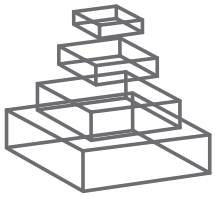
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CURRENT CHALLENGES IN PLANT CELL WALLS

Hosted by
Jose M. Estevez and Seth DeBolt

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CURRENT CHALLENGES IN PLANT CELL WALLS

Hosted By:
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Cell morphogenesis and the arrangement of diverse cell shapes form the foundation for building a multicellular organism. In plants, cells are surrounded by a rigid cell wall that extends in a highly controlled manner to constrain the cells internal turgor pressure and define cell shape. Despite the importance of the cell wall for plant physiology, a major challenge remains to characterize the fundamental organization of all key wall components and determine how their biosynthesis and structure-function relationship are regulated. This special topics feature in *Frontiers in Plant Physiology* encompasses the ongoing research effort to meet this challenge. A series of perspective papers highlight the increasingly wide range of disciplinary and multidisciplinary approaches, organisms and cell types that are being employed to decode cell wall biosynthesis. This special feature welcomes outstanding contributions addressing fundamental questions in cell wall biology.

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Current challenges in plant cell walls: editorial overview

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EXPANDING METHODOLOGIES AND MODELS TO STUDY THE PLANT CELL WALLS

An exciting feature of this special issue was the injection of a series of papers dealing with developed and developing methodological approaches applied to cell wall biosynthesis. For example, our understanding of cell wall synthesis and remodeling has been limited by a lack of suitable chemical probes that are compatible with live cell imaging. Here, Wallace and Anderson (2012) summarize the currently available molecular tool box of probes available for cell wall polysaccharide imaging in plants, with particular emphasis on recent advances in small molecule-based fluorescent probes such as the cellulose-binding dyes S4B and xyloglucan-binding dye 7GFE. In addition, these authors investigated the potential of "click" chemistry to label specific cell wall polysaccharides. The term "Click" chemistry describes chemistry tailored to generate substances quickly and reliably by joining small units together. They explore selectivity issues and highlight how structure – activity relationships of click sugar variants may be used to elucidate cell wall structural associations. Gonneau et al. (2012) carried on with live cell reporter theme and explored a large panel of fluorescent imaging techniques and reporters currently available for the study of tissues, cells, and cellular components in three dimensions (3D) or even in 4D (3D + time) in plants. These imaging techniques hold promise to greatly improve our ability to ask cause and effect questions regarding the dynamic construction and deconstruction of cell wall architecture.

N-glycosylation is one of the most common and complex post-translational modifications of secreted eukaryotic proteins. Yet in plants, the functional significance of *N*-glycosylation has been poorly explored and lags far behind other eukaryotic models. Ruiz-May et al. (2012a) provide a seminal review on the range of existing analytical technologies for large-scale *N*-glycoprotein analysis. This review provides insight into the myriad of hurdles one may expect during glycopeptide amino acid sequencing by mass spectral approaches. In turn, the authors present a step-by-step guide to selective enrichment, improved analytical platforms, and software that can be used to wade through MS fragmentation data. This framework is then employed in a review of plant *N*-glycoprotein synthesis and trafficking (Ruiz-May et al., 2012b), which exemplifies those proteins that are cell wall localized. Thus, the contribution of Ruiz-May and coworkers provides a targeted resource for researchers who meet a cell wall localized glycoprotein. Bauer (2012) then provides a review of MS analytical techniques to identify structural information about complex carbohydrate (non-protein) composition of the plant cell wall. Here, powerful MS methods are detailed for the elucidation of the structure of oligosaccharides derived from hemicelluloses and pectins. The author illustrates how information on sequence, linkages, branching, and modifications can be obtained from characteristic fragmentation patterns. This method is of further note when combined with molecular genetics (mutant analysis) to elucidate the function of a target gene.

Global transcript analyses based on a wealth of available microarray datasets have revealed that genes that are in the same pathway are often transcribed in a coordinate manner. Here Ruprecht and Persson (2012) have summarized recent developments in software as well as combining transcriptional data from multiple plant species to learn about how particular cell wall processes are different, rather than the same. This exciting update provides successful examples of where expression profiling has helped to identify genes involved in the formation of cellulose, hemicelluloses, and lignin. In addition, they illustrate potential pitfalls and future perspectives. The broad employment of next generation sequencing for RNAseq experiments will surely expand the available data (as not all coding sequences are represented on a given microarray chip). This will require some software refinement, but also see further gains resulting from coexpression profiling in the near future. The use of deep sequencing platforms like Illumina's Genome Analyzer or ABI's SOLiD was reviewed by Vidaurre and Bonetta (2012) in the context of map based cloning. Here, a path to rapidly accelerating forward genetics in the context of cell wall biosynthesis and deconstruction was paved. Anyone who has laboriously mapped a missense mutation will greatly appreciate the ability to pinpoint a mutation selected from within an EMS (ethyl methyl suflonate) population in a single sequencing run. This combination of a classical genetics approach and cutting edge technology can holds immediate promise for gene identification.

To assemble plant cell wall polysaccharides and glycan structures on glycoproteins, the plant needs extensive biosynthetic machinery, and it has been estimated that over 2000 gene products are involved in making and maintaining the wall. The Carbohydrate Active Enzyme (CAZy) database is an invaluable resource for glycobiology and currently contains 45 glycosyltransferase (GT) families that are represented in plants. Hansen et al. (2012) describe the putative GTs that are not currently classified in the CAZy database. These families include proteins with domain of unknown function (DUF). The evidence for certain members of the DUF class proteins being GTs and their possible roles in cell wall biosynthesis are discussed.

Model organisms have been instrumental in fundamental discovery based science over the past several decades. A model for grass cell wall biology has lagged behind its dicotyledonous counterpart, *Arabidopsis*. Handakumbura and Hazen (2012) provide a review of the transcriptional wiring of secondary cell wall biosynthesis in grasses. Many grasses have abundant sequence information including Corn (*Zea mays* L.), barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.) and currently *Brachypodium distachyon* is emerging as a model for functional genomics and gene discovery. Handakumbura and Hazen (2012) focus on NAC and MYB transcription factors and review methods for establishing functionality of these regulatory genes. Characterization of the transcriptional regulatory networks of eudicots and grasses remains of high priority in efforts to optimize biomass quality. Perhaps better still than a model organism, is a model cell type in order to genetically decode a target process. Haigler et al. (2012) and Haughn and Western (2012) examine this notion using cotton fibers and *Arabidopsis* seed coats respectively. Both cell types display intriguing polarized differentiation characteristics and represent models to study the role of cell walls in morphogenesis as well as general cell wall biosynthesis. The *Arabidopsis* seed coat differentiation involves the deposition of a large pectin rich mucilage pocket on the outer side of the seed coat (Haughn and Western, 2012). Therefore, the cell type lends itself to investigating the chemical and physical properties of different cell wall components and their interactions *in vivo*, especially with respect to its major pectic component rhamnogalacturonan I (RGI). Similarly, cotton fibers are single-celled extensions of the seed epidermis. They can be isolated in pure form as they undergo staged differentiation including primary cell wall synthesis during elongation and nearly pure cellulose synthesis during secondary wall thickening. Haigler et al. (2012) exemplify the potential to combine virus induced gene silencing as a genetic tool with systematically staging of developmental phenotypes to provide a highly relevant tool to dissect open questions in cellulose biosynthesis.

CONCEPTS IN CELL WALL BIOLOGY

Plant cell walls provide structural support during development and represent the first line of defense against biotic and abiotic stress. In recent years evidence has accumulated that a dedicated plant cell wall integrity maintenance mechanism exists. Here, Hamann (2012) reviewed the available evidence regarding the mode of action of the cell wall integrity maintenance mechanism and discuss its role in the context of biotic plant stress response mechanisms. Recent strides in our understanding of surface defense or cell wall-associated defenses induced by pathogen perception have been made. Underwood (2012) reviews these in context of the impact of these changes in cell wall polymers and highlights significant unanswered questions driving future research in the area. These include plasma membrane-cell wall adhesion, a critical site for pathogen recognition and signal transduction. Along this theme, the wall-associated kinases (WAKs, receptor-like kinases) that associate with pectin in the cell wall and contain a cytoplasmic kinase domain may play a role in such a transducing signal on wall development and or pathogen ingress. Indeed, Kohorn and Kohorn (2012) delve into the world of WAKs and investigate a role in receptor sensing for both short oligogalacturonic acid fragments generated during pathogen exposure or wounding, and longer pectins resident in plant cell wall biosynthesis. The authors look into the capability of WAKs to bind and respond to several types of pectins and highlight the many remaining unanswered questions.

