION AGAINST *Botrytis cinerea* INFECTION

Five-weeks old plants of two different tomato cultivars (Castlemart and Moneymaker) inoculated with *T. harzianum* were challenged with the foliar pathogen *B. cinerea*. The progress of the disease was recorded and data corresponding to 96 hpi are shown. *T. harzianum*-inoculated plants resulted in a statistically significant reduction of lesion diameter in both cultivars, compared with untreated control plants (**Figures 1A,B**).

THE SYSTEMIC PROTECTION TRIGGERED BY *Trichoderma harzianum* IN TOMATO IS NOT RELATED TO IMPROVED NUTRITION OR GROWTH PROMOTION

In order to determine the effect of *T. harzianum* on plant development, shoot and root fresh weighs were evaluated and nitrogen, phosphorous, and potassium shoot content were measured on the tomato lines Castlemart and Moneymaker 5 weeks after inoculation with *T. harzianum*. There were no significant differences in growth associated to *T. harzianum* inoculation in any of the tomato lines (**Table 2**). Except for a moderate decrease in potassium levels in Castlemart, the nutrient analyses in shoots showed no differences in the main macronutrients nitrogen and phosphorous between *Trichoderma*-inoculated and control plants, suggesting that *Trichoderma* effects on disease development cannot be regarded as a consequence of improved plant growth or nutrition improvement.

Trichoderma harzianum-INDUCED SYSTEMIC RESISTANCE IS DEPENDENT ON THE PHYTOHORMONES JA, SA, and ABA

In order to analyze the involvement of different defense-related pathways in *Trichoderma*-mediated ISR, we investigated the effect of *T. harzianum* on *B. cinerea* infection in different tomato mutant lines and their corresponding backgrounds. Mutants affected in the biosynthesis of specific defense-related hormones were selected, including the JA-deficient *defenseless1* (*def1*), the SA-deficient *NahG*, the ABA-deficient *sitiens* and the ET-underproducing ACC deaminase *ACD*. Additionally, we also analyzed the disease development in the tomato lines over-expressing the *prosystemin* gene in the sense (*PS*+) and antisense (*PS*−) orientation. Prosystemin is the precursor of the peptide defense hormone systemin, a positive regulator of JA signaling. The evaluation of the lesions upon *Botrytis* inoculation revealed that disease development was significantly affected by the plant genotype ($P < 0.001$; $F = 7.43$), the fungal treatment ($P < 0.001$; $F = 10.98$)

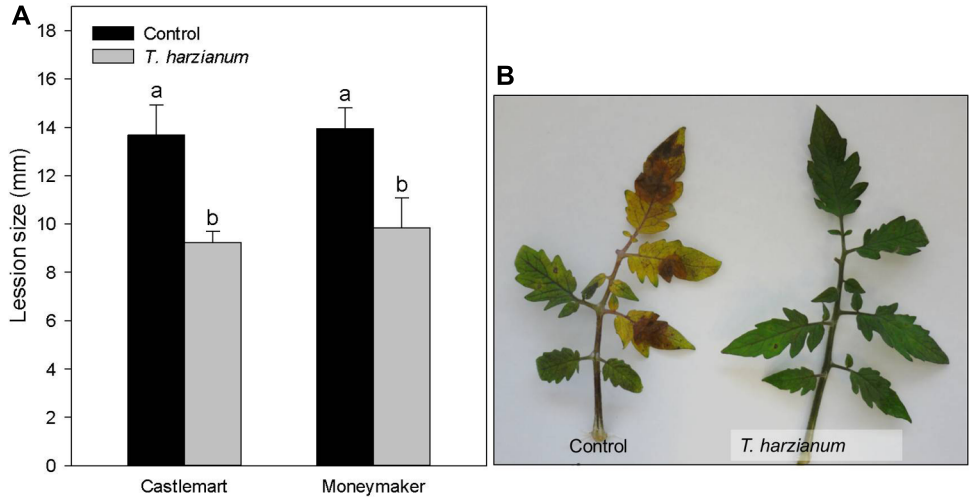


FIGURE 1 | *Trichoderma harzianum* induces systemic protection against the pathogen *Botrytis cinerea* in tomato plants. (A) Leaves of 5 weeks-old tomato plants (cv. Castlemart and Moneymaker) grown in soil containing or not *T. harzianum* were challenged with a conidial suspension of *B. cinerea*. Lesion diameter was determined 96 h after pathogen inoculation. The data show the lesion diameter (mm) ±SE (n = 12). Data not sharing a letter in common differ significantly ($P < 0.05$) according to Tukey's multiple-range test. **(B)** *B. cinerea* symptom development in *T. harzianum* inoculated and non-inoculated (control) plants (cv. Moneymaker).

Table 2 | Effect of *Trichoderma harzianum* on tomato plant development. Shoot and root fresh weight (in grams) and shoot nitrogen, phosphorous, and potassium content (g/100 g fresh weigh) of 5-weeks old tomato lines Castlemart and Moneymaker inoculated with *T. harzianum*.

Tomato Cv.	Treatment	Shoot fresh weight (g)	Root fresh weight (g)	Shoot nitrogen (g/100 g)	Shoot phosphorous (g/100 g)	Shoot potassium (g/100 g)
Castlemart	Control	9.90 ± 0.46	1.63 ± 0.16	2.69 ± 0.20	0.243 ± 0.054	2.54 ± 0.13
	T. harzianum	8.20 ± 0.30	1.67 ± 0.35	2.43 ± 0.21	0.174 ± 0.012	2.05 ± 0.09*
Moneymaker	Control	10.15 ± 0.57	1.77 ± 0.15	1.90 ± 0.14	0.164 ± 0.045	2.30 ± 0.09
	T. harzianum	10.05 ± 0.69	1.32 ± 0.19	2.02 ± 0.34	0.124 ± 0.008	2.66 ± 0.24

The data are the means of six replicates ± SE. For each tomato genotype asterisks indicate statistically significant differences between T. harzianum inoculated and non-inoculated plants (Student's t-test, P < 0.05).

and their interaction ($P < 0.01$; $F = 2.82$), as confirmed by two-way ANOVA analysis. As shown in **Figure 2A** the suppressive effect on *B. cinerea* disease observed in the wild-type Castlemart plants elicited with *T. harzianum* was absent in the JA-deficient *def1* mutant, indicating that JA-regulated pathway is required for TISR against *B. cinerea*. Similarly, the mutant lines impaired in SA (*NahG*) and ABA (*sitiens*) accumulation did not display the TISR against *B. cinerea* observed in their corresponding background Moneymaker (**Figure 2B**). In the transgenic *NahG* line SA-accumulation is blocked through the transformation of SA to catechol. Interestingly, we observed a lower susceptibility of *NahG* control plants toward *B. cinerea* infection compared to its parental wild-type Moneymaker ($P < 0.1$), which support the idea that SA affect negatively basal resistance against this necrotroph in tomato (**Figure 2B**). In contrast to Castlemart and Moneymaker, the wild-type UC82B plants were unable to develop *T. harzianum* ISR (**Figure 2C**). In the ET-underproducing ACC deaminase mutants (*ACD*), *T. harzianum* slightly, but not significantly reduced the pathogen lesion (above 20%). Concerning systemin, plants of the over-expressing mutant line *PS+*, elicited and non-elicited with *T. harzianum* were more resistant to the necrotroph than any other cultivar tested ($P < 0.05$), confirming the involvement of this molecule in tomato basal resistance against *B. cinerea*. Although *T. harzianum*-induced resistance in the wild-type Betterboy, *Trichoderma* colonization could not reduce further *B. cinerea* disease development in *PS+*. Remarkably, *T. harzianum* was also able to induce ISR in the tomato line silenced in prosystemin expression *PS−* (**Figure 2D**).

Trichoderma harzianum EFFECTIVELY COLONIZES THE RHIZOSPHERE AND ROOTS OF WILD-TYPE AND MUTANT TOMATO LINES

The biocontrol effect of *Trichoderma* is associated to its efficient colonization of the rhizosphere. To analyze if the unability of the mutants to mount TISR is related to a deficient *Trichoderma* colonization, we tested the ability of *T. harzianum* to colonize the rhizosphere of the different tomato mutant lines and their correspondent backgrounds. The number of *Trichoderma* colony-forming units (cfu) in the rhizosphere, determined after 5 weeks,

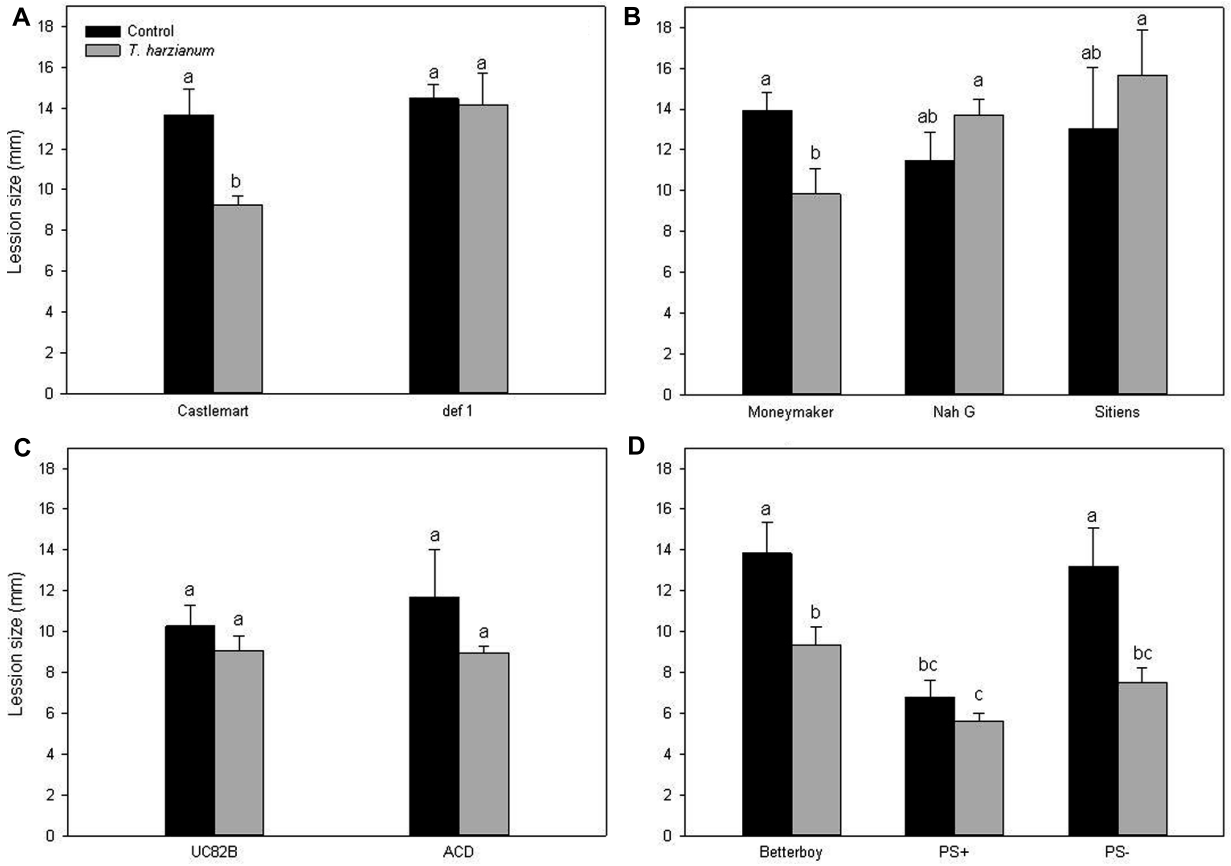


FIGURE 2 | *Trichoderma*-induced systemic resistance requires JA, SA, and ABA. Lesion diameter was measured 96 h after challenge with the pathogen **(A)** the wild-type tomato plants cv. Castlemart and the JA-impaired mutant *def1*; **(B)** in the wild-type cv. Moneymaker and the SA- and ABA-impaired mutants *NahG* and *sitiens*, respectively; **(C)** in the wild-type cv. UC82B and in the ET-impaired mutant *ACD*; and **(D)** in the wild-type cv. Betterboy and in the over-expressing mutant line *PS+* and the prosystemin silenced line *PS−*. The data show the lesion diameter (mm) ± SE ($n = 12$). Data not sharing a letter in common differ significantly ($P < 0.05$) according to Tukey's multiple-range test.

was similar to initial inoculation values in all the tested lines. We did not find significant ($P < 0.05$) differences in cfu numbers in the rhizosphere of the different tomato mutant lines compared to their corresponding genetic backgrounds. Moreover, endophytic colonization was also confirmed for all of the lines. Incubation of surface-sterilized roots under appropriate conditions revealed that *Trichoderma* could outgrow from inside the roots regardless of the plant genotype. The results indicated that the impairment on the production of the hormones JA, SA, ABA, ET, or systemin does not affect *T. harzianum* capacity for rhizosphere and root colonization.

Trichoderma harzianum PRIMES JASMONATE-DEPENDENT DEFENSES

Induced systemic resistance by beneficial microbes is commonly not associated with major changes in defense-related gene expression. Instead, a relatively mild systemic immune reaction is triggered that is frequently associated with priming for enhanced defense. In order to establish whether the enhanced resistance induced by *T. harzianum* in tomato was associated with priming of plant defense, we compared the plant response to *B. cinerea* in *Trichoderma* elicited and not elicited plants. We analyzed by RT-qPCR the expression of known marker genes for the main plant defense-related pathways in *B. cinerea* challenged plants. No significant differences were found for the marker genes of SA- (*PR1a* and *PAL*) or ET- (*gluB*) modulated pathways between *Trichoderma* induced and not induced plants (data not shown). In contrast, an enhanced expression of the JA responsive genes *PI II*, *MC*, and *PS*, coding for proteinase inhibitor II, multicystatin, and prosystemin, respectively, was found in *T. harzianum*-elicited compared to non-elicited plants (**Figure 3A**). Interestingly, *T. harzianum*-inoculated plants showed no or slight induction of those genes in the absence of the pathogen (**Figure 3B**), thus pointing at priming of the JA-dependent defense responses as the mechanism underlying the induction of resistance against *B. cinerea*. *T. harzianum*-colonized plants also displayed higher levels of expression of the ABA responsive marker gene *Le4* (coding for a desiccation protective protein) after pathogen challenge, but a similar increase was observed in *T. harzianum* induced plants in the absence of the pathogen (**Figures 3A,B**).

We further confirmed the priming of the JA-dependent defense responses against *B. cinerea* with the analysis of pathogen

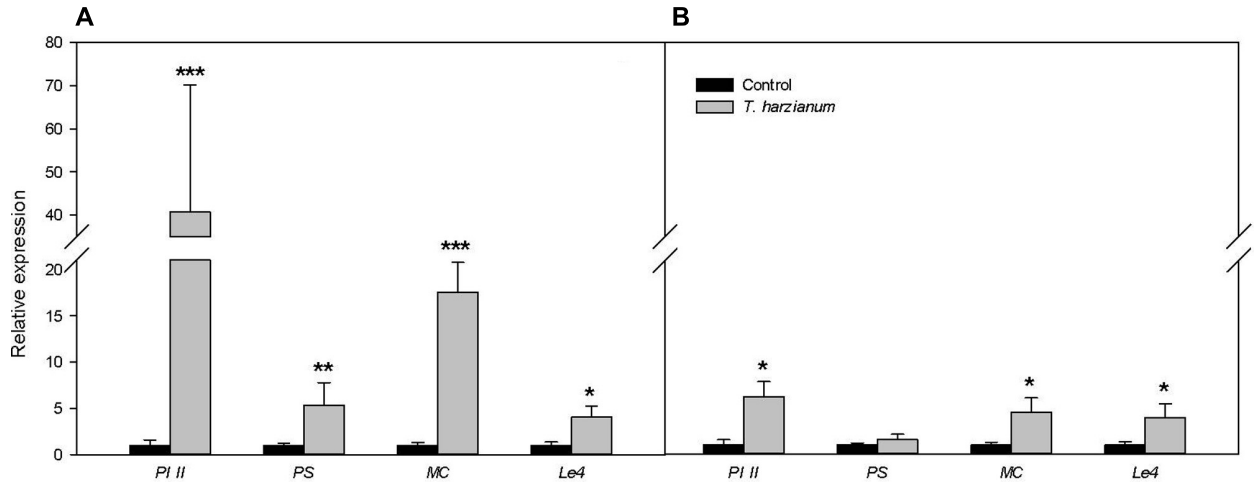


FIGURE 3 | *Trichoderma* primes JA-regulated responses. The expression of different defense-related marker genes was analyzed in *T. harzianum* inoculated and non-inoculated (control) plants (cv. Moneymaker) 96 h upon pathogen infection **(A)** and before infection **(B)**. Expression levels of the JA-related marker genes *PI II*, *MC*, and *PS*; and the ABA-related marker gene *Le4* is shown. The results were normalized to the *SlEF* gene expression levels. The expression levels are reported as the fold increase relative to that of the control plants not treated with *T. harzianum* ± SE ($n = 5$). Asterisks indicate statistically significant differences between *Trichoderma* induced and non-induced plants (Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

proliferation and the induction of JA responses in leaves of the wild-type Castlemart and the JA-deficient *def1*. Expression levels of a *B. cinerea* constitutive gene in leaves confirmed that the differences observed in symptom development (**Figure 2**) were due to differences in pathogen proliferation in the tissues, and confirmed that *def1* plants were unable to develop *Trichoderma*-induced resistance in contrast to the wild-type Castlemart (**Figure 4A**). The inability of *def1* plants to develop TISR correlated with a lack of priming of *PI II* expression (**Figure 4B**) further supporting the essential role of primed JA responses in the enhanced systemic resistance triggered by *Trichoderma*.

DISCUSSION

Selected *Trichoderma* species colonize plant roots and establish symbiotic relationships with the plant. As consequence, plant resistance against pathogens is frequently enhanced, even in aboveground tissues (Segarra et al., 2009; Fontenelle et al., 2011; Perazzolli et al., 2011; Brotman et al., 2012; Yoshioka et al., 2012). In this study we analyzed the effectiveness of *T. harzianum* T-78 root colonization in the enhancement of tomato resistance against the foliar necrotrophic pathogen *B. cinerea*. *T. harzianum* T-78 is an effective biocontrol agent in the soil (Martínez-Medina et al., 2011b), with high mycoparasitic capacity (López-Mondéjar et al.,

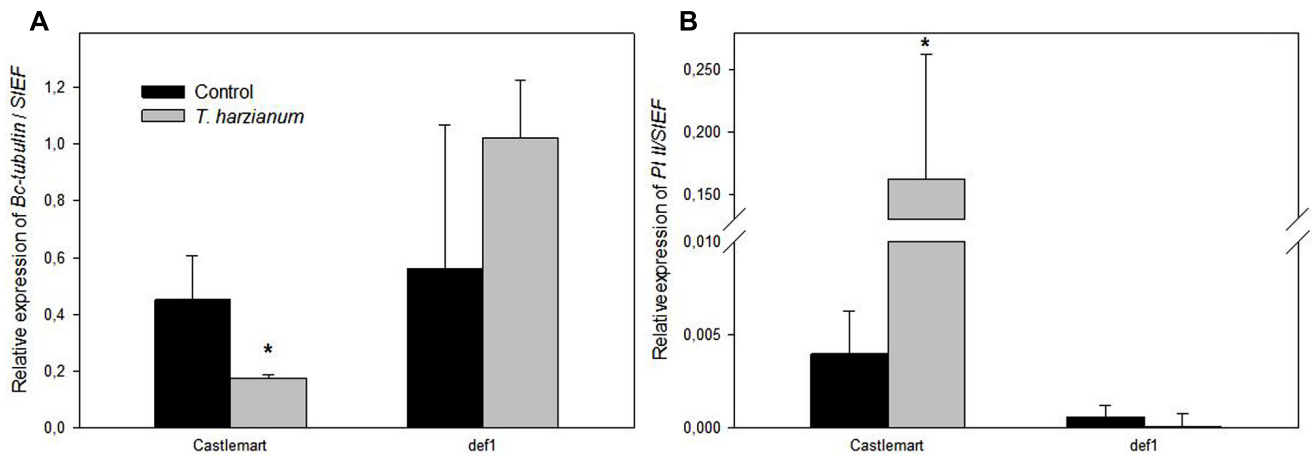


FIGURE 4 | *Trichoderma harzianum* priming of defenses requires JA signaling. The *Botrytis cinerea* constitutive gene *Bc-Tubulin* **(A)**, and the JA-related marker gene *PI II* **(B)** were analyzed in leaves of the wild-type tomato plants cv. Castlemart and the JA-impaired mutant *def1* upon 96 h of *B. cinerea* infection. Results were normalized to the *SlEF* gene expression in the same samples. Data show the relative expression level (±SE). For each tomato genotype asterisks indicate statistically significant differences between *Trichoderma* induced and non-induced plants (Student's *t*-test, $P < 0.05$, $n = 5$).

2011), but its ability to induce plant resistance was not previously tested.

We found that treatment of tomato roots with *T. harzianum* T-78 clearly reduced disease development upon inoculation with the necrotrophic pathogen *B. cinerea* in three out of four cultivars tested (Castlemart, Moneymaker, and Betterboy). We examined the presence of *Trichoderma* isolate T-78 in the shoots and we could not detect its presence in any of the tomato cultivars (data not shown). Therefore, *Trichoderma* and the pathogen remain physically separated, and accordingly, it can be concluded that *T. harzianum* T-78 activates a plant-mediated systemic response that is effective in restricting *B. cinerea* development. The dependence on the plant genotype of TISR against *Botrytis*, also shown for other tomato cultivars (Tucci et al., 2011), further confirms that the protection depends on plant-mediated mechanisms. Other studies have shown the ability of different *Trichoderma* strains to induce a plant-mediated effect against this necrotroph, mostly in *Arabidopsis* (Korolev et al., 2008; Contreras-Cornejo et al., 2011; Mathys et al., 2012) but also in other crop plants (De Meyer et al., 1998; Tucci et al., 2011).

Trichoderma colonization is reported to improve plant nutrition and growth in several plant species (Martínez-Medina et al., 2011b; Salas-Marina et al., 2011; Tucci et al., 2011). Since improved plant nutritional is considered one of the mechanisms responsible for bioprotection against pathogens by beneficial microorganisms (Whipps, 2001) we tried to analyze the contribution of this effect to the enhanced resistance observed. In our experimental conditions there was no increase in plant growth or nutrient content associated to *Trichoderma* colonization, probably because plants were grown under optimal conditions (Martínez-Medina et al., 2011b). Thus, our experimental system allows uncoupling nutritional from defense effects, and it can be concluded therefore that the protective effect observed in *Trichoderma* T-78-inoculated plants was related to mechanisms other than an improved nutrition, most likely related to plant defenses.

As for ISR by selected non-pathogenic rhizobacteria (Van Wees et al., 1999; Verhagen et al., 2004; Pozo et al., 2008), some studies have shown that the systemic resistance triggered by *Trichoderma* requires responsiveness to JA and ET (Shoresh et al., 2005; Segarra et al., 2009; Perazzolli et al., 2011; Tucci et al., 2011). However, phenotypic analysis of disease on *Arabidopsis* signaling mutants revealed that other small-molecule hormones such as SA or ABA could also play pivotal roles in the regulation of this network (Korolev et al., 2008; Mathys et al., 2012; Yoshioka et al., 2012). To determine the main signaling pathways involved in the induced systemic resistance elicited by *T. harzianum* T-78 in tomato against *B. cinerea*, we assessed the ability of different hormone-impaired tomato mutants for TISR development. The phenotypic analysis of disease development in the JA (*def1*)- and SA (*NahG*)-impaired mutants demonstrated that *T. harzianum*-induced systemic resistance against *B. cinerea* requires not only the JA but also the SA signaling pathways, as these mutant lines developed similar level of disease than non-induced control plants. Similarly, a recent study showed a role of the SA-pathway in *T. hamatum* T-382-induced ISR against *B. cinerea* in *Arabidopsis*, as TISR was blocked in the SA-impaired mutants *NahG* and *sid2* (Mathys et al., 2012). In contrast, *Trichoderma asperellum*-induced resistance in *Arabidopsis* against the hemibiotrophic leaf pathogen *Pseudomonas syringae* seems independent of SA, as TISR was fully expressed in the SA-impaired mutant *sid2* (Segarra et al., 2009). Thus experimental evidences support that induced resistance is a flexible process that may involve different signaling pathways depending on the mode of action of the pathogen, as it has been shown for some resistance-inducing chemicals (Flors et al., 2008). Our results demonstrate that in tomato, both SA and JA signaling pathways are required for TISR development against *B. cinerea*. Necrotrophic pathogens are usually controlled by JA-defense responses (Glazebrook, 2005), and JA signaling has been shown as key for basal resistance to *Botrytis* in tomato (AbuQamar et al., 2008; El-Oirdi et al., 2011). It is therefore not surprising the requirement of intact JA-related hormonal signaling pathway for boosted plant defenses against *Botrytis* by *Trichoderma*. The role of the SA signaling in plant resistance against *B. cinerea* is, however, more complex (Ferrari et al., 2003). Recently it has been shown that SA plays a regulatory role in the balance between disease and resistance as *Botrytis* induces SA signaling to promote disease in tomato through its negative interaction with the JA-dependent pathway (El-Oirdi et al., 2011).

In relation to ET, since the wild-type plants UC82B were unable to develop *T. harzianum*-induced ISR, we were unable to determine if ET signaling is required for TISR against *B. cinerea*. In contrast to earlier findings in rhizobacteria-mediated ISR (Pieterse et al., 1998; Knoester et al., 1999), Mathys et al. (2012) observed a limited role of the ET pathway in *T. hamatum* T382-induced resistance. Our results, although inconclusive, are in line with this finding as a reduced disease development on ET-mutants (*ACD*) was observed. It is noticeable that non-induced wild-type UC82B plants showed the lowest susceptibility to *B. cinerea* among all cultivars tested, and likely *T. harzianum* was unable to further boost plant resistance.

Additionally, analysis of the disease development in the ABA-deficient mutant *sitiens* showed that disruption of the ABA signaling results in the loss of ability to develop TISR against *B. cinerea*. Although ABA is commonly associated with plant development and abiotic stress, its role in plant immunity is now clear, as this hormone has been shown to be connected to the SA–JA–ET network (Anderson et al., 2004; Mauch-Mani and Mauch, 2005). The role of ABA in tomato resistance against pathogens is controversial, and indications for both a role in susceptibility and resistance have been given (Flors et al., 2008; Sánchez-Vallet et al., 2012). In tomato, a negative regulatory role of ABA in resistance to *B. cinerea* has been proposed, as the *sitiens* mutant showed reduced susceptibility than wild-type plants (Audenaert et al., 2002; Asselbergh et al., 2007, 2008). In our system *sitiens* plants did not show enhanced basal resistance compared to wild-type plants, indicating that ABA is not a major player in basal resistance, but it is important for *Trichoderma*-induced resistance. In line with these observations, Vicedo et al. (2009) found that ABA-deficient mutants were not affected in basal resistance against *B. cinerea*, but they were impaired in hexanoic acid-mediated protection against this pathogen, also based in primed JA responses.

Finally, systemin has been also shown to play a role in resistance against *B. cinerea* in tomato (Díaz et al., 2002; El-Oirdi et al., 2011). The disease examination in the over-expressing *PS+* mutant line confirmed a role of the polypeptide in the basal resistance against *B. cinerea*, as over-expressing *PS+* mutants were

highly resistant to the necrotroph. Probably because of this high resistance, *Trichoderma* was unable to boost further resistance in this line. Remarkably, the analysis of the line silenced in prosystemin expression *PS*- showed that TISR was fully expressed in the *PS*− mutants suggesting that *T. harzianum*-mediated systemic resistance against *B. cinerea* does not rely on systemin signaling.

Trichoderma effects on plant defenses have been related to the fungal ability for intercellular root colonization (Yedidia et al., 2000; Chacón et al., 2007; Djonovic et al., 2007; Velázquez-Robledo et al., 2011). Successful rhizosphere and root endophytic colonization by *T. harzianum* T-78 was confirmed for all genotypes. Accordingly, the defect in TISR observed in some of the mutants is not related to defects in colonization but to the requirement of the hormone in the regulation of the plant response to the pathogen. The above findings demonstrate that *T. harzianum*-mediated resistance against *B. cinerea* requires the JA-, SA-, and ABA-regulated pathways. Cross-talk between hormonal-related signaling pathways acts as a cost-efficient regulatory mechanism for inducible defense responses (reviewed in Pieterse et al., 2009), and our results suggest that cross-talk between JA, SA, and ABA signaling pathways is essential for the induction of resistance mechanisms by *Trichoderma* T-78 in tomato. Nevertheless, it remains to be determined if additional hormones such as auxin, gibberellin, cytokinin, and brassinosteroids may also contribute to the regulatory network behind *Trichoderma*-induced resistance to *B. cinerea*.

Once key elements in the regulation of the response during TISR were identified, we aim to identify the actual defense responses underlying the resistance in *Trichoderma*-inoculated plants. For that we compared the plant defense response to *Botrytis* infection in *Trichoderma* elicited and not elicited plants through the expression analysis of known defense genes, markers for the main defense-related pathways. *T. harzianum* colonization of the roots resulted in priming of the aboveground plant tissues for enhanced JA-responsive gene expression, as a boosted expression of the JA-regulated marker genes *PI II*, *PS*, and *MC* coding for the proteinase inhibitor II (Farmer and Ryan, 1992), prosystemin, the precursor of the hormone systemin (Farmer and Ryan, 1992) and multicystatin (Girard et al., 2007) was observed in *Trichoderma*-induced plants, upon *B. cinerea* infection. It has been recently reported that the proteinase inhibitor II encoded for *PI II* plays a major role for tomato resistance against *B. cinerea* (El-Oirdi et al., 2011). The induction of those genes in plant shoots by *Trichoderma* was relatively weak before *Botrytis* infection, thus pointing to priming of the JA-dependent defense responses as the mechanism underlying the induction of resistance against *B. cinerea*. Activation of a JA-related priming state in plants by *Trichoderma* has been observed previously in *Arabidopsis*, tomato, and grapevine plants (Segarra et al., 2009; Tucci et al., 2011; Brotman et al., 2012; Perazzolli et al., 2012) with no obvious costs for the plant. Indeed, priming by beneficial microorganisms offers broad-spectrum

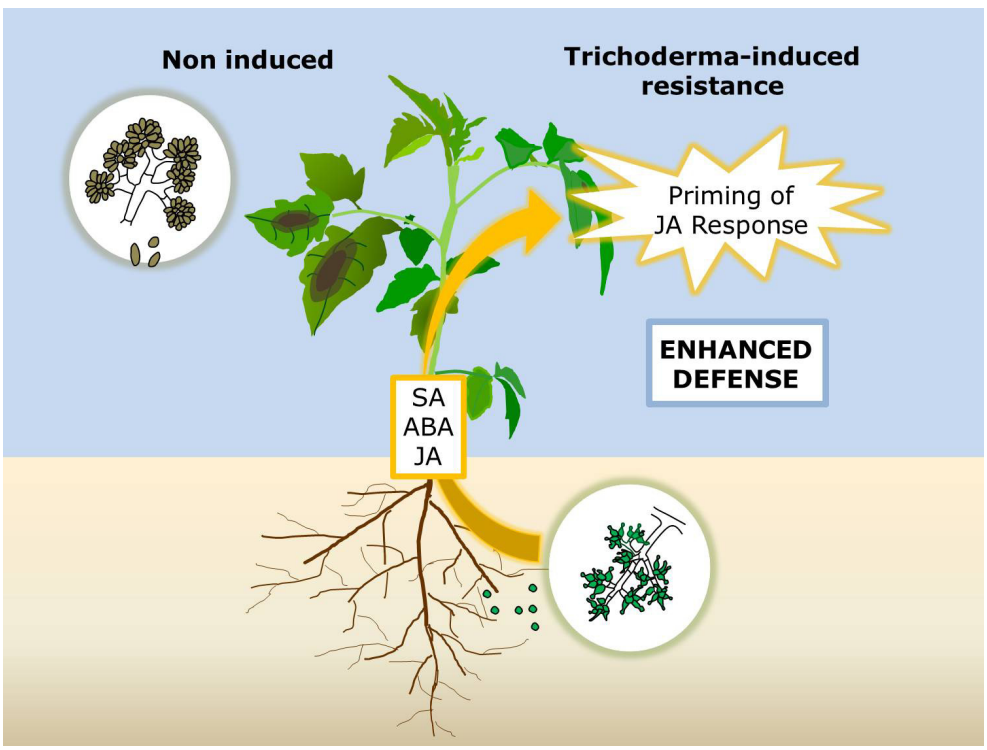


FIGURE 5 | Model for *Trichoderma*-induced resistance (TISR) against *Botrytis cinerea* in tomato. Root colonization with *Trichoderma* primes leaf tissues for enhanced activation of JA-regulated defense responses leading to a higher resistance to the necrotroph. Intact JA, SA, and ABA signaling pathways are required for TISR development.

protection whenever required (Van der Ent et al., 2009), without significant energy costs to plant metabolism and growth (Walters and Heil, 2007; Van Wees et al., 2008). *Trichoderma* elicitation, however, did not boost SA- or ET-related defense responses against *B. cinerea*, as no variation in the SA-marker genes *PR1a* and *PAL* nor in the ET regulated *gluB* were found in our study, in contrast to earlier studies (Yedidia et al., 2003; Shoresh et al., 2005). Nevertheless, as resistance to *B. cinerea* is JA-dependent (AbuQamar et al., 2008) and SA signaling is the target of the pathogen to interfere with JA-signaling and promote disease (El-Oirdi et al., 2011), priming of SA responses in this interaction would be detrimental for the plant. Notably, *Trichoderma* inoculation induced the expression of the ABA-marker gene *Le4* before *B. cinerea* infection, suggesting a moderate direct activation of ABA-signaling that could participate in the defense against the pathogen.