The plasma membrane-cell wall continuum also appears to require a membrane organizational matrix. Schrick et al. (2012) highlights the possible direct or indirect involvement of membrane structural sterols in facilitating the cellulose biosynthetic machinery. In one possible scenario, molecular interactions in sterol-rich plasma membrane microdomains or another form of sterol-dependent membrane scaffolding may be needed for correct subcellular localization, structural integrity, and/or activity of the cellulose synthase (CESA) machinery. In this context, Davis (2012) takes a biochemical view and rationalizes how polymers requires transport across a membrane and many polysaccharide biosynthetic enzymes appear to have both transporter and transferase activities. Drawing on what is known about such dual-function enzymes in other species, the author examines the required number of transmembrane helices, phosphorylation regulation, and the importance of the membrane environment surrounding the enzyme. This review does an exceptional job of highlighting where deficiencies reside in current models and sets up many questions for immediate elucidation. Of course, many cell wall enzymes modify the structurally diverse polymers of the cell wall, an example being the elusive enzymes responsible for extensive *O*-acetylation. Exactly how plants *O*-acetylate wall polymers and why has remained elusive until recently, when two protein families were identified in the model plant *Arabidopsis* that are involved in the *O*-acetylation of wall polysaccharides. Gille and Pauly (2012) provide a detailed review discussing the role of these two protein families in polysaccharide *O*-acetylation and outlines the differences and similarities of polymer acetylation mechanisms in plants, fungi, bacteria, and mammals.

Dropping beneath (literally) the plasma membrane-cell wall continuum, both the actin and microtubule cytoskeleton are involved in assuring the proper distribution, organization, and dynamics of CESA complexes (CSCs). The review of Lei et al. (2012) provides an update on the characterization of the composition, regulation, and trafficking of CSCs and also introduces a newly identified CESA interactive protein1 (CSI1). The CESA superfamily of proteins contains several sub-families of closely related CESA-LIKE (CSL) sequences. Among these, the CSLA and CSLC families are closely related to each other and are the most evolutionarily divergent from the CESA family. Liepman and Cavalier (2012) summarize some of the most significant progress that has been made with the functional characterization of CSLA and CSLC genes, which have been shown to encode enzymes with 1,4-β-glycan synthase activities involved in the biosynthesis of mannan and possibly xyloglucan backbones, respectively. The authors highlight a series of key questions, whose answers likely will provide further insight about the specific functions of members of the CSLA/C families.

Plant cell walls are not only composed by large amounts and diverse types of polysaccharides but also *N*- and *O*-glycoproteins. Within *O*-glycoproteins, Arabinogalactan-glycoproteins (AGPs) and Extensins (EXTs) are commonly glycosylated. The genetic set up and the enzymes that define the *O*-glycosylation sites and transfer the activated sugars to cell wall glycoprotein EXTs have remained unknown for a long time. Velasquez et al. (2012)

summarizes several of the genes recently discovered that confers the posttranslational modifications, i.e., proline hydroxylation and subsequent *O*-glycosylation, of the EXTs. The effects of posttranslational modifications on the structure and function of EXTs in plant cell walls is also discussed. The location and diversity of AGPs have made them attractive targets as modulators of plant development but definitive proof of their direct role(s) in biological processes remains elusive. Tan et al. (2012) further reviews the current state of knowledge on AGPs. These authors identify key challenges impeding progress in the field and propose approaches using modern bioinformatic, (bio)chemical, cell biological, molecular, and genetic techniques that could be applied to address these conceptual gaps.

Pectins are one of the most complex polymers within the plant cell wall. Bar-Peled et al. (2012) draws attention to the evidence showing that the pectic polysaccharide rhamnogalacturonan II (RG-II) exists in the primary cell wall as a borate cross-linked dimer and that this dimer is required for the assembly of a functional wall and for normal plant growth and development. RG-II structure and crosslinking is well conserved in vascular plants and that RG-II likely appeared early in the evolution of land plants. Although many of the genes involved in the generation of the nucleotide sugars used for RG-II synthesis have been functionally characterized, only one GT involved in the assembly of RG-II has been identified. In addition, these authors provide an overview of the formation of the activated sugars required for RG-II synthesis and point to the possible cellular and metabolic processes that could be involved in assembling and controlling the formation of a borate cross-linked RG-II molecule. They also discuss how nucleotide sugar synthesis is compartmentalized and how this may control the flux of precursors to facilitate and regulate the formation of RG-II. Still focusing on pectin, Peaucelle et al. (2012) provide a compelling investigation into the role of pectin in cell wall mechanics. Specifically, in the past most studies have focused on the role of the cellulose/xyloglucan network (reviewed in Zabotina, 2012) and the enigmatic wall-loosening agents expansins. By contrast, cell wall synthesis in the land plants close evolutionary progenitor, the Charophycean algae, is coupled to cell wall extensibility by a chemical Ca^{2+}-exchange mechanism between Ca^{2+}–pectate complexes. In land plants, this mechanism would provide an intriguing alternative. Authors review the current evidence for the existence in terrestrial plants of a "primitive" Ca^{2+}-pectate-based growth control mechanism in parallel to the more recent, land plant-specific, expansin-dependent process.

Plant cell walls are vey dynamic and change in both structure and composition over time, cell development, and differentiation, environmental conditions, etc. On the other hand, despite differences in composition and structure between dicots and monocots, all flowering plants respond to a defined suite of growth regulators in similar tissue-specific ways and also exhibit similar growth physics. Using this rationale, Benatti et al. (2012) examine the physiological control of cell expansion emerging from genetic functional analyses, mostly in *Arabidopsis* and other dicots, and a few examples of genes of potential functions in grass species. These authors discuss examples of cell wall architectural features that impact growth, independent of composition, and progress in identifying proteins involved in transduction of growth signals, and the integration of their outputs in the molecular machinery of wall expansion. In another insightful minireview, Cosgrove and Jarvis (2012) discuss the essential differences between primary and secondary cell walls and identify crucial gaps in our knowledge of their structure and biomechanics. The authors provide compelling evidence that there is a particular need to revise and correct current "cartoon depictions" of plant cell walls, so that they are more consistent with present data available from diverse approaches.

PLANT CELL WALLS: STRUCTURAL DIVERSITY AND EVOLUTIONARY ASPECTS

While all of the contributions in this special issue provide important insights into cell walls that ultimately fit into an evolutionary context, three reviews attempt to piece together cell wall evolutionary processes (Domozych et al., 2012; Fangel et al., 2012; Roberts et al., 2012). The first examines the green algae, which represent a large group of morphologically diverse photosynthetic eukaryotes that display much diversity in their cell walls. They compare and contrast the Charophycean green algae, which possess cell walls compositionally similar embryophyte walls with that of the Ulvophycean seaweeds, which have cell wall components whose most abundant fibrillar constituents may change from cellulose to β-mannans to β-xylans and during different life cycle phases. Cell wall composition is incorporation of emerging genomic and transcriptomic data to divulge evolutionary trends. The second review grapples with the diversity of plant cell wall structures. An additional review by Roberts et al. (2012) presents the molecular and physiological features of cell wall biosynthesis of the moss *Physcomitrella patens*. The cell walls of mosses and vascular plants are composed of the same classes of polysaccharides as found in moss, but with differences inside chain composition and structure. Similarly, the genomes of *P. patens* and angiosperms encode the same families of cell wall GTs, yet, in many cases these families have diversified independently in each lineage. Combined, these three reviews capture the spectrum of cell wall evolutionary processes and highlight numerous underexplored cell walls types and processes that are underexplored and could provide a rational starting point in isolating industrially useful bioproducts.