The identification of primed JA responses as the control mechanism underlying TISR in tomato- *B. cinerea* pathosystem was further corroborated in the JA-impaired mutant *def1* through the quantification of pathogen biomass and the induction of plant defenses. The failure of *def1* plants to develop TISR correlated with a lack of priming for *PI II* expression. These results confirm the essential role of the boosted expression of JA responses in the enhancement of resistance by *Trichoderma*against *B. cinerea.*

In summary, this study provides evidence that *T. harzianum* induces systemic resistance against *B. cinerea* in tomato through a boosted JA-dependent defense response, which reduce pathogen proliferation and disease development in plant leaves. The regulation of the response requires not only JA but also at least SA and ABA signaling (**Figure 5**). All in all, our results support the consistent central role of JA in the induction of resistance by different *Trichoderma* strains, and illustrate the requirement of other signaling pathways probably shaping the final response adapted to the challenging pathogen.

ACKNOWLEDGMENTS

We thank Dr. Saskia Van Wees and Dr. Juan A. López Ráez for useful comments on the manuscript, and Juan M. García for technical assistance. This research was supported by grants AGL2009-07691 from the Spanish Ministry of Science and Technology and by the CYCIT project AGL2010 21073 from the Spanish Ministry of Economy and Competitiveness.

REFERENCES

AbuQamar, S., Chai, M.-F., Luo, H., Song, F., and Mengiste, T. (2008). Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. *Plant Cell* 20, 1964–1983. doi: 10.1105/tpc.108.059477

Alfano, G., Lewis Ivey, M. L., Cakir, C., Bos, J. I. B., Miller, S. A., Madden, L. V., et al. (2007). Systemic modulation of gene expression in tomato by *Trichoderma hamatum* 382. *Phytopathology* 97, 429–437. doi: 10.1094/PHYTO-97-4-0429

Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16, 3460–3479. doi: 10.1105/tpc.104.025833

Asselbergh, B., Achuo, A. E., Höfte, M., and Van Gijsegem, F. (2008). Abscisic acid deficiency leads to rapid activation of tomato defence responses upon infection with *Erwinia chrysanthemi*. *Mol. Plant Pathol.* 9, 11–24. doi: 10.1111/j.1364-3703.2007.00437.x

Asselbergh, B., Curvers, K., França, S. C., Audenaert, K., Vuylsteke, M., Van Breusegem, F., et al. (2007). Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiol.*144, 1863–1877.

Audenaert, K., De Meyer, G. B., and Höfte, M. M. (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. *Plant Physiol.* 128, 491–501. doi: 10.1104/pp.010605

Bailey, B. A., Bae, H., Strem, M. D., Roberts, D. P., Thomas, S. E., Crozier, J., et al. (2006). Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta* 224, 1449–1464. doi: 10.1007/s00425-006-0314-0

Brading, P. A., Hammond-Kosack, K. E., Parr, A., and Jones, J. D. G. (2000). Salicylic acid is not required for cf-2- and cf-9-dependent resistance of tomato to *Cladosporium fulvum*. *Plant J.* 23, 305–318. doi: 10.1046/j.1365-313x.2000.00778.x

Brotman, Y., Lisec, J., Méret, M., Chet, I., Willmitzer, L., and Viterbo, A. (2012). Transcript and metabolite analysis of the *Trichoderma*-induced systemic resistance response to *Pseudomonas syringae* in *Arabidopsis thaliana*. *Microbiology* 158, 139–146. doi: 10.1099/mic.0.052621-0.

Brouwer, M., Lievens, B., Van Hemelrijck, W., Van Den Ackerveken, G., Cammue, B. P. A., and Thomma, B. P. H. J. (2003). Quantification of disease progression of several microbial pathogens on *Arabidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiol. Lett.* 228, 241–248. doi: 10.1016/S0378-1097(03)00759-6

Chacón, M. R., Rodriguez-Galan, O., Benítez, T., Sousa, S., Rey, M., Llobell, A., et al. (2007). Microscopic and transcriptome analyses of early colonization of tomato roots by *Trichoderma harzianum*. *Int. Microbiol.* 10, 19–27.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., et al. (2006). Priming: getting ready for battle. *Mol. Plant Microbe Interact.* 19, 1062–1071. doi: 10.1094/MPMI-19-1062

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Beltrán-Peña, E., Herrera-Estrella, A., and López-Bucio, J. (2011). *Trichoderma*-induced plant immunity likely involves both hormonal- and camalexin dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungus *Botrytis cinerea*. *Plant Signal.Behav.* 6, 1554–1563. doi: 10.4161/psb.6.10.17443.

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., et al. (2012). The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.*13, 414–430. doi: 10.1111/j.1364-3703.2011.0783.x

De Meyer, G., Bigirimana, J., Elad, Y., and Höfte, M. (1998). Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *Eur. J. Plant Pathol.*104, 279–286. doi: 10.1023/A:1008628806616

Díaz, J., Ten Have, A., and van Kan, J. A. L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. *Plant Physiol.* 129, 1341–1351. doi: 10.1104/pp.001453

Djonovic, S., Pozo, M. J., Dangott, L. J., Howell, C. R., and Kenerley, C. M. (2006). Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. *Mol. Plant Microbe Interact.* 19, 838–853. doi: 10.1094/MPMI-19-0838

Djonovic, S., Vargas, W. A., Kolomiets, M. V., Horndeski, M., Wiest, A., and Kenerley, C. M. (2007). A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiol.* 145, 875–899. doi: 10.1104/pp.107.103689

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

El-Oirdi, M., El-Rahman, T. A., Rigano, L., El-Hadrami, A., Rodriguez, M. C., Daayf, F., et al. (2011). *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* 23, 2405–2421. doi: 10.1105/tpc.111.083394

Farmer, E. E., and Ryan, C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4, 129–134. doi: 10.1105/tpc.4.2.129

Ferrari, S., Plotnikova, J. M., De Lorenzo, G., and Ausubel, F. M. (2003). *Arabidopsis* local resistance

to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J.* 35, 193–205. doi: 10.1046/j.1365-313X.2003.01794.x

Flors, V., Ton, J., Van Doorn, R., Jakab, G., García-Agustín, P., and Mauch-Mani, B. (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. *Plant J.* 54, 81–92. doi: 10.1111/j.1365-313X.2007.03397.x

Fontenelle, A. D. B., Guzzo, S. D., Lucon, C. M. M., and Harakava, R. (2011). Growth promotion and induction of resistance in tomato plant against *Xanthomonas euvesicatoria* and *Alternaria solani* by *Trichoderma* spp. *Crop Prot.* 30, 1492–1500. doi: 10.1016/j.cropro.2011.07.019

Girard, C., Rivard, D., Kiggundu, A., Kunert, K., Gleddie, S. C., Cloutier, C., et al. (2007). A multicomponent, elicitor-inducible cystatin complex in tomato, *Solanum lycopersicum*. *New Phytol.*173, 841–851. doi: 10.1111/j.1469-8137.2007.01968.x

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Hanson, L. E., and Howell, C. R. (2004). Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. *Phytopathology* 94, 171–176. doi: 10.1094/PHYTO.2004.94.2.171

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Hewitt, E. J. (1966). *Sand and Water Culture Methods Used in the Study of Plant Nutrition. Technical communication no. 22.* London: Commonwealth Agriculture Bureau. doi: 10.1007/s00294-006-0091-0

Howe, G. A., Lightner, J., Browse, J., and Ryan, C. A. (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell* 8, 2067–2077. doi: 10.1105/tpc.8.11.2067

Klee, H. J., Hayford, M. B., Kretzmer, K. A., Barry, G. E., and Kishore, G. M. (1991). Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. *Plant Cell* 3, 1187–1193. doi: 10.1105/tpc.3.11.1187

Knoester, M., Pieterse, C. M. J., Bol, J. F., and Van Loon, L. C. (1999). Systemic resistance in *Arabidopsis* induced by Rhizobacteria requires ethylene-dependent signaling at the site of application. *Mol. Plant Microbe Interact.* 12, 720–727. doi: 10.1094/MPMI.1999.12.8.720

Korolev, N., Rav David, D., and Elad, Y. (2008). The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Biocontrol* 53, 667–683. doi: 10.1007/s10526-007-9103-3

López-Mondéjar, R., Ros, M., and Pascual, J. A. (2011). Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. *Biol. Control* 56, 59–66. doi: 10.1073/pnas.91.21.9799

López-Ráez, J. A., Verhage, A., Fernández, I., García, J. M., Azcón-Aguilar, C., Flors, V., et al. (2010). Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *J. Exp. Bot.* 61, 2589–2601. doi: 10.1093/jxb/erq089

Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., et al. (2006). Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. *Curr. Genet.* 50, 307–321. doi: 10.1007/s00294-006-0091-0

Martínez-Medina, A., Pascual, J. A., Pérez-Alfocea, F., Albacete, A., and Roldán, A. (2010). *Trichoderma harzianum* and *Glomus intraradices* modify the hormone disruption induced by *Fusarium oxysporum* infection in melon plants. *Phytopathology* 100, 682–688. doi: 10.1094/PHYTO-100-7-0682

Martínez-Medina, A., Roldán, A., Albacete, A., and Pascual, J. A. (2011a). The interaction with arbuscular mycorrhizal fungi or *Trichoderma harzianum* alters the shoot hormonal profile in melon plants. *Phytochemistry*72, 223–229. doi: 10.1016/j.phytochem.2010.11.008

Martínez-Medina, A., Roldán, A., and Pascual, J. A. (2011b). Interaction between arbuscular mycorrhizal fungi and *Trichoderma harzianum* under conventional and low input fertilization field condition in melon crops: growth response and Fusarium wilt biocontrol. *Appl. Soil Ecol.* 47, 98–105. doi: 10.1016/j.apsoil.2010.11.010

Martínez-Medina, A., Roldán, A., and Pascual, J. A. (2009). Performance of a *Trichoderma harzianum* bentonite–vermiculite formulation against Fusarium wilt in seedling nursery melon plants. *HortScience* 44, 2025–2027. doi: 10.1016/j.phytochem.2010.11.008

Masunaka, A., Hyakumachi, M., and Takenaka, S. (2011). Plant growth-promoting fungus, *Trichoderma koningi* suppresses isoflavonoid phytoalexin vestitol production for colonization on/in the roots of *Lotus japonicus*. *Microbes Environ.* 26, 128–134. doi: 10.1264/jsme2.ME10176

Mathys, J., De Cremer, K., Timmermans, P., Van Kerckhove, S., Lievens, B.,Vanhaecke, M., et al. (2012). Genome-wide characterization of ISR induced in *Arabidopsis thaliana* by *Trichoderma hamatum* T382 against *Botrytis cinerea* infection. *Front. Plant Sci.* 3:108. doi: 10.3389/fpls.2012.00108

Mauch-Mani, B., and Mauch, F. (2005). The role of abscisic acid in plant-pathogen interactions. *Curr.Opin. Plant Biol.* 8, 409–414. doi: 10.1016/j.pbi.2005.05.015

McGurl, B., Orozco-Cardenas, M., Pearce, G., and Ryan, C. A. (1994). Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 91, 9799–9802. doi: 10.1073/pnas.91.21.9799

Morán-Diez, E., Rubio, B., Domínguez, S., Hermosa, R., Monte, E., and Nicolás, C. (2012). Transcriptomic response of *Arabidopsis thaliana* after 24h incubation with the biocontrol fungus *Trichoderma harzianum*. *J. Plant Physiol.* 169, 614–620. doi: 10.1016/j.jplp.2011.12.016

Orozco-Cardenas, M., McGurl, B., and Ryan, C. A. (1993). Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8273–8276. doi: 10.1073/pnas.90.17.8273

Perazzolli, M., Moretto, M., Fontana, P., Ferrarini, A., Velasco, R., Moser, C., et al. (2012). Downy mildew resistance induced by *Trichoderma harzianum* T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genomics* 13:660. doi: 10.1186/1471-2164-13-660

Perazzolli, M., Roatti, B., Bozza, E., and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. *Biol. Control* 58, 74–82. doi: 10.1016/j.biocontrol.2011.04.006

Petutschnig, E. K., Jones, A. M. E., Serazetdinova, L., Lipka, U., and Lipka, V. (2010). The Lysin Motif Receptor-Like Kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J. Biol. Chem.* 285, 28902–28911. doi: 10.1074/jbc.P110.116657

Pieterse, C. M. J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. M. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Pieterse, C. M. J., Van Wees, S. C. M., Hoffland, E., Van Pelt, J. A., and Van Loon, L. C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8, 1225–1237. doi: 10.1016/j.pbi.2008.05.005

Pieterse, C. M. J., Van Wees, S. C. M., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., et al. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* 10, 1571–1580. doi: 10.1105/tpc.10.9.1571

Pozo, M. J., Van Der Ent, S., Van Loon, L. C., and Pieterse, C. M. J. (2008). Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytol.* 180, 511–523. doi: 10.1111/j.1469-8137.2008.02578.x

Ron, M., and Avni, A. (2004). The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 16, 1604–1615. doi: 10.1105/tpc.022475

Rotenberg, D., Thompson, T. S., German, T. L., and Willis, D. K. (2006). Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *J. Virol. Methods* 138, 49–59. doi: 10.1016/j.jviromet.2006.07.017

Salas-Marina, M. A., Silva-Flores, M. A., Uresti-Rivera, E. E., Castro-Longoria, E., Harrera-Estrella, A., and Casas-Flores, S. (2011). Colonization of *Arabidopsis* roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonate and salicylate pathways. *Eur. J. Plant Pathol.* 131, 15–26. doi: 10.1007/s10658-011-9782-6

Sánchez-Vallet, A., López, G., Ramos, B., Delgado-Cerezo, M., Riviere, M. P., Llorente, F., et al. (2012). Disruption of abscisic acid signaling constitutively activates *Arabidopsis* resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant Physiol.* 160, 2109–2124. doi: 10.1016/S0981-9428(00)01198-0

Segarra, G., Van der Ent, S., Trillas, I., and Pieterse, C. M. J. (2009). MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biol.* 11, 90–96. doi: 10.1111/j.1438-8677.2008.00162.x

Shoresh, M., and Harman, G. E. (2008). The relationship between increased growth and resistance induced in plants by root colonizing microbes. *Plant Signal. Behav.* 3, 737–739. doi: 10.4161/psb.3.9.6605

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi: 10.1146/annurev-phyto-073009-114450

Shoresh, M., Yedidia, I., and Chet, I. (2005). Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology* 95, 76–84. doi: 10.1094/PHYTO-95-0076

Taylor, I. B., Linforth, R. S. T., Al-Naieb, R. J., Bowman, W. R., and Marples, B. A. (1988). The wilty tomato *mutants flacca* and sitiens are impaired in the oxidation of ABA-aldehyde to ABA. *Plant Cell Environ.* 11, 739–745. doi: 10.1111/j.1365-3040.1988.tb01158.x

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Uppalapati, S. R., Ayoubi, P., Weng, H., Palmer, D. A., Mitchell, R. E., Jones, W., et al. (2005). The phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant J.* 42, 201–217. doi: 10.1111/j.1365-313X.2005.02366.x

Van der Ent, S., Van Wees, S. C. M., and Pieterse, C. M. J. (2009). Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* 70, 1581–1588. doi: 10.1016/j.phytochem.2009.06.009

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483. doi: 10.1146/annurev.phyto.36.1.453

Van Wees, S. C. M., Luijendijk, M., Smoorenburg, I., Van Loon, L. C., and Pieterse, C. M. J. (1999). Rhizobacteria-mediated induced systemic resistance (ISR) Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. *Plant Mol. Biol.* 41, 537–549. doi: 10.1023/A:1006319216982

Van Wees, S. C. M., Van der Ent, S., and Pieterse, C. M. J. (2008). Plant immune responses triggered by beneficial microbes. *Curr.Opin. Plant Biol.* 11, 443–448 doi: 10.1016/j.pbi.2008.05.005

Velázquez-Robledo, R., Contreras-Cornejo, H. A., Macías-Rodríguez, L., Hernández-Morales, A., Aguirre, J., Casas-Flores, S., et al. (2011). Role of the 4-phosphopantetheinyl transferase of *Trichoderma virens* in secondary metabolism and induction of plant defense responses. *Mol. Plant Microbe Interact.* 24, 1459–1471.

Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H. S., Van Loon, L. C., and Pieterse, C. M. J. (2004). The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Mol. Plant Microbe Interact.*17, 895–908. doi: 10.1094/MPMI.2004.17.8.895

Vicedo, B., Flors, V., Leyva, M. D. O., Finiti, I., Kravchuk, Z., Real, M. D., et al. (2009). Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. *Mol. Plant Microbe Interact.* 22, 1455–1465. doi: 10.1094/MPMI-22-11-1455

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. (2008). *Trichoderma*-plant-pathogen interactions. *Soil Biol. Biochem.* 40, 1–10. doi: 10.1016/j.soilbio.2007.07.002

Viterbo, A., Wiest, A., Brotman, Y., Chet, I., and Kenerley, C. (2007). The 18merpeptaibols from *Trichoderma virens* elicit plant defense responses. *Mol. Plant Pathol.* 8, 737–746. doi: 10.1111/j.1364-3703.2007.00430.x

Walters, D., and Heil, M. (2007). Costs and trade-offs associated with induced resistance. *Physiol. Mol. Plant Pathol.*, 71, 3–17. doi: 10.1016/j.pmpp.2007.09.008

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511. doi: 10.1093/jexbot/52.suppl_1.487

Yedidia, I., Benhamou, N., and Chet, I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* 65, 1061–1070.

Yedidia, I., Benhamou, N., Kapulnik, Y., and Chet, I. (2000). Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. *Plant Physiol. Biochem.* 38, 863–873. doi: 10.1016/S0981-9428(00)01198-0

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y., and Chet, I. (2003). Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Appl. Environ. Microbiol.* 69, 7343–7353. doi: 10.1128/AEM.69.12.7343-7353.2003

Yoshioka, Y., Ichikawa, H., Naznin, H. A., Kogure, A., and Hyakumachi, M. (2012). Systemic resistance induced in *Arabidopsis thaliana* by *Trichoderma asperellum* SKT-1, a microbial pesticide of seedborne diseases of rice. *Pest Manag. Sci.* 68, 60–66. doi: 10.1002/ps.2220

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 March 2013; accepted: 31 May 2013; published online: 24 June 2013.

*Citation: Martínez-Medina A, Fernández I, Sánchez-Guzmán MJ, Jung SC, Pascual JA and Pozo MJ (2013) Deciphering the hormonal signaling network behind the systemic resistance induced by Trichoderma harzianum in tomato. Front. Plant Sci. **4**:206. doi: 10.3389/fpls.2013.00206*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Martínez-Medina, Fernández, Sánchez-Guzmán, Jung, Pascual and Pozo. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 30 July 2013
doi: 10.3389/fpls.2013.00258

Investigating the beneficial traits of *Trichoderma hamatum* GD12 for sustainable agriculture—insights from genomics

David J. Studholme[1], Beverley Harris[1], Kate Le Cocq[1], Rebecca Winsbury[1], Venura Perera[1], Lauren Ryder[1], Jane L. Ward[2], Michael H. Beale[2], Chris R. Thornton[1] and Murray Grant[1]*

[1] Biosciences, Molecular Plant Pathology, College of Life and Environmental Sciences, University of Exeter, Exeter, UK
[2] Plant Biology and Crop Science, Rothamsted Research, Harpenden, UK

Edited by:
Corné M. J. Pieterse, Utrecht University, Netherlands

Reviewed by:
Bruno Cammue, Katholieke Universiteit Leuven, Belgium
Juan A. Lopez Raez, Consejo Superior de Investigaciones Científicas, Spain

***Correspondence:**
Murray Grant, Biosciences, College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope Building, Stocker Road, Exeter EX4 4QD, UK
e-mail: m.r.grant@exeter.ac.uk

Trichoderma hamatum strain GD12 is unique in that it can promote plant growth, activate biocontrol against pre- and post-emergence soil pathogens and can induce systemic resistance to foliar pathogens. This study extends previous work in lettuce to demonstrate that GD12 can confer beneficial agronomic traits to other plants, providing examples of plant growth promotion in the model dicot, *Arabidopsis thaliana* and induced foliar resistance to *Magnaporthe oryzae* in the model monocot rice. We further characterize the lettuce-*T. hamatum* interaction to show that bran extracts from GD12 and an *N*-acetyl-β-D-glucosamindase-deficient mutant differentially promote growth in a concentration dependent manner, and these differences correlate with differences in the small molecule secretome. We show that GD12 mycoparasitises a range of isolates of the pre-emergence soil pathogen *Sclerotinia sclerotiorum* and that this interaction induces a further increase in plant growth promotion above that conferred by GD12. To understand the genetic potential encoded by *T. hamatum* GD12 and to facilitate its use as a model beneficial organism to study plant growth promotion, induced systemic resistance and mycoparasitism we present *de novo* genome sequence data. We compare GD12 with other published *Trichoderma* genomes and show that *T. hamatum* GD12 contains unique genomic regions with the potential to encode novel bioactive metabolites that may contribute to GD12's agrochemically important traits.

Keywords: *Trichoderma hamatum*, comparative genomics, secretome, plant growth promotion, induced systemic resistance

INTRODUCTION

With the global population estimated to reach 9 billion by 2050, current plant breeding approaches alone will not support the increased demand for food. There is an urgent need to investigate alternative, sustainable approaches to enhance agricultural production. Additional pressures on food production such as existing and emerging pathogens (Anderson et al., 2004; Fisher et al., 2012), soil erosion (Montgomery, 2007), reduced water and nutrient availability (Sauer et al., 2010; Powlson et al., 2011), climate change (Schmidhuber and Tubiello, 2007) and competition for available land from other sectors such as house building and biofuels (Harvey and Pilgrim, 2011), will add further pressure on agricultural systems to maximize crop productivity. Moreover, hazard-based criteria for assessing pesticides could lead to a range of agrochemicals being withdrawn from European markets, leading to the potential loss of the only effective fungicide groups against major crop diseases.

These challenges have lead to research into alternative sustainable agricultural strategies, with a strong focus on exploiting beneficial organisms. Members of the fungal genus *Trichoderma* have the potential for reducing existing dependence on the use of environmentally damaging and unsustainable chemicals required for disease control and fertilizers (Fantke et al., 2012), by providing an opportunity to sustainably improve crop productivity while reducing the likelihood of development of fungicide resistant pathogens.

Trichoderma is a member of the Ascomycota, the largest group of fungi. Asexual reproduction occurs through the production and germination of asexual conidia (Steyaert et al., 2010) and in some species of *Trichoderma*, sexual teleomorphic stages (*Hypocrea* spp.) have been identified (Seidl et al., 2009), although *Trichoderma* is now the accepted holomorph nomenclature (International Botanical Congress, 2011). *Trichoderma* has been exploited in many industries including paper, textile, biofuel and agriculture due to its prolific secretion of degrading enzymes and biocontrol activities (Pere et al., 2001; Miettinen-Oinonen and Suominen, 2002; Chaverri et al., 2003; Giraldo et al., 2007; Kuhad et al., 2011).

Biocontrol encompasses a variety of mechanisms working singularly or synergistically during the interaction between a biological control agent, plant pathogen and plant to achieve effective disease control (Howell, 2003). These mechanisms can be either indirect, via competition for nutrients and space, antibiosis and stimulation of plant-defense mechanisms or direct mycoparasitism, or they can be a combination of both. Mycoparasitism involves direct antagonism of soil-borne pathogens by a combination of enzymatic lysis through secretion of chitinases, glucanases, proteases, antibiotic production, and competition for

space and substrates (Harman, 2006; Lorito et al., 2010). Since the 1930's, *Trichoderma*'s mycoparasitic biocontrol activities have been extensively used in agriculture. Research has focussed predominately on *Trichoderma virens, T. atroviride, T. asperelloides*, *T. asperellum* and *T. harzianum* (Howell, 2003; Benitez et al., 2004). However, mycoparasitism is widespread. More than 1100 *Trichoderma* strains from 75 molecularly defined species displayed mycoparasitic potential against the pathogens *Alternaria alternata, Botrytis cinerea* and *Sclerotinia sclerotiorum* [reported in Druzhinina et al. (2011)]. Yet, despite its agronomic importance, our current knowledge about the mechanistic basis for mycoparasitism is rudimentary.

Certain *Trichoderma* strains have been shown to stimulate plant growth through the production of plant-growth-promoting (PGP) compounds (Chang et al., 1986; Ousley et al., 1994; Contreras-Cornejo et al., 2009; Vinale et al., 2009) although both biological control and PGP traits are rarely found together. Often, PGP is unpredictable and is influenced by environmental factors (Maplestone et al., 1991; Ousley et al., 1993). The mechanisms for PGP are thought to variously arise from direct effects on plants, decreased activity of microflora and inactivated toxic compounds in the root zone (Harman et al., 2004). *Trichoderma* species can also ameliorate a wide range of abiotic stresses such as salinity, temperature and drought; they can improve photosynthetic efficiency, enhance nutrient uptake and significantly increase nitrogen use efficiency in crops. These are all attributes that can contribute to enhanced PGP characteristics often evident upon inoculation (Harman et al., 2004; Djonovic et al., 2006; Bae et al., 2009; Shoresh et al., 2010). Strains stimulate PGP through the production of, yet to be defined, PG compounds (Contreras-Cornejo et al., 2009; Vinale et al., 2009; Ryder et al., 2012), most likely through a combination of one or more of the remarkably diverse array of secondary metabolites and proteins such as pyrones, peptaibols, and terpenes (Lorito et al., 2010) that *Trichoderma* produces.

In addition to mycoparasitism and PGP, some *Trichoderma* strains can induce broad spectrum systemic resistance (ISR) in leaves (Shoresh et al., 2010). Generally it is accepted that, in agricultural systems, the activation of defense responses generates a "trade-off" in terms of reduced growth or enhanced susceptibility to other stresses (Heidel et al., 2004; van Hulten et al., 2006). Remarkably, however, *Trichoderma* inoculation can ameliorate these traditionally perceived "costs" suggesting that it can locally suppress MAMP (Microbe Associated Molecular Pattern) triggered immunity (MTI) and systemically activate or prime induced plant immunity. Suppression of MTI has been recently demonstrated for the plant growth promoting rhizobacterium (PGPR) *Pseudomonas fluorescens* strain WCS417r, which grows endophytically or on root surfaces (Millet et al., 2010).

Modifications of hormonal balance by host or microbe are key drivers in determining the outcome of plant-pathogen interactions, including suppression of MTI (Grant and Jones, 2009). ISR induced by *P. fluorescens* WCS417r is mediated through jasmonic acid/ethylene (JA/ET) signaling (Ton et al., 2002; Santner and Estelle, 2007). Evidence for engagement of specific hormone signaling in *Trichoderma* ISR is often contradictory, with various *Trichoderma* strains activating ISR through different signaling modules. The ISR mediated by *T. asperellum* T34 appears to parallel the JA/ET-based "priming" events observed for *P. fluorescens* WCS417r, resulting in enhanced resistance to obligate biotrophs, hemi-biotrophs and necrotrophs (Segarra et al., 2009). By contrast, maize inoculated with *T. harzianum* T22 showed constitutive expression of some PR proteins in the absence of a pathogen. In melon, *T. harzianum* can control *Fusarium* wilt through induction of basal resistance and the attenuation of hormonal disruption of abscisic acid (ABA), salicylic acid (SA) and ET signaling that *Fusarium oxysporum* induces (Martinez-Medina et al., 2010, 2011). Recently, ISR, induced by *T. hamatum* T382 against *Botrytis cinerea* in *A. thaliana* was reported to involve both an initial priming and a post-infection response (Mathys et al., 2012). Thus, current knowledge suggests that induction of ISR depends upon the specific strain. Host genotype also contributes since genetic variability between tomato lines determines the outcome of PGP and biocontrol interactions with *T. atroviride* and *T. harzianum* (Tucci et al., 2011).

Trichoderma hamatum is a naturally occurring rhizosphere dwelling member of the genus which has attracted academic and industrial interest due to its ability to increase plant biomass and its potential as a biological control agent (Chet et al., 1981; Elad, 2000; Harman, 2006). A previously described strain of *Trichoderma hamatum* (GD12) isolated from soil in Devon, UK, promotes plant growth in low pH, nutrient poor peat and displays biological protection against pre- and post-emergence diseases of lettuce seedlings caused, respectively, by *Sclerotinia sclerotiorum* and *Rhizoctonia solani,* under the same conditions (Thornton, 2005, 2008; Ryder et al., 2012). While plant growth promotion and biocontrol by *Trichoderma* have been well-documented, both traits rarely occur together (Contreras-Cornejo et al., 2009; Vinale et al., 2009). Whole genome sequences are becoming increasingly available, with the industrial strain *T. reesei*, and biological control strains *T. atroviride, T. harzianum*, *T. virens, T. longibrachiatum, T. citrinoviride, T. asperellum* now accessible in public repositories (http://tinyurl.com/trichoderma). Genome comparisons between the mycoparasitic *T. atroviride* and *T. virens* species vs. the saprophytic *T. reesei* identified components predicted to contribute to a parasitic lifestyle and a genome reduction in *T. reesei* (Kubicek et al., 2011). Availability of the genome sequence of GD12 would provide a valuable insight into the genetic potential underlying these important agronomic traits.

Here we present further characterization of the dual PGP and biocontrol strain *T. hamatum* GD12. To demonstrate the broad utility of GD12 as an experimental system we extend previous work to show (i) GD12 induced PGP of *Arabidopsis thaliana*, (ii) GD12 mycoparasitized isolates of the pre-emergent soil pathogen, *Sclerotinia sclerotiorum* and this antagonistic interaction resulted in further enhanced lettuce PGP, (iii) PGP of lettuce by sterile bran extracts from GD12 which is further enhanced by extracts from the GD12 *N*-acetyl-β-D-glucosaminidase deficiency mutant, (iv) clear differences in GD12 and the *N*-acetyl-β-D-glucosaminidase deficient mutant secretome fingerprint which may account for the difference in biocontrol and PGP and (v) induction of induced systemic resistance in rice to rice blast by both GD12 and the *N*-acetyl-β-D-glucosaminidase deficiency. To provide genomic resource to predict important components

involved in PGP and biological control fitness of *T. hamatum* GD12 we undertook whole genome sequencing of this strain and compared it to sequenced *Trichoderma* strains (*T. atroviride. T. harzianum* and *T. virens)*. This work revealed substantial differences between strains which allowed us to identify genomic regions/clusters unique to GD12 that can be further studied to gain a more comprehensive understanding of genetic basis for PGP, biocontrol against pre- and post-emergence soil pathogens and induced systemic resistance to foliar pathogens. In sum, this study provides a foundation for further dissection of GD12's ability to promote beneficial attributes.

MATERIALS AND METHODS

PLANT GROWTH PROMOTION AND BIOLOGICAL CONTROL ASSAYS

Peat microcosms

One litre of sieved sphagnum moss peat (Shamrock, Scotts Professional, UK) was mixed with 400 ml dH_2O and sterilized by autoclaving. Twenty-five lettuce seeds (*Lactuca sativa* cultivar Webb's Wonderful) were sown into triplicate 120 mm × 120 mm × 12 mm square plastic culture dishes (Greiner, Bio-One, UK) each containing 100 g sterile peat. For plant growth promotion and biological control assays, microcosms 300 g of peat was supplemented with: 8 g *T. hamatum* bran inoculum, *Sclerotinia* poppy seed inoculum or both (Ryder et al., 2012). Microcosms were maintained at 24°C under a 16 h light 8 h dark cycle at 90% humidity. Following the removal of lids after 48 h, microcosms were watered daily with sterile dH_2O. After 21 days, plants were harvested, washed and oven dried (75°C) to a constant weight. Shoot and root fresh and dry weights were determined and the data analysed by using ANOVA and *t*-tests.

Bran extracts

Bran inoculum was prepared by inoculating a sterile bran mixture (250 ml conical flask containing 10 g wheat bran (Badminton Horse Feeds, UK) and 30 ml sterile dH_2O with five 4 mm plugs of agar from the leading edge of a 3-day-old *T. hamatum* culture, grown on Potato Dextrose Agar (Sigma-Aldrich). The inoculum was incubated for 5 days at 26°C under a 16 h light regime.

S. sclerotiorum poppy seed inoculum

A sterile poppy seed mixture (250 ml conical flask containing 10 g black poppy seeds with 10 ml sterile dH_2O) was inoculated with ten 1 mm plugs of agar from the leading edge of a 3-day-old *S. sclerotiorum* culture grown on PDA. The inoculum was incubated for 10 days at 26°C under a 16 h light regime. The four *S. sclerotiorum* isolates used in this study, BFS, GFR1, GFR11 and M488 were obtained from Dr Jon West, Rothamsted Research, Harpenden, UK.

REWATERING ASSAY

One Hundred microliters dH_2O was added to triplicate 5 day old *Trichoderma hamatum* bran inoculum flasks (prepared as above) for each strain to be tested. Samples were mixed for 1 h and filtered through miracloth (Calbiochem) into 2 × 50 ml aliquots. Samples were centrifuged at 10,000 g for 10 min. and vacuum filtered through 5 μm filter paper (Whatman) and autoclaved for 15 min. at 121°C. Seedlings were watered with filtrate on alternate days for 21 days.

BIOCONTROL ASSAY

Magnaporthe oryzae leaf infection assays were carried out using dwarf Indica rice (*Oryza sativa* cultivar CO-39, which is susceptible to rice blast. Eight seedlings of CO-39 were planted in 15 pots (7 cm) and grown for 14 days (2–3 leaf stage) in soil containing *Trichoderma*-bran inoculum prior to *M. oryzae* strain Guy-11 infection. Disease symptoms were scored after 5 days according to Valent et al. (1991).

BIOINFORMATICS METHODS

We used Velvet version 1.1.04 (Zerbino and Birney, 2008) for *de novo* assembly of genome sequence. For ab initio gene prediction we used FgenesH (http://linux1.softberry.com/berry.phtml?topic=fgenesh). SignalP 3.0 (Bendtsen et al., 2004) and Phobius (Kall et al., 2004) were used for prediction of signal peptides and transmembrane domains. Alignments were visualized using the Artemis Comparison Tool (Carver et al., 2005). To generate Venn diagrams we used the Venn Diagram Generator (http://bioinformatics.psb.ugent.be/webtools/Venn/). PfamScan (Punta et al., 2012) was used to search for conserved domains in protein sequences.

LINKED ^{1}H-NMR FINGERPRINTING AND DIRECT INFUSION ELECTROSPRAY-MASS SPECTROMETRY

Extracts from three replicate flasks of bran inoculum were prepared with equal 50 mL volumes of dH_20:acetic acid:methanol (80:20 vol/vol) and left to mix for 1 h at room temperature, prior to centrifugation at 12,000 × *g*, and subsequent filtration through 0.2 μm membranes (Millipore). Samples were snap frozen in liquid nitrogen and then lyophilized. Triplicate samples (15 mg) of freeze-dried *Trichoderma*-bran extract were processed for ^{1}H-NMR-MS using modified protocols described by Ward et al. (2010). For ^{1}H-NMR fingerprinting samples were extracted (10 min, 50°C) with 1 mL of deuterated sodium phosphate buffer (300 mM pH6) containing 0.05% w/v TSP-d4 (sodium salt of trimethylsilylpropionic acid). After centrifugation the supernatant was heated to 90°C (2 min). After cooling and centrifugation, the supernatant (600 μL) was transferred to an NMR tube for analysis. Samples for direct infusion electrospray mass spectrometry (DI-ESI-MS) were prepared exactly as described in Ward et al. (2010) and accompanying supplementary material using a mixture (20:80) methanol:water.

^{1}H-NMR spectra were acquired under automation at a temperature of 300 K on an Avance spectrometer (Bruker Biospin) operating at 600.0528 MHz using a 5 mm SEI probe. ESI spectra were acquired, in both positive and negative ionization modes, under automation using Esquire 3000 (Bruker Daltonics) ion trap mass spectrometer.

^{1}H-NMR spectra were automatically reduced to CSV files using AMIX (v3.0, Bruker Biospin) and DI-ESI-MS data were processed using Data Analysis v3.2 (Bruker Daltonics). Spectral processing for both ^{1}H-NMR and DI-ESI-MS was carried out using routines described previously in Ward et al. (2010). Unsupervised multivariate analyses by PCA and PLS-DA were performed using SIMCA-P 11.0 (Umetrics, http://www.umetrics.com), using mean-centered scaling throughout the modeling. The signals resulting from the NMR internal standard

(trimethylsilylpropionic acid; TSP-d_4) were removed prior to importing the data set into SIMCA-P 11.0 for multivariate analysis.

RESULTS

Trichoderma hamatum PLANT GROWTH PROMOTION

T. hamatum promotes growth of lettuce (Ryder et al., 2012). Here we also demonstrate *T. hamatum* GD12 also promotes growth of *Arabidopsis thaliana* (**Figure 1A**) using acidic, nutrient-poor, organic peat soil microcosms (Thornton, 2005). In previous work, we hypothesized that the plant-growth-promotion (PGP) properties of GD12 might occur through the enzymatic release of nitrogen from chitin. Remarkably, however, rather than reduced or loss of PGP, disruption of the GD12 *N*-acetyl-b-D-glucosaminidase gene (Δ*Thnag1::hph* strain) dramatically enhanced the growth of lettuce seedlings, indicating

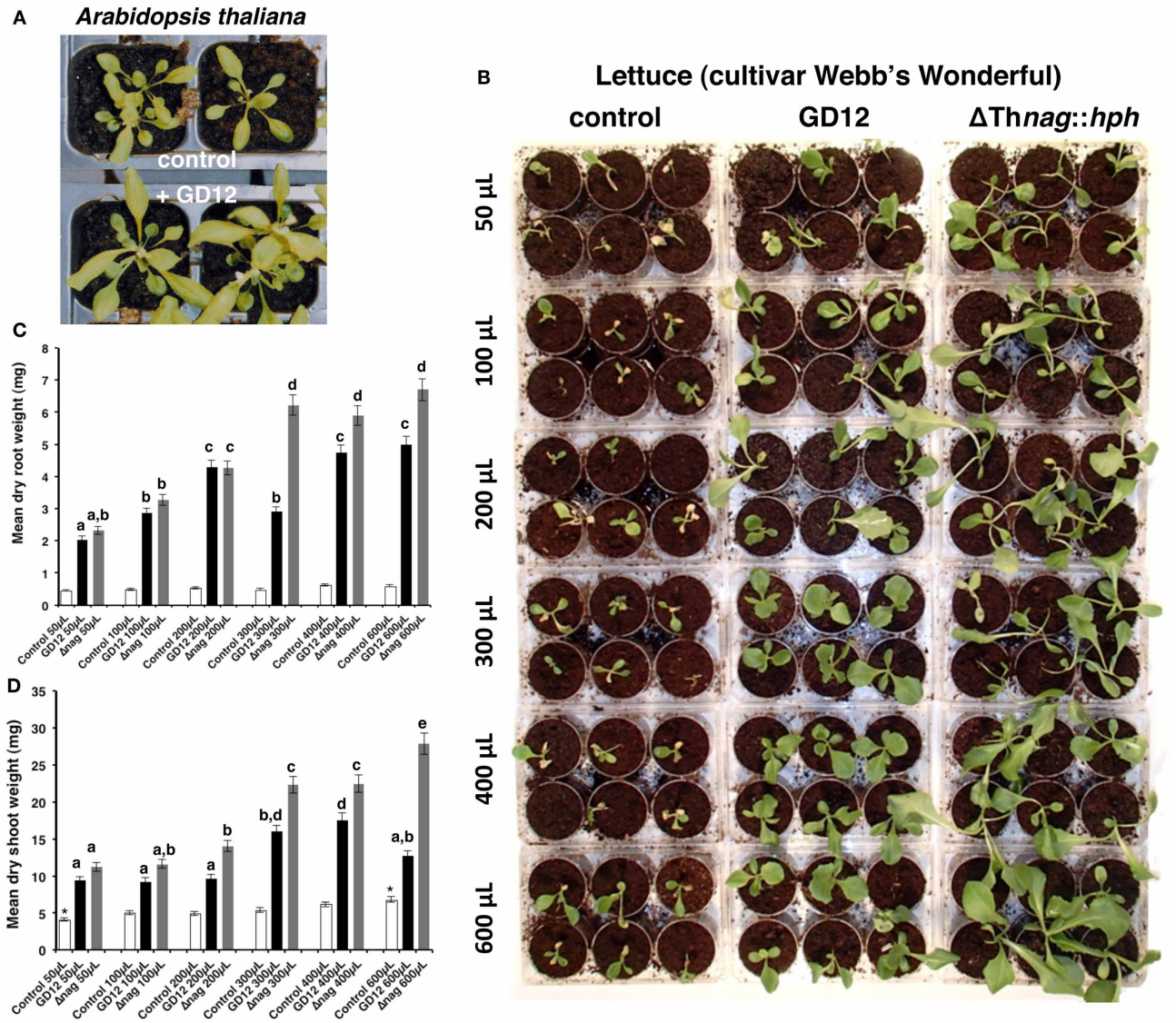


FIGURE 1 | Plant growth promotion by *Trichoderma hamatum* GD12. **(A)** Amendment of peat compost with *T. hamatum* GD12 promotes growth of *Arabidopsis thaliana* accession Landsberg—erects. Photographed at 3 weeks. **(B)** Soluble, autoclaved bran extracts from GD12 or the *N*-acetyl-β-D-glucosaminidase knockout mutant (Δ*Thnag1::hph*) promote growth of lettuce (*Lactuca sativa* cultivar Webb's Wonderful) in sterile peat. Lettuce were supplemented with the indicated amount of sterilized bran exudate on alternate days. Control plants were watered with a corresponding aliquot of dH_2O. After 21 days growth plants were harvested to determine root and shoot weights. Each microcosm diameter is 15 mm. The photograph, taken 21 days after sowing is representative of a microcosm replicate. Histogram showing dry weights of lettuce root **(C)** or shoot **(D)** biomass 21 days after growth in peat microcosms supplemented by application of sterilized bran extracts. Each plant was treated with the indicated amount of metabolite extract from either *T. hamatum* strain GD12 (black bars) or Δ*Thnag::hph* (Δnag; gray bars) on alternate days. Control plants (white bars) were watered with dH_2O. Each bar represents the mean of 25 samples, each with 3 experimental replicates ± SE. Same letter denote no significant difference and *denote significant difference at 95% confidence level (*t*-test).

that increased production of stimulatory compound(s) was due to *N*-acetyl-β-D-glucosaminidase deficiency (Ryder et al., 2012). Here we further demonstrate that this PGP activity was found to be present in water-soluble extracts derived from both bran grown GD12 and Δ*Thnag1::hph.* PGP activity, and that the PGP activity was heat stable, withstanding autoclaving to 121°C for 15 min (**Figure 1B**). Δ*Thnag1::hph* bran extracts promoted enhanced lettuce growth compared to GD12, which is consistent with the predicted hyper-secretion capability of Δ*Thnag1::hph*. These data were quantified by measurement of dry weights of treated material (**Figures 1C,D**). Application of either extract significantly increased PGP, even at the lowest addition (50 μL). The root dry weight of both GD12 and Δ*Thnag1::hph* treated lettuce showed significant increases to 200 μL aliquots application whereas Δ*Thnag1::hph* treatment root dry weight increased up to 300 μL aliquot applications (**Figure 1C**). Dry shoot weights followed a similar trend except PGP induced by GD12 extracts plateaued at 300 μL application and lettuce shoot dry weight continued to increase with 600 μL of extract (**Figure 1D**).

It has been previously shown that in contrast to enhanced PGP, loss of *N*-acetyl-β-D-glucosaminidase activity drastically impaired GD12's competitive saprotrophic ability and biocontrol against a strain of the pre-emergent pathogen *S. sclerotiorum* (Ryder et al., 2012). We extended this study to show that GD12, but not Δ*Thnag1::hph,* effectively mycoparasitises four geographically distinct *Sclerotinia sclerotiorum* strains (**Figure 2A**). *T. hamatum* GD12 not only showed strong biocontrol of *S. sclerotiorum*, but strikingly PGP was also dramatically enhanced compared to GD12 amendment alone (**Figure 2B**). We interpret these data to suggest that cryptic metabolomic pathways, ordinarily silent in GD12 in axenic culture, are induced during antagonistic interactions in soil.

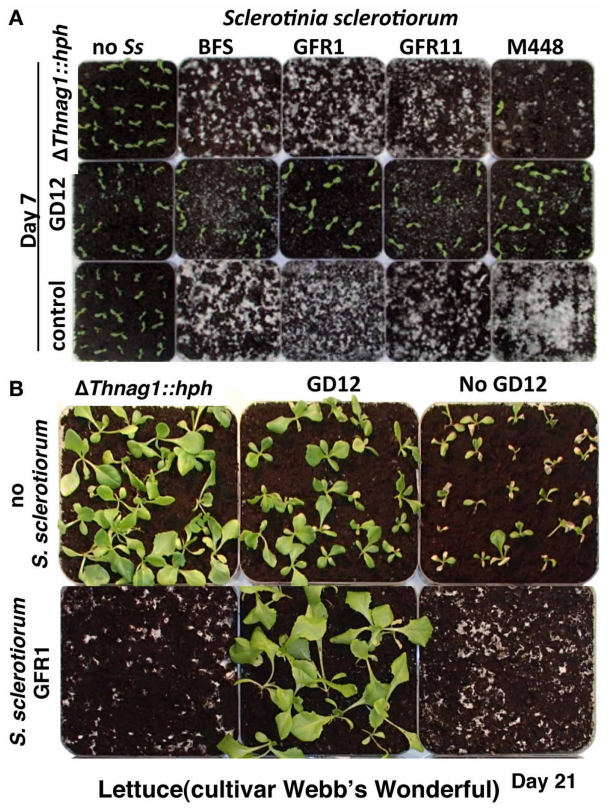


FIGURE 2 | Active biocontrol of the pre-emergence pathogen, *Sclerotinia sclerotiorum* by *T. hamatum,* GD12 results in additional plant growth promotion. (A) *T. hamatum,* GD12 but not the *N*-acetyl-β-D-glucosaminidase mutant Δ*Thnag1::hph* is able to suppress *S. sclerotiorum* and allow germination of lettuce seedlings. Photograph taken 7 days post sowing. **(B)** Mycoparasitism of *Sclerotinia sclerotiorum* by *T. hamatum,* GD12 results in enhanced plant growth promotion, compared to amendment with GD12 alone.

T. hamatum INDUCES RESISTANCE TO RICE BLAST

Production of plant-growth-promoting compounds and mycoparasitism by certain strains of *Trichoderma* are well-documented (Contreras-Cornejo et al., 2009; Vinale et al., 2009) although both traits are rarely found together. Some *Trichoderma* strains additionally possess the ability to activate ISR to a broad range of pathogens. To investigate the robustness of *T. hamatum* GD12 biocontrol properties we examined the ability of GD12 and the Δ*Thnag1::hph* mutant to confer resistance to the rice blast pathogen *Magnaporthae oryzae*. GD12 and, to a greater extent, Δ*Thnag1::hph* both reduced lesion formation compared to non-inoculated rice plants. Thus, although Δ*Thnag1::hph* has lost the ability to mycoparasitize *S. sclerotiorum* it has the capacity to elicit a strong induced systemic resistance response in rice to *M. oryzae* (**Figure 3**). Unlike the lettuce response, we did not see any clear increase in foliar growth, but observed an increase in root development following *Trichoderma* amendment.