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REFERENCES

Bar-Peled, M., Urbanowicz, B. R., and O'Neill, M. A. (2012). The synthesis and origin of the pectic polysaccharide rhamnogalacturonan II – insights from nucleotide sugar formation and diversity. *Front. Plant Sci.* 3:92. doi: 10.3389/fpls.2012.00092

Bauer, S. (2012). Mass spectrometry for characterizing plant cell wall polysaccharides. *Front. Plant Sci.* 3:45. doi: 10.3389/fpls.2012.00045

Benatti, M. R., Penning, B. W., Carpita, N. C., and McCann, M. C. (2012). We are good to grow: dynamic integration of cell wall architecture with the machinery of growth. *Front. Plant Sci.* 3:187. doi: 10.3389/fpls.2012.00187

Cosgrove, D. J., and Jarvis, M. C. (2012). Comparative structure and biomechanics of plant primary and secondary cell walls. *Front. Plant Sci.* 3:204. doi: 10.3389/fpls.2012.00204

Davis, J. K. (2012). Combining polysaccharide biosynthesis and transport in a single enzyme: dual-function cell wall glycan synthases. *Front. Plant Sci.* 3:138. doi: 10.3389/fpls.2012.00138

Domozych, D. S., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P., and Willats, W. G. T. (2012). The cell walls

of green algae: a journey through evolution and diversity. *Front. Plant Sci.* 3:82. doi: 10.3389/fpls.2012.00082

Fangel, J. U., Ulvskov, P., Knox, J. P., Mikkelsen, M. D., Harholt, J., Popper, Z. A., et al. (2012). Cell wall evolution and diversity. *Front. Plant Sci.* 3:152. doi: 10.3389/fpls.2012.00152

Gille, S., and Pauly, M. (2012). O-acetylation of plant cell wall poly saccharides. *Front. Plant Sci.* 3:12. doi: 10.3389/fpls.2012.00012

Gonneau, M., Höfte, H., and Vernhettes, S. (2012). Fluorescent tags to explore cell wall structure and dynamics. *Front. Plant Sci.* 3:145. doi: 10.3389/fpls.2012.00145

Haigler, C. H., Betancur, L., Stiff, M. R., and Tuttle, J. R. (2012). Cotton fiber: a powerful single-cell model for cell wall and cellulose research. *Front. Plant Sci.* 3:104. doi: 10.3389/fpls.2012.00104

Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms. *Front. Plant Sci.* 3:77. doi: 10.3389/fpls.2012.00077.

Handakumbura, P. P., and Hazen, S. P. (2012). Transcriptional regulation of grass secondary cell wall biosynthesis: playing catch-up with *Arabidopsis thaliana*. *Front. Plant Sci.* 3:74. doi: 10.3389/fpls.2012.00074

Hansen, S. F., Harholt, J., Oikawa, A., and Scheller, H. V. (2012). Plant glycosyltransferases beyond CAZy: a perspective on DUF families. *Front. Plant Sci.* 3:59. doi: 10.3389/fpls.2012.00059

Haughn, G. W., and Western, T. L. (2012). Arabidopsis seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function. *Front. Plant Sci.* 3:64. doi: 10.3389/fpls.2012.00064

Kohorn, B. D., and Kohorn, S. L. (2012) The cell wall-associated kinases, WAKs, as pectin receptors. *Front. Plant Sci.* 3:88. doi: 10.3389/fpls.2012.00088

Lei, L., Li, S., and Gu, Y. (2012). Cellulose synthase complexes: composition and regulation. *Front. Plant Sci.* 3:75. doi: 10.3389/fpls.2012.00075

Liepman, A. H., and Cavalier, D. M. (2012). The CELLULOSE SYNTHASE-LIKE A and CELLULOSE SYNTHASE-LIKE C families: recent advances and future perspectives. *Front. Plant Sci.* 3:109. doi: 10.3389/fpls.2012.00109

Peaucelle, A., Braybrook, S., and Höfte, H. (2012). Cell wall mechanics and growth control in plants: the role of pectins revisited. *Front. Plant Sci.* 3:121. doi: 10.3389/fpls.2012.00121

Roberts, A. W., Roberts, E. M., and Haigler, C. H. (2012). Moss cell walls: structure and biosynthesis. *Front. Plant Sci.* 3:166. doi: 10.3389/fpls.2012.00166.

Ruiz-May, E., Thannhauser, T. W., Zhang, S., and Rose, J. K. C. (2012a). Analytical technologies for identification and characterization of the plant N-glycoproteome. *Front. Plant Sci.* 3:150. doi: 10.3389/fpls.2012.00150.

Ruiz-May, E., Kim, S.-J., Brandizzi, F., and Rose, J. K. C. (2012b). The secreted plant N-glycoproteome and associated secretory pathways. *Front. Plant Sci.* 3:117. doi: 10.3389/fpls.2012.00117

Ruprecht, C., and Persson, S. (2012). Co-expression of cell wall-related genes: new tools and insights. *Front. Plant Sci.* 3:83. doi: 10.3389/fpls.2012.00083

Schrick, K., DeBolt, S., and Bulone, V. (2012). Deciphering the molecular functions of sterols in cellulose biosynthesis. *Front. Plant Sci.* 3:84. doi: 10.3389/fpls.2012.00084

Tan, L., Showalter, A. M., Egelund, J., Hernandez-Sanchez, A., Doblin, M. S., and Bacic, A. (2012). Arabinogalactan- proteins and the research challenges for these enigmatic plant cell surface proteoglycans. *Front. Plant Sci.* 3:140. doi: 10.3389/fpls.2012.00140

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. *Front. Plant Sci.* 3:85. doi: 10.3389/fpls.2012.00085

Velasquez, M., Salter, J. S., Dorosz, J. G., Petersen, B. L., and Estevez, J. M. (2012). Recent advances on the post-translational modifications of EXTs and their roles in plant cell walls. *Front. Plant Sci.* 3:93. doi: 10.3389/fpls.2012.00093

Vidaurre, D., and Bonetta, D. (2012). Accelerating forward genetics for cell wall deconstruction. *Front. Plant Sci.* 3:119. doi: 10.3389/fpls.2012.00119

Wallace, I. S., and Anderson, C. T. (2012). Small molecule probes for plant cell wall polysaccharide imaging. *Front. Plant Sci.* 3:89. doi: 10.3389/fpls.2012.00089

Zabotina, O. A. (2012). Xyloglucan and its biosynthesis. *Front. Plant Sci.* 3:134. doi: 10.3389/fpls.2012.00134.

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O-acetylation of plant cell wall polysaccharides

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Plant cell walls are composed of structurally diverse polymers, many of which are *O*-acetylated. How plants *O*-acetylate wall polymers and what its function is remained elusive until recently, when two protein families were identified in the model plant *Arabidopsis* that are involved in the *O*-acetylation of wall polysaccharides – the reduced wall acetylation (RWA) and the trichome birefringence-like (TBL) proteins. This review discusses the role of these two protein families in polysaccharide *O*-acetylation and outlines the differences and similarities of polymer acetylation mechanisms in plants, fungi, bacteria, and mammals. Members of the TBL protein family had been shown to impact pathogen resistance, freezing tolerance, and cellulose biosynthesis. The connection of TBLs to polysaccharide *O*-acetylation thus gives crucial leads into the biological function of wall polymer *O*-acetylation. From a biotechnological point understanding the *O*-acetylation mechanism is important as acetyl-substituents inhibit the enzymatic degradation of wall polymers and released acetate can be a potent inhibitor in microbial fermentations, thus impacting the economic viability of, e.g., lignocellulosic based biofuel production.

Keywords: cell wall, polysaccharides, *O*-acetylation, acetyltransferase

INTRODUCTION

The cells of higher plants are encased in a wall consisting of multiple, structurally complex, highly substituted polymers. One of the substituents found on many of these polymers is *O*-linked acetate. How the *O*-acetyl-substituent is transferred to the wall polymers and its role in the life cycle of a plant is not known. From an industrial perspective *O*-acetyl-groups on plant polymers contribute significantly to their viscosity and gelation properties and hence applicability, e.g., in the food industry (Rombouts and Thibault, 1986; Huang et al., 2002). Recently, *O*-acetylation of cell wall polysaccharides has received increased attention, as wall polymers, the primary constituent of plant biomass, can be used as a renewable source for the production of biofuels and other commodity chemicals (Pauly and Keegstra, 2008; Carroll and Somerville, 2009). During the processing of plant biomass acetylation can reduce saccharification yields, if not removed by pre-treatments (Selig et al., 2009). Even if acetate is released during processing it inhibits some sugar-to-ethanol fermenting organisms (Helle et al., 2003). Techno-economic models predict that a 20% reduction in biomass *O*-acetylation could result in a 10% reduction in ethanol price (Klein-Marcuschamer et al., 2010). Hence, a major goal in plant research in the biofuel area is to try to reduce *O*-acetylation in the walls of plant feedstocks.