DRAFT GENOME SEQUENCE OF *T. hamatum* GD12

We hypothesized that the genome sequence of *T. hamatum* GD12 might provide valuable insight into the genetic potential underlying the unique PGP, mycoparasitism and ISR inducing properties of this saprotrophic fungus. A GD12 genome sequence could facilitate secondary metabolite pathway predictions, mapping of mRNA-seq data to genomic clusters and capture unique genes and gene families not coded by other *Trichoderma* genomes.

We therefore assembled a draft genome sequence of GD12 from 12 million pairs of Illumina GA2 paired-end 73-bp reads using Velvet 1.1.04. This yielded 2770 scaffolds with a N_{50} length of 41.6 Kb. The total length of the assembly was 38.2 Mb. The whole genome shotgun data have been deposited at DDBJ/EMBL/GenBank under the accession ANCB00000000. Using FgenesH (trained on *Neurospora crassa*) we predicted 12391 protein-coding genes in GD12.

COMPARISON WITH OTHER *Trichoderma* GENOMES

The genome sequence of GD12 shares little similarity with previously sequenced *Trichoderma* genomes at the nucleotide sequence level. The three sequenced *Trichoderma* genomes analysed in detail, *T. atroviride* (∼36.4 Mb), *T. virens* (∼38.8 Mb) and *T. reesei* (∼34 Mb) show remarkably conserved gene order (78–96%), with >50% of annotated genes having orthologues in the related ascomycetes *Neurospora crassa* and *Gibberella zeae.* (Kubicek et al.,

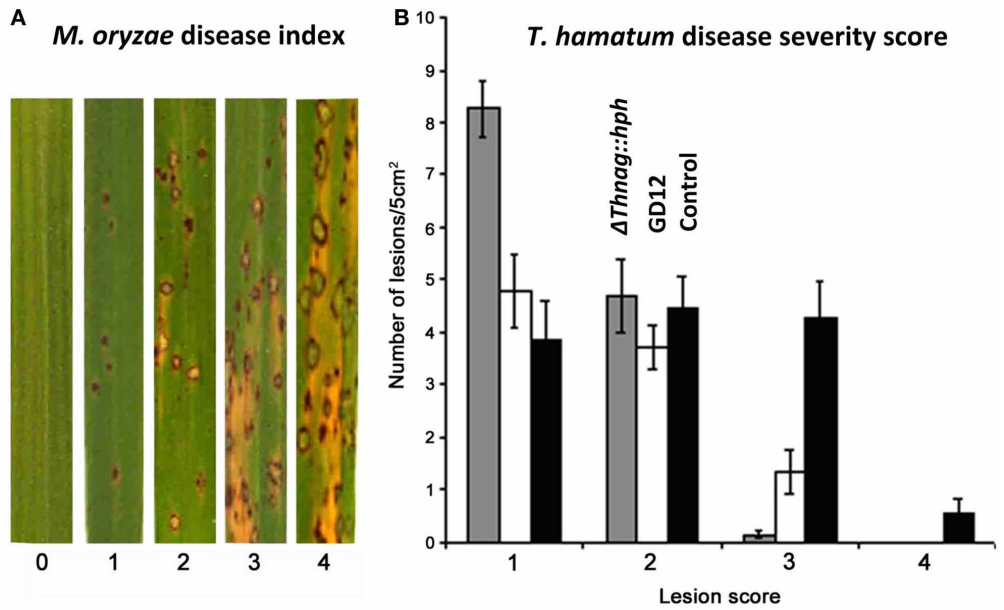


FIGURE 3 | *T. hamatum* biocontrol and plant disease suppression. **(A)** Leaf segments of rice (cultivar CO-39) showing rice blast symptoms. **(B)** Suppression of rice blast disease by *T. hamatum*. Growth of rice cultivar CO-39 in soil amended with *T. hamatum* GD12 (white bars) and the *N*-acetyl-β-D-glucosaminidase deficient mutant Δ*Thnag1::hph* (gray bars) reduced the size of the lesions caused by the rice blast *Magnaporthe oryzae*. Lesions were scored as previously documented (Valent et al., 1991) according to the following range. Type 1 (lesion 0.5 mm in length); type 2 (lesion ∼1 mm in length); type 3 (lesions ∼2 mm in length) and type 4 (lesions ∼3–4 mm in length) lesions. Each bar represents the mean of 8 samples, each with 5 experimental replicates ± SE.

2011). Strikingly, only 52% of the GD12 genome sequence aligned against that of *T. atroviride* and only 6% aligned against the more distantly related *T. reesei* (using the *dnadiff* tool from the Mummer package). To ensure this limited sequence similarity was not due to sample contamination, a geographically distinct *T. hamatum* isolate, strain 11, was sequenced. Strain 11 showed 98% sequence identify to GD12.

At the level of amino acid sequence, 62.4% of the GD12 predicted proteins (*i.e.* 7773 proteins; **Figure 4A**) had a close homologue in at least one of *T. atroviride*, *T. harzianum*, *T. reesei* or *T. virens* species compared (here, we define a close homologue as sharing at least 80% sequence identity over at least 90% of the length of the query sequence). Of the GD12 predicted proteins, only 5531 (59%) are highly conserved in *T. atroviride* (at least 80% amino acid sequence identity over at least 90% of the sequence length; **Figure 4A**). Thus, GD12 contains novel genomic regions with the potential to encode novel, agrochemically important gene products leading to unique bioactive metabolites that may contribute to GD12's PGP and biocontrol activities.

These *T. hamatum*-specific genomic regions likely hold the key to the unique biological interactions observed in this species. For example, we identified a 47-kbp *T. hamatum*-specific region described in **Table 1** and illustrated in **Figure 5**, which appears to encode several enzymes (Genes 3, 5, 6, 7) and transporters (Genes 2, 4) that might contribute to novel secondary metabolism pathways. Predicted gene 3 encodes a protein containing an attachment site for phosphopantetheine, a prosthetic group that acts as a "swinging arm" for the attachment of activated fatty acid and amino-acid groups. It also contains a domain characteristic of AMP-binding enzymes. Gene 5 encodes a protein with an ATP-grasp domain, characteristic of enzymes that possess ATP-dependent carboxylate-amine ligase activity. Gene 6 encodes a putative aminotransferase while Gene 7 is predicted to encode a polyketide synthase, which provide important sources of naturally occurring small molecules such as antibiotics and other industrially important polyketides.

While the vast majority of the unique GD12 genes were of unknown function, there were some interesting candidate genes that might contribute to both PGP and biocontrol activities. These included enzymes with homology to benzene and benzoate oxidases and nicotinate (nicotinamidase) degradation; potential synthesis of the plant phytohormone zeatin (adenylate isopentenyltransferase) and the insect hormone biosynthetic hormone ecdysone oxidase. Most notable are the non-ribosomally synthesized cyclic lipopetide antibiotics such as surfactin and three of the five *Bacillus subtilis* fengycin synthetases that non-ribosomally synthesize fengycin, a lipopeptidic antibiotic (Wu et al., 2007). Thus, a rich reservoir of metabolic potential exists in the unique genomic regions of *T. hamatum*.

THE SECRETOME

Given the dual plant growth promotion and biocontrol properties of *T. hamatum,* constituents of the "secretome" represent candidates in the molecular dialogue between soil pathogens and the plant rhizosphere. Of the 12391 hypothetical GD12 genes, 1014 (8.2%) were predicted to be encode secreted proteins

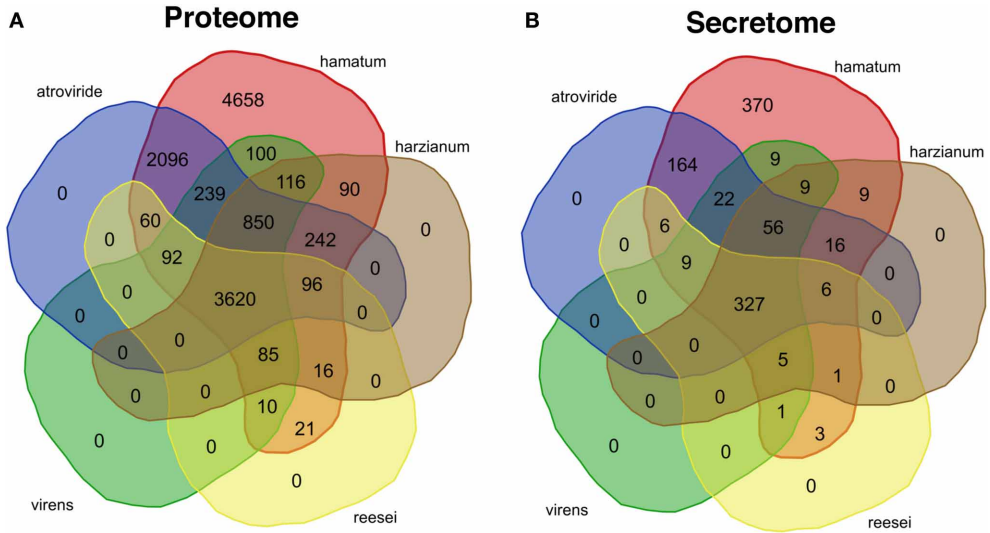


FIGURE 4 | Venn diagrams showing the conservation of the *Trichoderma hamatum* GD12 predicted proteome and secretome in previously sequenced *Trichoderma* species. BLASTP was used to search for similar sequences to each of the 12,391 predicted GD12 proteins. We performed BLASTP searches against the previously published predicted proteomes of *T. atroviride*, *T. virens*, *T. reesei*, and *T. harzianum* as well as against the GD12 predicted proteome. A protein was counted as conserved in a species if there was a BLASTP hit with least 80% amino acid sequence identity covering at least 90% of the query sequence. The predicted proteins and a subset comprising the predicted secreted proteins were compared to other sequenced *Trichoderma* isolates. **(A)** *T. hamatum* GD12 shares a core proteome of 3620 predicted proteins, with *T. hamatum* with *T. atroviride*, *T. harzianum*, *T. reesei* and *T. virens* and has 4658 unique proteins. The GD12 proteome is most homologous to that of *T. atroviride*. **(B)** The 1,014 proteins predicted to encode secreted proteins based upon secretion signals (SignalP) and lack of a typical transmembrane domain (Phobius) were compared to similarly derived secretomes from *T. atroviride*, *T. harzianum*, *T. reesei* and *T. virens*. GD12 shares a core secretome of 327 proteins and has 370 predicted unique secreted proteins.

based upon SignalP 3.0 (Bendtsen et al., 2004) and absence of a typical transmembrane domain as determined by Phobius (Kall et al., 2004) (**Supplementary File S1**). Of these, 370 were unique to *T. hamatum,* more than entire the "core" secretome shared by the 5 *Trichoderma* species (**Figure 4B**). Only 469 (55.5%) of *T. hamatum* secretome proteins are conserved in *T. atroviride* suggesting some divergence in the nature of the secreted bioactive proteins. The secretome of *T. atroviride* is, surprisingly, enriched for 26 proteins containing the fungal-specific Zn(2)Cys(6) transcription factor domain (Pfam: PF04082; http://genomebiology.com/2011/12/4/R40). The predicted secretome of GD12 is also similarly enriched, with 11 proteins containing this transcription-factor domain. One striking feature of the GD12 secretome is the enrichment for putative AMP-binding enzymes; 14 of the GD12 secreted proteins contain an AMP-binding domain (Pfam: PF00501; **Supplementary File S2**).

SMALL SECRETED (CYSTEINE-RICH) PROTEINS (SSCRPs)

Fungi manipulate the immune systems of their plant hosts via effector proteins, many of which are small secreted cysteine-rich proteins (Stergiopoulos and de Wit, 2009). Furthermore, it was recently shown that SSCRPs are upregulated in *Trichoderma* species during mycoparasitic interaction (Atanasova et al., 2013). We identified potential SSCRPs in the predicted secretomes of *T. hamatum* and *T. atroviride* as proteins whose length was 300 amino acids or fewer and which contained at least four cysteine residues, as defined in (Kubicek et al., 2011). There were 153 proteins in *T. hamatum* satisfying these criteria (**Supplementary File S3**), of which 83 had no close homologue in *T. harzianum*, *T. reesei*, *T. virens* or *T. atroviride* (i.e., no BLASTP hit with at least 80% sequence identity over at least 90% of the protein's length). For comparison, in *T. atroviride* there were 170 proteins satisfying these criteria, of which 106 had no close homologue in *T. harzianum*, *T. reesei*, *T. virens* or *T. hamatum*. Thus, there is a complement of ∼60–70 SSCRPs that may constitute a "core" effector complement. The *T. hamatum* SSCRP's contained a diverse range of Pfam domains (**Supplementary Files S4**, **S5**) suggesting a complex array of biological activities associated with these SSCRPs.

LysM MOTIFS

The LysM motif binds different peptidoglycans in bacteria and chitin-like compounds in eukaryotes (Buist et al., 2008; de Jonge and Thomma, 2009). Recent studies have shown that fungal LysM motifs can bind and suppress chitin oligomers that would be recognized by plant pattern recognition receptors, preventing the activation of an innate immune response (de Jonge et al., 2010). Seven hypothetical GD12 proteins contain a LysM domain (Pfam: PF01476) although none of these are predicted to be secreted. This is similar to the numbers reported previously for *T. reesei* (6), *T. virens* (7) and *T. atroviride* (9).

METABOLITE PROFILING OF THE GD12 AND ΔThnag1::hph MUTANT SECRETED METABOLOME

As a foundation to establish the underlying chemical differences that may collectively contribute to the PGP and biocontrol

Table 1 | Genes encoded in a 47-kbp genomic region unique to *Trichoderma hamatum* GD12.

Gene	Start–end (orientation)	Best hit in SwissProt (amino acid sequence identity)	Best hit in NCBI Proteins	Pfam domains
1	6,264–7,541 (+)	Q9PKX8.1 *Chlamydia muridarum* tyrosine-tRNA ligase (28%)	EGU81361.1 *Fusarium oxysporum* hypothetical protein (39%)	None
2	8,951–13,442 (+)	P11636.2 *Neurospora crassa* Quinate permease (32%)	EHK41798.1 *Trichoderma atroviride* hypothetical protein (60%)	Sugar (and other) transporter (PF00083)
3	17,805–21,557 (+)	Q4WYG2.2 *Aspergillus fumigatus* nonribosomal peptide synthetase 5 (31%)	XP_001262961.1 *Neosartorya fischeri* nonribosomal peptide synthase (54%)	AMP-binding enzyme (PF00501); Phosphopantetheine attachment site (PF00550)
4	21,747–23,653 (−)	Q864R9.1 *Macaca fascicularis* multidrug resistance-associated protein 1 (32%)	EHK16312.1 *Trichoderma virens* hypothetical protein (82%)	ABC transporter (PF00664); ABC transporter transmembrane region (PF00005)
5	26,142–30,603 (+)	Q6ZPS2.2 *Mus musculus* carnosine synthase 1 (26%)	XP_001262963.1 *Neosartorya fischeri* hypothetical protein (56%)	ATP-grasp domain (PF13535)
6	32,148–35,686 (−)	Q635G4.1 *Bacillus cereus* L-alanine–pimeloyl-CoA ligase (35%)	XP_003298955.1 *Pyrenophora teres f. teres* hypothetical protein (64%)	Aminotransferase class I and II (PF00155)
7	36,727–45,336 (+)	Q4WAZ9.2 *Aspergillus fumigatus* nonribosomal peptide synthetase 14 (38%)	ELA23575.1 *Colletotrichum gloeosporioides* polyketide synthase (49%)	Beta-ketoacyl synthase, N-terminal domain (PF00109); Beta-ketoacyl synthase, C-terminal domain (PF02801); Acyl transferase domain (PF00698); Alcohol dehydrogenase GroES-like domain (PF08240); Zinc-binding dehydrogenase (PF00107); KR domain (PF08659); Phosphopantetheine attachment site (PF00550)

This region (GenBank: KB232787) has no detectable nucleotide sequence similarity to previously sequenced Trichoderma genomes.

properties of *T. hamatum* we used two metabolite fingerprinting approaches, ^{1}H-NMR and direct infusion electrospray ionization mass spectrometry (DI-ESI-MS). These methodologies sample a subset of overlapping, largely polar, chemistry which is ideally suited to analysis of the *T. hamatum* amended bran extracts showing PGP activity (**Figure 2**). Freeze dried, bran culture filtrates from uninoculated or GD12 and Δ*Thnag1::hph* amended cultures were initially extracted in 20% deuterated methanol in deuterium oxide solvent which is selective for polar metabolites but also has the advantage of being suitable for direct ^{1}H-NMR analysis (Ward et al., 2003) and which we have successfully used in fingerprinting *Arabidopsis-Pseudomonas syringae* interactions (Ward et al., 2010). However, due to some shifts in peaks, presumably due to differing pHs of the samples, the freeze-dried bran culture filtrates were re-extracted using deuterated sodium phosphate buffer which aligned all peaks in the spectrum.