MANY PLANT CELL WALL POLYMERS ARE *O*-ACETYLATED

O-acetyl-substituents are present on nearly all wall polymers with the exception of cellulose, β-1,3-1,4-linked glucan in grasses, and the wall glycoproteins. In particular the various hemicelluloses, the pectic polysaccharides and the polyphenol lignin can be *O*-acetylated (**Table 1** and references therein). The degree of *O*-acetylation can vary depending on species, tissue type, and developmental state as has been observed with any other wall substituent (Pauly and Keegstra, 2010). In the dicot hemicellulose xyloglucan (XyG) only the sidechain galactosyl-residue is *O*-acetylated (Pauly, 1999; Scheller and Ulvskov, 2010; Gille et al., 2011b). In contrast, in members of the *Solanaceae* and the *Poaceae* the glucosyl-residues of the XyG backbone are *O*-acetylated (Gibeaut et al., 2005; Jia et al., 2005). Presumably, polymer backbone *O*-acetylation has a larger effect on the polymer properties such as conformation and hydrophobicity, while on the sidechain *O*-acetates might present just an alternative substituent. Other polymers, whose glycan-backbones can be *O*-acetylated, include arabinoxylan (Bastawde, 1992; Van Dongen et al., 2011), glucomannans present in softwood (Lundqvist et al., 2002) or in storage polymers (Gille et al., 2011a), as well as the pectic polysaccharides homogalacturonan (Liners et al., 1994) and rhamnogalacturonan I (Schols and Voragen, 1994). Lignin can also be *O*-acetylated; up to 58% of the phenolic units were found to be *O*-acetylated in species such as Kenaf (Del Río et al., 2008).

The position of the *O*-acetyl-group has been identified in nearly all of the polymers (**Table 1**). Some monosaccharides present in wall polymers can be substituted with two *O*-acetyl-groups such as XyG, arabinoxylan, and some of the pectic polysaccharides. It should be noted that *O*-acetyl-groups can migrate in aqueous solutions to non-substituted hydroxyl-groups within the same glycosyl-residue by a chemical, non-protein mediated mechanism forming an equilibrium of acetylation on the various positions, e.g., O-2 and O-3 on xylosyl-residues in xylan (Kabel et al., 2003). Migration has also been observed on the galactosyl-residue in XyG (Pauly, 1999).

MECHANISMS OF CELL WALL POLYMER *O*-ACETYLATION: COMPARISON TO OTHER ORGANISMS

Despite the abundance of *O*-acetyl-substituents on various wall polymers virtually nothing was known about the molecular mechanism of *O*-acetylation in plants until recently. More information

Table 1 | *O*-acetylated cell wall polysaccharides.

Wall polymer	Species	Backbone/ sidechain	Residue	Position	Mono-/ Di-acetylated	Reference(s)
HEMICELLULOSE						
Xyloglucan	Dicots (with the exceptions below)	Sidechain	Gal	O-6; (O-3,O-4)	Mono/di	Kiefer et al. (1989); Pauly (1999)
	Solanaceae	Backbone	Glc	O-6	Mono	Hoffman et al. (2005); Jia et al. (2005)
		Sidechain	Ara*f*	O-5	Mono	
	Poaceae	Backbone	Glc	O-6	Mono	Gibeaut et al. (2005); Hsieh and Harris (2009)
(Glucurono)-arabinoxylan		Backbone	Xyl	O-2, O-3	Mono/Di	Kabel et al. (2003) Teleman et al. (2000)
		Sidechain	Ara*f*	O-2	Mono	Ishii (1991)
Gluco(galacto-) mannan		Backbone	Man	O-2, O-3		Lundqvist et al. (2002)
PECTIC POLYSACCHARIDES						
Homogalacturonan		Backbone	GalA	O-2, O-3	Mono/di	Ishii (1997)
RGI		Backbone	GalA	O-2, O-3	Mono/di	Ishii (1997)
		Backbone	Rha	O-3	Mono	Sengkhamparn et al. (2009)
RGII		Sidechain	2-*O*-Me-Fuc	n.d.	Mono	O'Neill et al. (2004)
LIGNIN						
			Syringyl-/Guaiacyl-units	γ-C of sidechain	Mono	Del Río et al. (2007)

n.d., not determined.

had been amassed from the polysaccharide *O*-acetylation systems in bacteria, fungi, and mammals. In the fungal pathogen *Cryptococcus neoformans* the mannosyl-residues of the capsular polysaccharide glucuronoxylomannan can be *O*-acetylated (Cherniak and Sundstrom, 1994). The responsible protein, CnCas1p, has been identified as essential for the *O*-acetylation of this capsular polysaccharide (Janbon et al., 2001). CnCas1p contains 12 transmembrane domains as well as a 347 aa loop predicted to face the Golgi lumen between the first and second transmembrane domain from the N-terminus (**Figure 1**). Orthologs to CnCas1p were also identified in the human and drosophila genomes (Janbon et al., 2001). The human ortholog HsCasD1 has a very similar protein structure; multiple (13) transmembrane domains and a large loop, also predicted to face a membrane enclosed lumen (Arming et al., 2011; **Figure 1**). Ectopic expression of HsCasD1 leads to an increase in the *O*-acetylation of sialic acids and subcellular localization experiments located the protein to the Golgi apparatus (Arming et al., 2011). In bacteria such as *Staphylococcus*, *Campylobacter*, *Helicobacter*, *Neisseria*, and *Bacillus* the muramyl-residue in the wall polymer peptidoglycan is *O*-acetylated (Clarke and Dupont, 1992; Moynihan and Clarke, 2010). In Gram-positive bacteria *O*-acetylation of peptidoglycan is facilitated by OatA (Bera et al., 2005; Aubry et al., 2011; Bernard et al., 2011). Similar to the fungal and human Cas1 proteins, also the bacterial OatA proteins consist of multiple transmembrane domains and a large loop (Bernard et al., 2011; see LpOatA in **Figure 1**). In contrast, in Gram-negative bacteria such as *Neisseria gonorrhoeae* or *Bacillus anthracis*, the peptidoglycan acetylation is facilitated by two separate proteins – PatA and PatB (Moynihan and Clarke, 2010; Laaberki et al., 2011). While PatA represents a protein with multiple transmembrane domains, PatB is a membrane bound periplasmic protein reminiscent of the loop present in the Cas1 proteins in other organisms (**Figure 1**). Another *O*-acetylated bacterial polysaccharide is alginate – a viscous exopolysaccharide produced by certain bacteria such as the human pathogen *Pseudomonas aeruginosa* (Franklin and Ohman, 1996). Here, three proteins were shown to be required for alginate acetylation; PaAlgI, PaAlgJ, and PaAlgF (Franklin and Ohman, 1993, 1996; Shinabarger et al., 1993; **Figure 1**). The PaAlgI protein contains multiple transmembrane domains while the PaAlgJ protein is a membrane-anchored protein with one transmembrane domain and a large loop facing the extracellular space (**Figure 1**). The function of the periplasmic PaAlgF protein is unknown but it is thought to interact with PaAlgI and PaAlgJ in the alginate biosynthetic complex (Franklin and Ohman, 2002).

Recent findings indicate that plants harbor a similar *O*-acetylation system. Based on protein homology to the CnCas1p protein from *Cryptococcus*, a family of four proteins has been identified in *Arabidopsis* that lead to a reduced cell wall acetylation phenotype (*rwa*-mutants; Manabe et al., 2011; Lee et al., 2011). RWA consists of 10 transmembrane domains but lacks the extended loop found in CnCas1p (**Figure 1**). Mutants affected only in RWA2 where shown to have an overall reduction of 20% in wall polymer acetylation levels with multiple polymers being affected including pectin, XyG, and xylan (Manabe et al., 2011).