Principal components analysis (PCA) of full unfiltered data at 95% confidence intervals was used to evaluate differences in chemistry between the bran extracts. **Figure 6** show that both chemistries clearly showed statistically significant separation between the three treatments. Notably, the GD12 amended bran culture extract fingerprint is significantly different from Δ*Thnag1::hph* confirming differences in the secreted metabolome between the two that may contribute to the PGP differences illustrated in **Figure 2**. As part of a large scale metabolite profiling study of GD12 secreted metabolites we undertook unbiased metabolite profiling on GD12 bran extracts. Bran extracts prepared as above were first tested for plant growth promotion activity then three independent experiments, each containing 4 replicates were analysed by liquid chromatography mass spectrometry on a C18 column as described in **Supplementary File S6**. Spectra were extracted and features aligned from biological replicates using a density maximisation approach as previously described (Perera et al., 2012). This study confirmed a large number of potentially novel metabolites were secreted by GD12. **Supplementary File S7** reports the top 30 ranked features identified in both positive and negative ionisation mode from GD12 bran extract showing at least a 5-fold enrichment in a minimum of 10 out of 12 samples analysed.

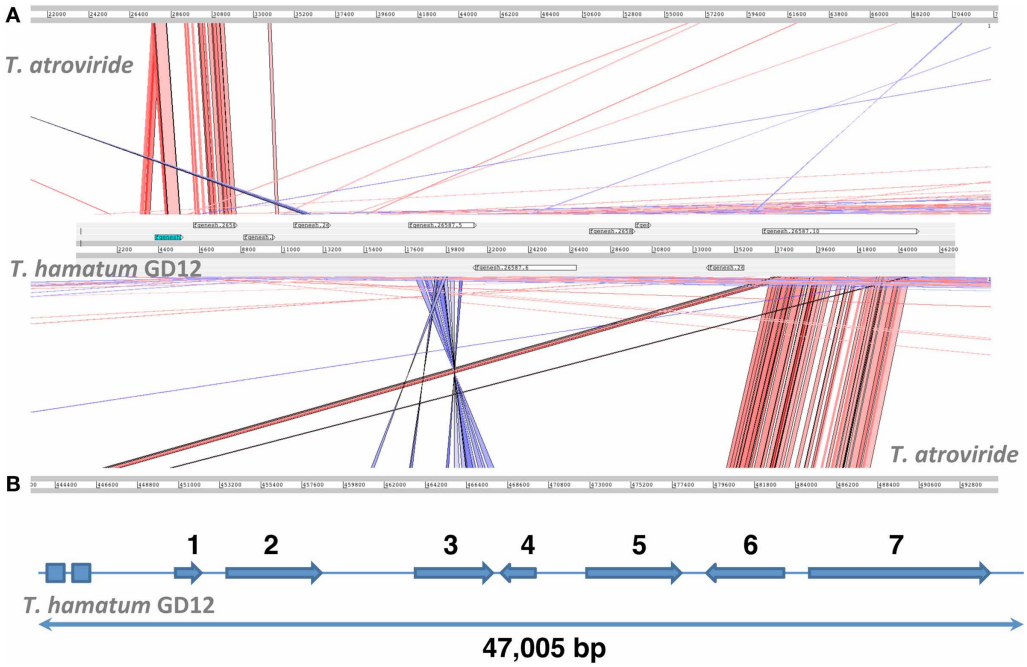


FIGURE 5 | A genomic region unique to *Trichoderma hamatum* GD12 with the capacity to encode novel secondary metabolites. (A) This 47-kbp region (GenBank: KB232787) has no detectable nucleotide sequence similarity to previously sequenced *Trichoderma* genomes except for the two short regions indicated by rectangles, which share 85% and 78% nucleotide sequence identity with *T. atroviride* scaffold 19. **(B)** Arrows indicating predicted protein-coding genes, which are described in **Table 1**.

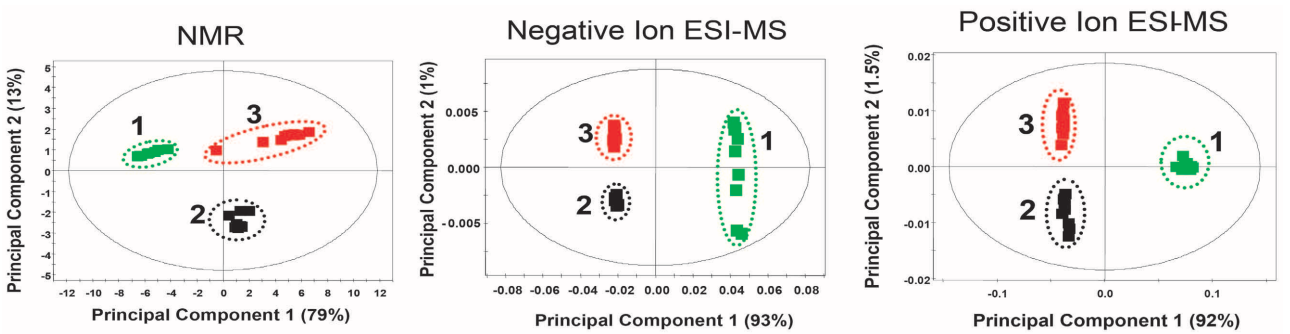


FIGURE 6 | Metabolite fingerprinting of bran extracts using NMR and DI-ESI-MS datasets. Extracts from 5 day old *T. hamatum* bran inoculum were analysed by ^{1}H-NMR and direct infusion electrospray ionization mass spectrometry. Principal components analysis (PCA) of full unfiltered data at 95% confidence intervals was used to evaluate differences in chemistry between the bran extracts. PCA data showed clear separation of GD12 (black) and Δ*Thnag1::hph* (red) from control bran extract green indicating both chemistries captured differences in the secreted metabolome. This unsupervised multivariate PCA analysis was performed using SIMCA-P 11.0, using mean-centered scaling.

DISCUSSION AND CONCLUSIONS

Here we extend our initial characterization of *T. hamatum* strain GD12 (Ryder et al., 2012) to examine further beneficial agronomic traits of GD12. We show plant growth promotion in the model dicot, *Arabidopsis thaliana* and the ability to induce foliar biocontrol in rice, a model monocot. We further characterized the lettuce-*T. hamatum* interaction to show that bran extracts from GD12 and the *N*-acetyl-β-D-glucosamindase-deficient mutant Δ*Thnag1::hph* differentially promote growth in a concentration dependent manner. Both ^{1}H-NMR and direct infusion electrospray ionization mass spectrometry fingerprinting show that these differences in growth are also correlated with differences in the small molecule secretome extracted from bran cultures. This finding was extended by unbiased comparative metabolite profiling of GD12 amended and un-amended bran extracts. We also expanded our recent finding of GD12 mycoparasitism of a strain of the pre-emergence soil pathogen *Sclerotinia sclerotiorum* to show mycoparasitism across a range

of *S. sclerotiorum* strains. This interaction induces a further increase in plant growth promotion above that conferred by GD12 suggesting that cryptic metabolomic pathways, ordinarily silent in GD12 in axenic culture, are induced during antagonistic interactions in soil.

Fossil evidence predicts that the mycoparasitic lifestyle evolved more than 400 million years ago (Taylor and Berbee, 2006). We sequenced the *T. hamatum* genome and demonstrate that like *T. virens* and *T. atroviride*, *T. hamatum* encodes a vast arsenal of cell wall degrading enzymes such as chitinases, glycoside hydrolases, β-1,3-glucanases and *N*-acetyl-β-D-glucosaminidases that are presumably deployed to degrade the carbohydrate defenses of its biocontrol targets. It also encodes a wealth of proteases, polyketide synthases and non-peptide synthases consistent with its mycoparasitic lifestyle. Strikingly, however, approximately half of the *T. hamatum* proteome and its constituent secretome is unique to GD12 and *vice versa*. To ensure these data were not the result of unexpected contamination we sequenced the related *T. hamatum* strain 11 and verified ∼98% identity.

Recent comparative genomic experiments have revealed that the *T. reesei* genome contraction has occurred, with consequent loss of mycoparasitic ability. At 38.8 Mbp, the *T. virens* genome is nearly 5 Mbp larger than *T. reesei* and 2.7 Mbp larger than *T. atroviride*. Unique to *T. virens* and *T. atroviride* are secondary metabolite gene clusters localized on non-syntenic islands that are likely to contribute to mycoparasitism. Notable in the larger *T. virens* genome, are a repertoire of non-ribosomal peptide synthetases (NRPS) expanded to 28, twice that present in other fungi (Martinez et al., 2008; Kubicek et al., 2011).

T. hamatum is phylogenetically most closely related to *T. atroviride* (Kubicek et al., 2011) yet the incomplete GD12 assembly at ∼38.1 Mbp is nearly as large as the *T. virens* genome. Despite the close phylogenetic relationship, there were striking differences between *T. atroviride* and *T. hamatum* homology, with approximately 40% of the GD12 proteome being unique. The extent of these differences are interesting and most likely reflect the strong evolutionarily genomic potential and additional beneficial traits of plant growth promotion and induced systemic resistance encoded by GD12, as well as components required for niche differentiation. A simple analysis of GD12 unique regions identified a range of components implicated in secondary metabolism including evidence for production of non-ribosomally synthesized lipopetide antibiotics such as surfactin and the antibiotic fengycin (Wu et al., 2007).

T. hamatum encoded over 4658 unique proteins and shared a core proteome of 3620 predicted proteins with the four other *Trichoderma*'s (*T. atroviride, T. virens, T. reesei, T. harzianum*). An additional 2096 proteins were unique to GD12 and *T. atroviride* reflecting the closer evolutionarily relationship between these two species. Some of these genes may specify enzymes responsible for the breakdown of polymeric organic molecules into a form that can be absorbed, or in the secretion of fungal synthesized compounds that have roles in antibiosis or are signals molecules facilitating communication with mutualistic partners. How the unique component of the GD12 genome has been acquired and is deployed remains to be determined. **Figure 5** highlights a 47-kbp *T. hamatum*-specific region that encodes several biosynthetic enzymes and transporters with potential to contribute to novel secondary chemistries, including two NRPS components. Moreover, like *T. atroviride* (Baker et al., 2012), the *T. hamatum* genome has a number gene clusters encoding polyketide synthases. PKSs play important roles in synthesis of secondary metabolites such as in the plant pathogen *Ustilago maydis* (Kamper et al., 2006) and a hybrid NRPS/PKS has recently been implicated in ISR in maize (Mukherjee et al., 2012).

We predicted 370 unique proteins in the secretome of *T. hamatum* and a core of 327 proteins shared across *T. atroviride, T. virens, T. reesei* and *T. harzianum*. GD12 and *T. atroviride* shared 164 unique putative secreted proteins, nearly 20 times as many as any of the other species (**Figure 4**). The distinct genomic potential is also reflected in the deployment of small secreted cysteine rich peptides which function as potential fungal effector proteins to suppress host immunity and modulate host signalling networks (Stergiopoulos and de Wit, 2009). Approximately 50% of the SSCRPs were shared between the two species.

A striking feature of the GD12 secretome was the enrichment for putative AMP-binding enzymes; 14 of the GD12 secreted proteins contain the AMP-binding domain (Pfam: PF00501; **Supplementary File S1**). Interestingly, many of these proteins are capable of acyl:adenyl ligase activities that can positively or negatively modulate bioactivity through the ligation of residues such as amino acids. Plant acyl:adenyl ligases include enzymes generating bioactive amide hormone conjugates such as JA-Ile and JA-Trp from JA and IAA-Trp from indole acetic acid (Staswick and Tiryaki, 2004; Staswick, 2009). Virulent phytopathogens such as *Pseudomonas syringae* synthesize IAA-lysine synthetase which can inactivate plant IAA to IAA-lysine (Romano et al., 1991). The AMP-binding domain containing *Cochliobolus carbonum* race 1 HC-toxin synthetase produces the HC-toxin cyclic tetrapeptide (Scott-Craig et al., 1992; Walton, 2006). Thus enrichment for putative secreted AMP-binding enzymes may highlight a possible role in plant-microbe communication in the rhizosphere.

Overall, it is an exciting and opportune time to exploit the remarkable genetic and chemical potential for beneficial for sustainable agriculture. Co-evolution with hosts has endowed *Trichoderma* spp. with a range of agronomically important traits. The genome of *T. hamatum* GD12 encodes the genetic potential to promote growth and induce ISR in a range of plants. The arsenal of genes in GD12 also enables it to effectively mycoparasitize *S. sclerotiorum,* a successful and persistent pathogen of agronomic crops. Strikingly, mycoparasitism of *S. sclerotiorum* results in additional PGP. Importantly, culture filtrates that promote plant growth (**Figure 1B**) are incapable of suppressing the pathogenic effects of *Sclerotinia* suggesting that, additional enhanced plant growth stimulation occurs during interactions with soil pathogens. We hypothesize that antagonism between GD12 and root pathogens in the plant rhizosphere leads to transcriptional activation of cryptic secondary metabolite pathways that are phenotypically silent in axenic culture. This is in agreement with recent reports of activation of silent gene clusters in *Aspergillus nidulans* following co-cultivation of the fungus with

other microorganisms which has led to the identification of novel secondary metabolites (Schroeckh et al., 2009). However, it is also possible that mycoparasitism gives rise to new compounds from degrading tissue or releases pre-existing components with PGP activity. Thus, the genome sequence of GD12, and comparisons with other *Trichoderma* genomes will facilitate genetic dissection of these traits. Genome informed predictions will help to identify and experimentally validate novel secondary metabolism implicated in adaptation to specific ecological niches and promotion of beneficial traits.

ACKNOWLEDGMENTS

This work was supported by a Biotechnology and Biological Sciences Research Council grant BB/I014691/1 to Murray Grant and Chris R. Thornton.

SUPPLEMENTARY MATERIALS

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant-Microbe_Interaction/10.3389/fpls.2013.00258/abstract

Supplementary File S1 | GD12.secretome.no-TMs.faa.pfamscan.html. Frequencies of Pfam domains in predicted secreted proteins encoded in the GD12 genome.

Supplementary File S2 | Supp2_AMP-binding_secreted.faa.txt. AMP-binding domain proteins are over-represented in the secretome.

Supplementary File S3 | Supp3_GD12.secretome.no-TMs.SSCRPs.faa.txt. Sequences of candidate small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome. Amino acid sequences are given in FastA format.

Supplementary File S4 | Supp4_GD12.secretome.no-TMs.SSCRPs.faa.pfamscan.txt. Pfam domains in each predicted small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome.

Supplementary File S5 | Supp5_GD12.secretome.noTMs.SSCRPs.faa.pfamscan.html. Frequencies of Pfam domains in predicted small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome.

Supplementary File S6 | Method for unbiased profiling of GD12 and bran extracts by HPLC-MS.

Supplementary File S7 | Table of top 30 putative metabolites identified in GD12 bran extracts showing at least a 5 fold change compared to unamended bran extract.

REFERENCES

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R., and Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends Ecol. Evol.* 19, 535–544. doi: 10.1016/j.tree.2004.07.021

Atanasova, L., Le Crom, S., Gruber, S., Coulpier, F., Seidl-Seiboth, V., Kubicek, C. P., et al. (2013). Comparative transcriptomics reveals different strategies of Trichoderma mycoparasitism. *BMC Genomics* 14:121. doi: 10.1186/1471-2164-14-121

Bae, H., Sicher, R. C., Kim, M. S., Kim, S. H., Strem, M. D., Melnick, R. L., et al. (2009). The beneficial endophyte Trichoderma hamatum isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *J. Exp. Bot.* 60, 3279–3295. doi: 10.1093/jxb/erp165

Baker, S. E., Perrone, G., Richardson, N. M., Gallo, A., and Kubicek, C. P. (2012). Phylogenomic analysis of polyketide synthase-encoding genes in Trichoderma. *Microbiology* 158, 147–154. doi: 10.1099/mic.0.053462-0

Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340, 783–795. doi: 10.1016/j.jmb.2004.05.028

Benitez, T., Rincon, A. M., Limon, M. C., and Codon, A. C. (2004). Biocontrol mechanisms of Trichoderma strains. *Int. Microbiol.* 7, 249–260.