A second protein involved in plant polysaccharide *O*-acetylation was identified using a forward genetic screen for mutants with altered xyloglucan structures (*axy*; Gille et al., 2011b). Knock-out mutants of the identified gene *AXY4* lack *O*-acetyl-substituents on XyG completely in non-seed tissues,

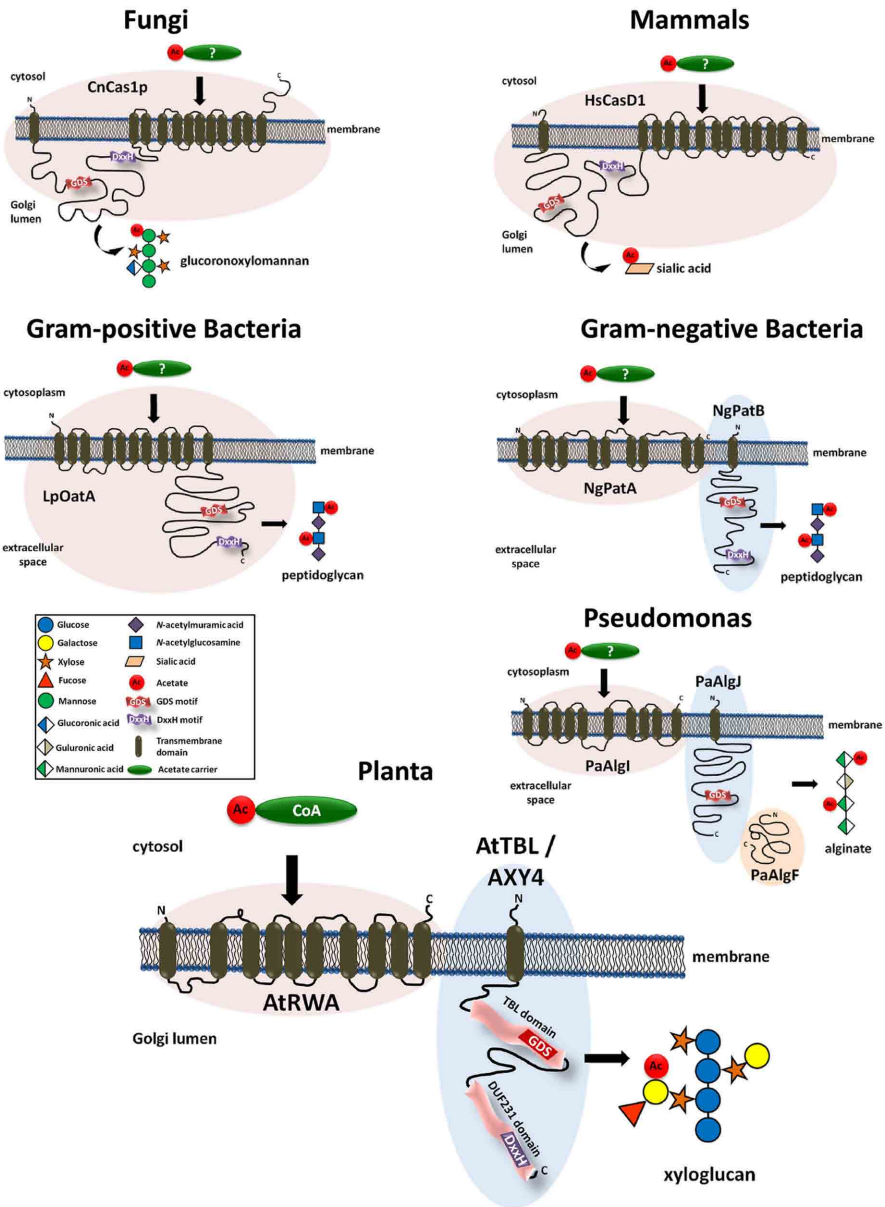


FIGURE 1 | Models of proteins involved in the *O*-acetylation of carbohydrates in various organisms. Presented are proteins that have been shown to impact *O*-acetylation of glucuronoxylomannan in *Cryptococcus neoformans* (Fungi), sialic acid in humans (Mammals), peptidoglycan in Gram-positive and Gram-negative bacteria, alginate in *Pseudomonas aeruginosa*, and xyloglucan in *Arabidopsis thaliana* (Plants). The structural organization of the proteins and protein complexes seems to be conserved among those organisms. All systems have in common a multi-transmembrane domain, proposed to be involved in the translocation of an acetyl-carrier across a membrane. In addition, they contain a large globular domain with highly conserved GDS and DxxH peptide motifs (see also **Figure 2**) representing the presumed acetyltransferase. While in fungi, mammals, and Gram-positive bacteria this mechanism is realized in a single protein, in Gram-negative bacteria and plants this mechanism is split into two proteins. Alginate *O*-acetylation in *Pseudomonas* seems to be a special case as no DxxH motif can be found and three proteins were shown to be essential.

while the *O*-acetylation level of other polysaccharides is not affected. These findings indicate that unlike RWA, AXY4 acts in a polysaccharide specific manner as a likely XyG specific *O*-acetyltransferase. The *O*-acetyltransferase activity of AXY4 and the position, to which the *O*-acetyl-group is transferred has yet to be experimentally demonstrated. However, overexpression of the AXY4 gene leads not only to an increase in XyG acetylation but there is also evidence that one galactosyl-unit of XyG becomes di-acetylated (Gille et al., 2011b). Hence, AXY4 is sufficient to add multiple acetyl-substituents to the same galactosyl-residue. AXY4 could accomplish this by adding acetyl-substituents onto two different positions. Alternatively, AXY4 adds the acetyl-group

to a single specific position. The acetyl-group is then distributed through chemical migration to the other position, leaving the initial *O*-acetylation site open for a further addition of a second *O*-acetate. However, overexpression of AXY4 did not lead to completely acetylated XyG, suggesting that the acetylated polymer can be further modified by acetylesterases, which remove the acetate, presumably after deposition in the apoplastic space (Gille et al., 2011b).

A homolog of AXY4, a second putative XyG *O*-acetyltransferase termed AXY4L for AXY4like, was identified as acting on XyG specifically in seeds (Gille et al., 2011b). AXY4 and AXY4L are both members of a large, plant-specific protein family annotated as trichome birefringence-like (TBL; Bischoff et al., 2010a). In *Arabidopsis* this family includes 46 members (TBR, TBL1-45). Based on the data for AXY4/TBL27 and AXY4L/TBL22 also other members of the TBL family are proposed to encode additional wall polysaccharide specific *O*-acetyltransferases (Gille et al., 2011b). All TBL proteins consist of one N-terminal transmembrane domain and two highly conserved plant-specific domains; a TBL domain and a DUF231 domain (Bischoff et al., 2010a). Both domains harbor conserved motifs; a Gly-Asp-Ser (GDS) peptide in the TBL domain and an Asp-x-x-His (DxxH) motif in the C-terminal DUF231 domain (**Figure 1**). These two motifs were shown to be conserved in esterases and lipases, including a fungal rhamnogalacturonan acetylesterase and seem to infer catalytic activity (Molgaard et al., 2000; Akoh et al., 2004; Bischoff et al., 2010a,b). One of the two domains could be involved in binding a particular polymer while the other domain facilitates the binding of the acetyl-donor substrate and transfer of the acetate group to the polymer. This proposed mechanism might require an esterase-like activity in order to hydrolyze the acetate from the donor substrate (Gille et al., 2011b). Sequence comparisons of selected TBL family proteins to the *O*-acetylation systems described in the various organisms above (**Figure 1**) indicates that despite significant sequence diversity both motifs, the GDS and the DxxH motif, are present in the large loops of nearly all *O*-acetylation systems (**Figure 2**). Solely in the *Pseudomonas* alginate *O*-acetylation system only a GDS-motif can be found, but not the DxxH motif, neither in PaAlgJ nor PaAlgF.

It thus appears that the *O*-acetylation mechanism is highly conserved among organisms and kingdoms. However, while in some organisms the responsible protein remains conserved as a single protein (mammals, fungi, Gram-positive bacteria) in other organisms this protein is split into two (Gram-negative bacteria, plants) or three proteins (*Pseudomonas*). Such a split allows the diversification of the proteins. For example, in plants there are four RWA and 46 TBL proteins perhaps reflecting the diversity of acetylated substrates and specificity in the various tissues and/or developmental stages. It has been proposed that the proteins harboring the multiple transmembrane domains such as PatA and AlgI are involved in translocation of an acetyl-donor molecule across the membrane (Franklin and Ohman, 2002), while the loop proteins PatB and AlgJ can be considered putative *O*-acetyltransferases that transfer the acetyl-group to the polysaccharide. By analogy in plants RWA would be an acetyl-donor translocator and the TBL proteins would represent the polysaccharide specific *O*-acetyltransferases



FIGURE 2 | Protein sequence alignment of domains containing the GDS and DxxH motives of proteins displayed in Figure 1. Alignment was performed using Kalign 2.0 (Lassmann and Sonnhammer, 2005). (Format: ClustalW, Gap open penalty: 30, Gap extension penalty: 0.2, Terminal Gap penalties: 0.45, Bonus score: 0). Proteins were trimmed to the amino acid residues given in bold numbers for the alignment. At, *Arabidopsis thaliana*; Cn, *Cryptococcus neoformans*; Pg, *Puccinia graminis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Lp, *Lactobacillus plantarum*; Sa, *Staphylococcus aureus*; Bs, *Bacillus subtilis*; Ng, *Neisseria gonorrhoeae*; Pa, *Pseudomonas aeruginosa.* (At_AXY4 – AT2G70230, At_PMR5 – AT5G58600, At_TBR – AT5G06700, At_TBL3 – AT5G01360, Cn_Cas1p – AF355592, Pg_Cas1 – XP_003331718, Hs_Cas1 – NP_075051, Mm_Cas1 – NP_663373, Lp_OatA – NP_784589, Sa_OatA – ZP_06313146, Bs_OatA – NP_390592, Ng_PatB – YP_207683, Pa_AlgJ – AAB09782).

(Gille et al., 2011b; Manabe et al., 2011). Analysis of the XyG *O*-acetylation status in an *rwa/axy4* (*tbl27*) double mutant does not exclude the possibility that both proteins act in the same pathway (Gille et al., 2011b). In mammals and fungi both functions of translocation and transfer are combined into a single protein (**Figure 1**). Further research is needed to ascertain the function of the two protein domains (putative translocator and putative transferase) as well as the role of the two motifs (GDS and DxxH). Moreover, there might be other proteins involved in the *O*-acetylation mechanism. For example, knocking out all four RWA proteins in *Arabidopsis* in a RWA1/2/3/4 quadruple mutant does not eliminate wall polysaccharide *O*-acetylation but leads only to a 40% reduction in xylan acetylation in the stem (Lee et al., 2011) demonstrating that other hitherto unknown proteins can substitute for the loss of the presumed translocator RWA.

There are several pieces of evidence that *O*-acetylation of wall polysaccharides in plants occurs in the endomembrane system, most likely the Golgi apparatus, where non-cellulosic polysaccharides are synthesized (Moore et al., 1986; Scheible and Pauly, 2004). First, *O*-acetylated XyG can be isolated from intact microsomes of *Arabidopsis* confirming that *O*-acetylation occurs prior to deposition of the wall polysaccharide in the apoplast (Obel et al., 2009). Second, when microsomal preparations of a potato cell suspension cultures are fed with radio-labeled acetyl-CoA, the radio-labeled acetate can be found in an esterified form on several polysaccharides including XyG and pectin (Pauly and Scheller, 2000). Third, RWA and AXY4/TBL27 have been localized to the endomembrane system and their loop containing the GDS and DxxH motives are predicted to face the lumen (Gille et al., 2011b; Lee et al., 2011; Manabe et al., 2011).

The microsome experiments in plants indicate that acetyl-CoA is an acetyl-donor for the acetylation of wall polysaccharides. Other additional acetyl-donors or acetyl-intermediates cannot be excluded. Acetyl-CoA is also the donor substrate for acetylation of sialic acids in the mammalian system (Vandamme-Feldhaus and Schauer, 1998). The nature of the *O*-acetyl-donor in the other systems has not been identified but acetyl-CoA is also a possibility there (Franklin and Ohman, 2002; Laaberki et al., 2011). Is acetyl-CoA translocated to act as a donor substrate for the *O*-acetyltransferases? This seems unlikely in bacteria as acetyl-CoA would be translocated into the extracellular space and thus lost in the culture medium. It seems thus likely that the acetyl-group is transferred to an intermediate preferably one that remains tightly bound to the membrane, potentially the translocator protein itself. If one hypothesizes similar *O*-acetylation mechanisms in plants/human/fungi, where *O*-acetylation occurs in the Golgi, the possibility of translocation of acetyl-CoA seems also improbable. However, future experimental evidence is needed to elucidate how and in what form the acetyl-group is translocated and transferred to the polysaccharide.

BIOLOGICAL FUNCTIONS OF PLANT WALL POLYMER *O*-ACETYLATION

The biological function of *O*-acetyl-substituents in plant development and morphology is not known. However, surveys of *O*-acetylation in various plant tissues and during plant development indicate that the degree of *O*-acetylation on wall polymers is modified by the plant (Liners et al., 1994; Pauly et al., 2001; Obel et al., 2009) suggesting an important functional role in the plant. Since *in vitro* polymer experiments indicate that *O*-acetylation impacts the rheological properties and hinders enzymatic breakdown of the polysaccharide presumably through steric hindrance and/or conformational changes of the polymer (Biely et al., 1986), it is possible that fine-tuning the acetylation level of wall polymers impacts non-covalent interaction of wall polymers and hinders degradation of the polysaccharide by invading pathogens. Adding *O*-acetyl-substituents (C2-unit) to wall polysaccharides instead of additional pentosyl- or hexosyl-units (C5- and C6-units, respectively) does not only increase the structural complexity of the polysaccharide but it is also energetically favorable as less carbon is deposited in the wall. It seems such a strategy has been pursued in the grass XyG, where compared to the structure of the dicot XyG a specific xylosyl-residue has been regularly replaced with an *O*-acetyl-group (Gibeaut et al., 2005; Jia et al., 2005).

The recent discoveries of the first plant mutants affected in wall polysaccharide *O*-acetylation give more insights in the biological function of this substituent. The *rwa2* single mutant exhibiting a 20% reduction in overall cell wall acetylation has a wild type like growth and morphology when grown under laboratory conditions (Manabe et al., 2011), while the RWA quadruple mutant exhibits collapsed xylem vessels (Lee et al., 2011). Consistent with observations made with mutants affected in the substitution pattern of XyG (Madson et al., 2003; Perrin et al., 2003; Cavalier et al., 2008; Günl et al., 2011) the *axy4* mutant containing non-acetylated XyG exhibits no obvious growth or developmental phenotypes. In fact, the *Arabidopsis* ecotype Ty-0 containing only non-acetylated XyG due to point mutations in AXY4 apparently thrives in its habitat, Taynuilt in northern Scotland (Gille et al., 2011b). Apparently, the lack of XyG *O*-acetylation does not impact the fitness of the plant and does not present a selective disadvantage under the environmental conditions at this location. Interestingly, Ty-0 has been shown to be highly susceptible to the fungal pathogen *Fusarium oxysporum* (Diener and Ausubel, 2005). Although the mapping of one of the QTLs in this study led to a wall-associated kinase-like kinase 22 (WAKL22) there is a possibility that XyG *O*-acetylation may play a role in the susceptibility of this pathogen. Evidence for a potential role of wall *O*-acetylation in pathogen defense is provided by *rwa2*, which was shown to have an increased resistance toward the necrotrophic fungal pathogen *Botrytis cinerea* (Manabe et al., 2011). Moreover, if one assumes that all TBL proteins are involved in wall polysaccharide *O*-acetylation, a mutant in one of its members, PMR5, exhibits increased resistance to powdery mildew, *Erysiphe spec* (Vogel et al., 2004). Indeed, the cell wall of *pmr5* exhibits a decrease in esterification as demonstrated by Fourier transform infrared (FTIR) analysis. If and which polysaccharide contains an alteration in *O*-acetylation has yet to be demonstrated in *pmr5*. Other TBL mutants also show significant phenotypes. The original mutant after which this class of genes was named, trichome birefringence (*tbr*), exhibits a defect in cellulose crystallinity in its trichomes (Potikha and Delmer, 1995; Bischoff et al., 2010a). The *tbr*-mutant and a mutant in its homolog TBL3 show alterations in their pectin esterification (Bischoff et al., 2010a). Another mutant affected in the expression of a gene in the TBL gene family is the *eskimo1* mutant that displays

strong freezing tolerance in the absence of cold acclimation (Xin et al., 2007). More detailed analysis of the mutant indicated collapsed xylem and alterations of the wall structure (Lefebvre et al., 2011). Although the precise nature of the wall structural change in *eskimo1* has not been elucidated it is tempting to speculate that it could be *O*-acetylation of a particular polysaccharide. Bridging the gap between those observed mutant phenotypes and the proposed *O*-acetyltransferases will be vital in understanding the function of *O*-acetylation in the life cycle of plants.