Buist, G., Steen, A., Kok, J., and Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol. Microbiol.* 68, 838–847. doi: 10.1111/j.1365-2958.2008.06211.x

Cartieaux, F., Thibaud, M. C., Zimmerli, L., Lessard, P., Sarrobert, C., David, P., et al. (2003). Transcriptome analysis of Arabidopsis colonized by a plant-growth promoting rhizobacterium reveals a general effect on disease resistance. *Plant J.* 36, 177–188. doi: 10.1046/j.1365-313X.2003.01867.x

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M. A., Barrell, B. G., and Parkhill, J. (2005). ACT: the Artemis Comparison Tool. *Bioinformatics* 21, 3422–3423. doi: 10.1093/bioinformatics/bti553

Chang, Y.-C., Baker, R., Kleifeld, O., and Chet, I. (1986). Increased growth of plants in the presence of the biological control agent Trichoderma harzianum. *Plant Dis.* 70, 145. doi: 10.1094/PD-70-145

Chaverri, P., and Gary J. S. (2003). *Hypocrea/Trichoderma* (*Ascomycota*, *Hypocreales*, *Hypocreaceae*): species with green ascospores. Centraalbureau voor Schimmelcultures.

Chet, I., Harman, G., and Baker, R. (1981). Trichoderma hamatum: its hyphal interactions with Rhizoctonia solani and Pythium spp. *Microb. Ecol.* 7, 29–38. doi: 10.1007/BF02010476

Contreras-Cornejo, H. A., Macias-Rodriguez, L., Cortes-Penagos, C., and Lopez-Bucio, J. (2009). Trichoderma virens, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiol.* 149, 1579–1592. doi: 10.1104/pp.108.130369

de Jonge, R., and Thomma, B. P. (2009). Fungal LysM effectors: extinguishers of host immunity? *Trends Microbiol.* 17, 151–157. doi: 10.1016/j.tim.2009.01.002

de Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., et al. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329, 953–955. doi: 10.1126/science.1190859

Djonovic, S., Pozo, M. J., Dangott, L. J., Howell, C. R., and Kenerley, C. M. (2006). Sm1, a proteinaceous elicitor secreted by the biocontrol fungus Trichoderma virens induces plant defense responses and systemic resistance. *Mol. Plant Microbe Interact.* 19, 838–853. doi: 10.1094/MPMI-19-0838

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B. A., Kenerley, C. M., Monte, E., et al. (2011). Trichoderma: the genomics of opportunistic success. *Nat. Rev. Microbiol.* 9, 749–759. doi: 10.1038/nrmicro2637

Elad, Y. (2000). Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19, 709–714. doi: 10.1016/S0261-2194(00)00094-6

Fantke, P., Wieland, P., Juraske, R., Shaddick, G., Itoiz, E. S., Friedrich, R., et al. (2012). Parameterization models for pesticide exposure via crop consumption. *Environ. Sci. Technol.* 46, 12864–12872. doi: 10.1021/es301509u

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–194. doi: 10.1038/nature10947

Giraldo, L. A., Tejido, M. L., Ranilla, M. J., and Carro, M. D. (2007). Effects of exogenous cellulase supplementation on microbial growth and ruminal fermentation of a high-forage diet in Rusitec fermenters. *J. Anim. Sci.* 85, 1962–1970. doi: 10.2527/jas.2006-318

Grant, M. R., and Jones, J. D. (2009). Hormone (dis)harmony moulds plant health and disease. *Science* 324, 750–752. doi: 10.1126/science.1173771

Harman, G. E. (2006). Overview of Mechanisms and Uses of Trichoderma spp. *Phytopathology*

96, 190–194. doi: 10.1094/PHYTO-96-0190

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). Trichoderma species–opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Harvey, M., and Pilgrim, S. (2011). The new competition for land: Food, energy, and climate change. *Food Policy* 36, S40–S51. doi: 10.1016/j.foodpol.2010.11.009

Heidel, A. J., Clarke, J. D., Antonovics, J., and Dong, X. (2004). Fitness costs of mutations affecting the systemic acquired resistance pathway in Arabidopsis thaliana. *Genetics* 168, 2197–2206. doi: 10.1534/genetics.104.032193

Howell, C. R. (2003). Mechanisms employed by trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* 87, 10. doi: 10.1094/PDIS.2003.87.1.4

Kall, L., Krogh, A., and Sonnhammer, E. L. (2004). A combined transmembrane topology and signal peptide prediction method. *J. Mol. Biol.* 338, 1027–1036. doi: 10.1016/j.jmb.2004.03.016

Kamper, J., Kahmann, R., Bolker, M., Ma, L. J., Brefort, T., Saville, B. J., et al. (2006). Insights from the genome of the biotrophic fungal plant pathogen Ustilago maydis. *Nature* 444, 97–101. doi: 10.1038/nature05248

Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D. A., Druzhinina, I. S., Thon, M., et al. (2011). Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of Trichoderma. *Genome Biol.* 12, R40. doi: 10.1186/gb-2011-12-4-r40

Kuhad, R. C., Gupta, R., and Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Res.* 2011:280696. doi: 10.4061/2011/280696

Lorito, M., Woo, S. L., Harman, Gary E., and Monte, E. (2010). Translational Research on Trichoderma: from 'Omics to the Field. *Annu. Rev. Phytopathol.* 48, 395–417. doi: 10.1146/annurev-phyto-073009-114314

Maplestone, P., Whipps, J., and Lynch, J. (1991). Effect of peat-bran inoculum of Trichoderma species on biological control of Rhizoctonia solani in lettuce. *Plant Soil* 136, 257–263. doi: 10.1007/BF02150057

Martinez, D., Berka, R. M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S. E., et al. (2008). Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nat. Biotechnol.* 26, 553–560. doi: 10.1038/nbt1403

Martinez-Medina, A., Pascual, J. A., Perez-Alfocea, F., Albacete, A., and Roldan, A. (2010). Trichoderma harzianum and Glomus intraradices modify the hormone disruption induced by Fusarium oxysporum infection in melon plants. *Phytopathology* 100, 682–688. doi: 10.1094/PHYTO-100-7-0682

Martinez-Medina, A., Roldan, A., Albacete, A., and Pascual, J. A. (2011). The interaction with arbuscular mycorrhizal fungi or Trichoderma harzianum alters the shoot hormonal profile in melon plants. *Phytochemistry* 72, 223–229. doi: 10.1016/j.phytochem.2010.11.008

Mathys, J., De Cremer, K., Timmermans, P., Van Kerckhove, S., Lievens, B., Vanhaecke, M., et al. (2012). Genome-Wide Characterization of ISR Induced in Arabidopsis thaliana by Trichoderma hamatum T382 Against Botrytis cinerea Infection. *Front. Plant Sci.* 3:108. doi: 10.3389/fpls.2012.00108

Miettinen-Oinonen, A., and Suominen, P. (2002). Enhanced production of Trichoderma reesei endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. *Appl. Environ. Microbiol.* 68, 3956–3964. doi: 10.1128/AEM.68.8.3956-3964.2002

Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., et al. (2010). Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell* 22, 973–990. doi: 10.1105/tpc.109.069658

Montgomery, D. R. (2007). Soil erosion and agricultural sustainability. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13268–13272. doi: 10.1073/pnas.0611508104

Mukherjee, P. K., Buensanteai, N., Moran-Diez, M. E., Druzhinina, I. S., and Kenerley, C. M. (2012). Functional analysis of non-ribosomal peptide synthetases (NRPSs) in Trichoderma virens reveals a polyketide synthase (PKS)/NRPS hybrid enzyme involved in the induced systemic resistance response in maize. *Microbiology* 158, 155–165. doi: 10.1099/mic.0.052159-0

Ousley, M. A., Lynch, J. M., and Whipps, J. M. (1994). Potential of Trichoderma spp. as consistent plant growth stimulators. *Biol. Fertil. Soils* 17, 85–90. doi: 10.1007/BF00337738

Ousley, M., Lynch, J., and Whipps, J. (1993). Effect of Trichoderma on plant growth: a balance between inhibition and growth promotion. *Microb. Ecol.* 26, 277–285. doi: 10.1007/BF00176959

Pere, J., Puolakka, A., Nousiainen, P., and Buchert, J. (2001). Action of purified Trichoderma reesei cellulases on cotton fibers and yarn. *J. Biotechnol.* 89, 247–255. doi: 10.1016/S0168-1656(01)00308-X

Perera, V., De Torres Zabala, M., Florance, H., Smirnoff, N., Grant, M., and Yang, Z.-R. (2012). Aligning extracted LC-MS peak lists via density maximization. *Metabolomics* doi: 10.1007/s11306-011-0389-x. [Epub ahead of print].

Powlson, D., Gregory, P., Whalley, W., Quinton, J., Hopkins, D., Whitmore, A., et al. (2011). Soil management in relation to sustainable agriculture and ecosystem services. *Food Policy* 36, S72–S87. doi: 10.1016/j.foodpol.2010.11.025

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., et al. (2012). The Pfam protein families database. *Nucleic Acids Res.* 40, D290–D301. doi: 10.1093/nar/gkr1065

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Romano, C. P., Hein, M. B., and Klee, H. J. (1991). Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of Pseudomonas savastanoi. *Genes Dev.* 5, 438–446. doi: 10.1101/gad.5.3.438

Ryder, L. S., Harris, B. D., Soanes, D. M., Kershaw, M. J., Talbot, N. J., and Thornton, C. R. (2012). Saprotrophic competitiveness and biocontrol fitness of a genetically modified strain of the plant-growth-promoting fungus Trichoderma hamatum GD12. *Microbiology* 158, 84–97. doi: 10.1099/mic.0.051854-0

Santner, A., and Estelle, M. (2007). The JAZ proteins link jasmonate perception with transcriptional changes. *Plant Cell* 19, 3839–3842. doi: 10.1105/tpc.107.056960

Sauer, T., Havlík, P., Schneider, U. A., Schmid, E., Kindermann, G., and Obersteiner, M. (2010). Agriculture and resource availability in a changing world: the role of irrigation. *Water Resour. Res.* 46, W06503. doi: 10.1029/2009WR007729

Schmidhuber, J., and Tubiello, F. N. (2007). Global food security under climate change. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19703–19708. doi: 10.1073/pnas.0701976104

Schroeckh, V., Scherlach, K., Nutzmann, H. W., Shelest, E., Schmidt-Heck, W., Schuemann, J., et al. (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14558–14563. doi: 10.1073/pnas.0901870106

Scott-Craig, J. S., Panaccione, D. G., Pocard, J. A., and Walton, J. D. (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus Cochliobolus carbonum is encoded by a 15.7-kilobase open reading frame. *J. Biol. Chem.* 267, 26044–26049.

Segarra, G., Van der Ent, S., Trillas, I., and Pieterse, C. M. (2009). MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biol.* 11, 90–96. doi: 10.1111/j.1438-8677.2008.00162.x

Seidl, V., Seibel, C., Kubicek, C. P., and Schmoll, M. (2009). Sexual development in the industrial workhorse Trichoderma reesei. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13909–13914. doi: 10.1073/pnas.0904936106

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi: 10.1146/annurev-phyto-073009-114450

Staswick, P. (2009). Plant hormone conjugation: a signal decision. *Plant Signal. Behav.* 4, 757–759. doi: 10.4161/psb.4.8.9180

Staswick, P. E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell* 16, 2117–2127. doi: 10.1105/tpc.104.023549

Stergiopoulos, I., and de Wit, P. J. (2009). Fungal effector proteins. *Annu. Rev. Phytopathol.* 47, 233–263. doi: 10.1146/annurev.phyto.112408.132637

Steyaert, J. M., Weld, R. J., Mendoza-Mendoza, A., and Stewart, A. (2010). Reproduction without

sex: conidiation in the filamentous fungus Trichoderma. *Microbiology* 156, 2887–2900. doi: 10.1099/mic.0.041715-0

Taylor, J. W., and Berbee, M. L. (2006). Dating divergences in the Fungal Tree of Life: review and new analyses. *Mycologia* 98, 838–849. doi: 10.3852/mycologia.98.6.838

Thornton, C. R. (2005). Use of monoclonal antibodies to quantify the dynamics of alpha-galactosidase and endo-1,4-beta-glucanase production by Trichoderma hamatum during saprotrophic growth and sporulation in peat. *Environ. Microbiol.* 7, 737–749. doi: 10.1111/j.1462-2920.2005.00747.x

Thornton, C. R. (2008). Tracking fungi in soil with monoclonal antibodies. *Eur. J. Plant Pathol.* 121, 347–353. doi: 10.1007/s10658-007-9228-3

Ton, J., De Vos, M., Robben, C., Buchala, A., Metraux, J. P., Van Loon, L. C., et al. (2002). Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance. *Plant J.* 29, 11–21. doi: 10.1046/j.1365-313x.2002.01190.x

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., Lorito, M. (2011). The beneficial effect of Trichoderma spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Valent, B., Farrall, L., and Chumley, F. G. (1991). Magnaporthe grisea genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127, 87–101.

van de Mortel, J. E., de Vos, R. C., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., et al. (2012). Metabolic and transcriptomic changes induced in Arabidopsis by the rhizobacterium Pseudomonas fluorescens SS101. *Plant Physiol.* 160, 2173–2188. doi: 10.1104/pp.112.207324

van Hulten, M., Pelser, M., van Loon, L. C., Pieterse, C. M., and Ton, J. (2006). Costs and benefits of priming for defense in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5602–5607. doi: 10.1073/pnas.0510213103

Verhagen, B. W., Glazebrook, J., Zhu, T., Chang, H. S., van Loon, L. C., and Pieterse, C. M. (2004). The transcriptome of rhizobacteria-induced systemic resistance in arabidopsis. *Mol. Plant Microbe Interact.* 17, 895–908. doi: 10.1094/MPMI.2004.17.8.895

Vinale, F., Flematti, G., Sivasithamparam, K., Lorito, M., Marra, R., Skelton, B. W., et al. (2009). Harzianic acid, an antifungal and plant growth promoting metabolite from Trichoderma harzianum. *J. Nat. Prod.* 72, 2032–2035. doi: 10.1021/np900548p

Walton, J. D. (2006). HC-toxin. *Phytochemistry* 67, 1406–1413. doi: 10.1016/j.phytochem.2006.05.033

Ward, J. L., Forcat, S., Beckmann, M., Bennett, M., Miller, S. J., Baker, J. M., et al. (2010). The metabolic transition during disease following infection of *Arabidopsis thaliana* by *Pseudomonas syringae*pv. *tomato*. *Plant J.* 63, 443–457. doi: 10.1111/j.1365-313X.2010.04254.x

Ward, J. L., Harris, C., Lewis, J., and Beale M. H. (2003). Assessment of ^{1}H-NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. *Phytochemistry* 62, 949–957. doi: 10.1016/S0031-9422(02)00705-7

Wu, C. Y., Chen, C. L., Lee, Y. H., Cheng, Y. C., Wu, Y. C., Shu, H. Y., et al. (2007). Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. *J. Biol. Chem.* 282, 5608–5616. doi: 10.1074/jbc.M609726200

Zerbino, D. R., and Birney, E. (2008). Velvet: algorithms for *de novo* short read assembly using de Bruijn graphs. *Genome Res.* 18, 821–829. doi: 10.1101/gr.074492.107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 March 2013; accepted: 25 June 2013; published online: 30 July 2013.

*Citation: Studholme DJ, Harris B, Le Cocq K, Winsbury R, Perera V, Ryder L, Ward JL, Beale MH, Thornton CR and Grant M (2013) Investigating the beneficial traits of Trichoderma hamatum GD12 for sustainable agriculture—insights from genomics. Front. Plant Sci. **4**:258. doi: 10.3389/fpls.2013.00258*

This article was submitted to Frontiers in Plant-Microbe Interaction, a specialty of Frontiers in Plant Science.

Copyright © 2013 Studholme, Harris, Le Cocq, Winsbury, Perera, Ryder, Ward, Beale, Thornton and Grant. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.