Homologs of RWA and TBLs can be found in crop plants (Gille et al., 2011b) and the recent finding that these genes are involved in the *O*-acetylation of wall polysaccharides opens the door to identify or generate crop plants whose biomass contains lower amounts of acetate. This can be achieved through genetic engineering of repressing *O*-acetylation or marker assisted breeding. The resulting plant biomass should represent an improved feedstock for the emerging lignocellulosic based biofuel industry.

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REFERENCES

Akoh, C. C., Lee, G.-C., Liaw, Y.-C., Huang, T.-H., and Shaw, J.-F. (2004). GDSL family of serine esterases/lipases. *Prog. Lipid Res.* 43, 534–552.

Arming, S., Wipfler, D., Mayr, J., Merling, A., Vilas, U., Schauer, R., Schwartz-Albiez, R., and Vlasak, R. (2011). The human Cas1 protein: a sialic acid-specific O-acetyltransferase? *Glycobiology* 21, 553–564.

Aubry, C., Goulard, C., Nahori, M.-A., Cayet, N., Decalf, J., Sachse, M., Boneca, I. G., Cossart, P., and Dussurget, O. (2011). OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. *J. Infect. Dis.* 204, 731–740.

Bastawde, K. (1992). Xylan structure, microbial xylanases, and their mode of action. *World J. Microbiol. Biotechnol.* 8, 353–368.

Bera, A., Herbert, S., Jakob, A., Vollmer, W., and Götz, F. (2005). Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.* 55, 778–787.

Bernard, E., Rolain, T., Courtin, P., Guillot, A., Langella, P., Hols, P., and Chapot-Chartier, M.-P. (2011). Characterization of O-acetylation of N-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. *J. Biol. Chem.* 286, 23950–23958.

Biely, P., Mackenzie, C., Puls, J., and Schneider, H. (1986). Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. *Biotechnology* 4, 731–733.

Bischoff, V., Nita, S., Neumetzler, L., Schindelasch, D., Urbain, A., Eshed, R., Persson, S., Delmer, D., and Scheible, W. R. (2010a). Trichome birefringence and its homolog AT5G01360 encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis*. *Plant Physiol.* 153, 590–602.

Bischoff, V., Selbig, J., and Scheible, W. R. (2010b). Involvement of TBL/DUF231 proteins into cell wall biology. *Plant Signal. Behav.* 5, 1057–1059.

Carroll, A., and Somerville, C. (2009). Cellulosic biofuels. *Annu. Rev. Plant Biol.* 60, 165–182.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Cherniak, R., and Sundstrom, J. (1994). Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. *Infect. Immun.* 62, 1507–1512.

Clarke, A. J., and Dupont, C. (1992). O-acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. *Can. J. Microbiol.* 38, 85–91.

Del Río, J. C., Marques, G., Rencoret, J., Martínez, Á. T., and Gutiérrez, A. (2007). Occurrence of naturally acetylated lignin units. *J. Agric. Food Chem.* 55, 5461–5468.

Del Río, J. C., Rencoret, J., Marques, G., Gutiérrez, A., Ibarra, D., Santos, J. I., Jiménez-Barbero, J., Zhang, L., and Martínez, Á. T. (2008). Highly acylated (acetylated and/or p-coumaroylated) native lignins from diverse herbaceous plants. *J. Agric. Food Chem.* 56, 9525–9534.

Diener, A. C., and Ausubel, F. M. (2005). Resistance to *Fusarium oxysporum* 1, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics* 171, 305–321.

Franklin, M., and Ohman, D. (1993). Identification of algf in the alginate biosynthetic gene-cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. *J. Bacteriol.* 175, 5057–5065.

Franklin, M., and Ohman, D. (1996). Identification of algI and algJ in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. *J. Bacteriol.* 178, 2186–2195.

Franklin, M. J., and Ohman, D. E. (2002). Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *J. Bacteriol.* 184, 3000–3007.

Gibeaut, D. M., Pauly, M., Bacic, A., and Fincher, G. B. (2005). Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. *Planta* 221, 729–738.

Gille, S., Cheng, K., Skinner, M. E., Liepman, A. H., Wilkerson, C. G., and Pauly, M. (2011a). Deep sequencing of voodoo lily (amorphophallus konjac): an approach to identify relevant genes involved in the synthesis of the hemicellulose glucomannan. *Planta* 234, 515–526.

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011b). O-acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Günl, M., Neumetzler, L., Kraemer, F., de Souza, A., Schultink, A., Pena, M., York, W. S., and Pauly, M. (2011). AXY8 encodes a fucosidase, underpinning the importance of apoplastic metabolism on the fine structure of plant cell wall polysaccharides. *Plant Cell* 23, 4025–4040.

Helle, S., Cameron, D., Lam, J., and White, B. (2003). Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of *S. cerevisiae*. *Enzyme Microb. Technol.* 33, 786–792.

Hoffman, M., Jia, Z., Pena, M., Cash, M., Harper, A., Blackburn, A., Darvill, A., and YORK, W. (2005). Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. *Carbohydr. Res.* 340, 1826–1840.

Hsieh, Y. S. Y., and Harris, P. J. (2009). Xyloglucans of monocotyledons have diverse structures. *Mol. Plant* 2, 943–965.

Huang, L., Takahashi, R., Kobayashi, S., Kawase, T., and Nishinari, K. (2002). Gelation behavior of native and acetylated konjac glucomannan. *Biomacromolecules* 3, 1296–1303.

Ishii, T. (1991). Acetylation at O-2 of arabinofuranose residues in feruloylated arabinoxylan from bamboo shoot cell-walls. *Phytochemistry* 30, 2317–2320.

Ishii, T. (1997). O-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiol.* 113, 1265–1272.

Janbon, G., Himmelreich, U., Moyrand, F., Improvisi, L., and Dromer, F. (2001). Cas1p is a membrane protein necessary for the O-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. *Mol. Microbiol.* 42, 453–467.

Jia, Z., Cash, M., Darvill, A., and York, W. (2005). NMR characterization of endogenously O-acetylated oligosaccharides isolated from tomato (*Lycopersicon esculentum*) xyloglucan. *Carbohydr. Res.* 340, 1818–1825.

Kabel, M. A., de Waard, P., Schols, H. A., and Voragen, A. G. J. (2003). Location of O-acetyl substituents in xylo-oligosaccharides obtained from hydrothermally treated eucalyptus wood. *Carbohydr. Res.* 338, 69–77.

Kiefer, L., York, W., Darvill, A., and Albersheim, P. (1989). Structure of plant-cell walls.27. Xyloglucan isolated from suspension-cultured sycamore cell-walls is o-acetylated. *Phytochemistry* 28, 2105–2107.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B. A., and Blanch, H. W. (2010). Technoeconomic analysis of biofuels, A wiki-based platform for lignocellulosic biorefineries. *Biomass Bioenergy* 34, 1914–1921.

Laaberki, M. H., Pfeffer, J., Clarke, A. J., and Dworkin, J. (2011). O-acetylation of peptidoglycan is required for proper cell separation and s-layer anchoring in *Bacillus anthracis*. *J. Biol. Chem.* 286, 5278–5288.

Lassmann, T., and Sonnhammer, E. L. L. (2005). Kalign – an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* 6, 298. doi:10.1186/1471-2105-6-298

Lee, C., Teng, Q., Zhong, R., and Ye, Z.-H. (2011). The four *Arabidopsis* reduced wall acetylation genes are expressed in secondary wall-containing cells and required for the acetylation of xylan. *Plant Cell Physiol.* 52, 1289–1301.

Lefebvre, V., Fortabat, M. N., Ducamp, A., North, H. M., Maia-Grondard, A., Trouverie, J., Boursiac, Y., Mouille, G., and Durand-Tardif, M. (2011). ESKIMO1 disruption in *Arabidopsis* alters vascular tissue and impairs water transport. *PLoS ONE* 6, e16645. doi:10.1371/journal.pone.0016645

Liners, F., Gaspar, T., and Vancutsem, P. (1994). Acetyl-esterification and methyl-esterification of pectins of friable and compact sugar-beet calli – consequences for intercellular-adhesion. *Planta* 192, 545–556.

Lundqvist, J., Teleman, A., Junel, L., Zacchi, G., Dahlman, O., Tjerneld, F., and Stålbrand, H. (2002). Isolation and characterization of galactoglucomannan from spruce (*Picea abies*). *Carbohydr. Polym.* 48, 29–39.

Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G., Calplan, J., Shoue, D., Carpita, N., and Reiter, W. (2003). The MUR3 gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *Plant Cell* 15, 1662–1670.

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., Pauly, M., Knox, J. P., Sakuragi, Y., and Scheller, H. V. (2011). Loss-of-function mutation of reduced wall acetylation2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078.

Molgaard, A., Kauppinen, S., and Larsen, S. (2000). Rhamnogalacturonan acetylesterase elucidates the structure and function of a new family of hydrolases. *Structure* 8, 373–383.

Moore, P., Darvill, A., Albersheim, P., and Staehelin, L. (1986). Immunogold localization of xyloglucan and rhamnogalacturonan-i in the cell-walls of suspension-cultured sycamore cells. *Plant Physiol.* 82, 787–794.

Moynihan, P. J., and Clarke, A. J. (2010). O-acetylation of peptidoglycan in Gram-negative bacteria: identification and characterization of peptidoglycan O-acetyltransferase in *Neisseria gonorrhoeae*. *J. Biol. Chem.* 285, 13264–13273.

Obel, N., Erben, V., Schwarz, T., Kuhnel, S., Fodor, A., and Pauly, M. (2009). Microanalysis of plant cell wall polysaccharides. *Mol. Plant* 2, 922–932.

O'Neill, M. A., Ishii, T., Albersheim, P., and Darvill, A. G. (2004). Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.* 55, 109–139.

Pauly, M. (1999). *Development of Analytical Tools to Study Plant Cell Wall Xyloglucan*, Ph.D. thesis, Shaker Verlag, Aachen.

Pauly, M., Eberhard, S., Albersheim, P., Darvill, A., and York, W. (2001). Effects of the mur1 mutation on xyloglucans produced by suspension-cultured *Arabidopsis thaliana* cells. *Planta* 214, 67–74.

Pauly, M., and Keegstra, K. (2008). Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant J.* 54, 559–568.

Pauly, M., and Keegstra, K. (2010). Plant cell wall polymers as precursors for biofuels. *Curr. Opin. Plant Biol.* 13, 304–311.

Pauly, M., and Scheller, H. (2000). O-acetylation of plant cell wall polysaccharides: identification and partial characterization of a rhamnogalacturonan O-acetyl-transferase from potato suspension-cultured cells. *Planta* 210, 659–667.

Perrin, R., Jia, Z., Wagner, T., O'Neill, M., Sarria, R., York, W., Raikhel, N., and Keegstra, K. (2003). Analysis of xyloglucan fucosylation in *Arabidopsis*. *Plant Physiol.* 132, 768–778.

Potikha, T., and Delmer, D. (1995). A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. *Plant J.* 7, 453–460.

Rombouts, F., and Thibault, J. (1986). Sugar-beet pectins – chemical structure and gelation through oxidative coupling. *ACS Symp. Ser.* 310, 49–60.

Scheible, W.-R., and Pauly, M. (2004). Glycosyltransferases and cell wall biosynthesis: novel players and insights. *Curr. Opin. Plant Biol.* 7, 285–295.

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289.

Schols, H., and Voragen, A. (1994). Hairy (ramified) regions of pectins occurrence of pectic hairy regions in various plant-cell wall materials and their degradability by rhamnogalacturonase. *Carbohydr. Res.* 256, 83–95.

Selig, M., Adney, W., and Himmel, M. (2009). The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. *Cellulose* 16, 711–722.

Sengkhamparn, N., Bakx, E. J., Verhoef, R., Schols, H. A., Sajjaanantakul, T., and Voragen, A. G. J. (2009). Okra pectin contains an unusual substitution of its rhamnosyl residues with acetyl and alpha-linked galactosyl groups. *Carbohydr. Res.* 344, 1842–1851.

Shinabarger, D., May, T., Boyd, A., Ghosh, M., and Chakrabarty, A. (1993). Nucleotide-sequence and expression of the *Pseudomonas aeruginosa* algf gene controlling acetylation of alginate. *Mol. Microbiol.* 9, 1027–1035.

Teleman, A., Lundqvist, J., Tjerneld, F., Stålbrand, H., and Dahlman, O. (2000). Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing 1H and 13C NMR spectroscopy. *Carbohydr. Res.* 329, 807–815.

Van Dongen, F., Van Eylen, D., and Kabel, M. (2011). Characterization of substituents in xylans from corn cobs and stover. *Carbohydr. Polym.* 86, 722–731.

Vandamme-Feldhaus, V., and Schauer, R. (1998). Characterization of the enzymatic 7-O-acetylation of sialic acids and evidence for enzymatic O-acetyl migration from C-7 to C-9 in bovine submandibular gland. *J. Biochem.* 124, 111–121.

Vogel, J., Raab, T., Somerville, C., and Somerville, S. (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978.

Xin, Z., Mandaokar, A., Chen, J., Last, R. L., and Browse, J. (2007). *Arabidopsis* ESK1 encodes a novel regulator of freezing tolerance. *Plant J.* 49, 786–799.

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Mass spectrometry for characterizing plant cell wall polysaccharides

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Mass spectrometry is a selective and powerful technique to obtain identification and structural information on compounds present in complex mixtures. Since it requires only small sample amount it is an excellent tool for researchers interested in detecting changes in composition of complex carbohydrates of plants. This mini-review gives an overview of common mass spectrometry techniques applied to the analysis of plant cell wall carbohydrates. It presents examples in which mass spectrometry has been used to elucidate the structure of oligosaccharides derived from hemicelluloses and pectins and illustrates how information on sequence, linkages, branching, and modifications are obtained from characteristic fragmentation patterns.

Keywords: mass spectrometry, cell wall, carbohydrates, oligosaccharides

INTRODUCTION

Plant cell wall carbohydrates are the most abundant biopolymers on Earth. In addition to specialized functions in plant development and physiology, the use of specific cell wall polysaccharides and proteoglyans in food chemistry and nutrition, pharmaceuticals, biomaterials, biofuels, and green chemistry is increasing. Determination of, and detection of changes in, polymer composition is of interest to an ever-widening array of researchers studying their synthesis or degradation. Even with only a limited number of monosaccharides used as building blocks, variations in sequence, linkage, branching, distribution of side chains, and functional group modifications result in a wide structural diversity. No single analytical method alone is capable of resolving carbohydrate structures, especially when present in complex mixtures. Nuclear magnetic resonance (NMR) spectroscopy is widely used in structural elucidation but requires rather pure samples and lacks the ability to reveal sequence information (Duus et al., 2000). Although some methods (e.g., nanoprobe-NMR) have significantly lowered concentration requirements for analysis, mass spectrometry (MS) provides high sensitivity and can even be applied to mixtures of carbohydrates. This allows detailed characterization of changes in cell wall polymers due to changes in environmental, developmental, and/or genetic factors. Because the technique is mass-based, isotope labeling can be used in a variety of creative ways to elucidate time-resolved changes in polymer synthesis and modification.

MASS SPECTROMETRY FOR POLYSACCHARIDE ANALYSIS

To be amenable for MS polysaccharides must first be converted into smaller oligosaccharides either by partial acid hydrolysis or enzymatic degradation. The latter has the advantage of being specific to one polymer alone and can therefore be applied to a mixture of polysaccharides or even the whole cell wall eliminating the need for purification which often removes modifications (e.g., acetylation). Enzymes are also specific for certain cleavage sites and anomeric linkages, information which can be exploited in structure elucidation. The key to the analysis of oligosaccharides is to find suitable MS fragments that are specific for one structure and allow the exclusion of alternatives. Mass spectra give ambiguous results when a product ion can originate from two or more precursor ions. Uncertainties in MS/MS spectra can often be removed by applying MS^n where product ions can be further examined and assigned to a specific structure.

In recent years, fast atom bombardment (FAB) has been more and more displaced by techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). In ESI, the sample solution is highly charged and sprayed through a capillary into a strong electric field to form a fine mist of charged droplets. The solvent evaporates and produces gas-phase ions. Because samples have to be crystallized with a matrix (e.g., 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid) that absorbs the energy of the laser and ionizes the sample, MALDI cannot be coupled directly with separation systems (Brüll et al., 1998). MALDI is relatively salt tolerant and offers a large mass range and high sensitivity when coupled to time-of-flight (TOF) analyzers. MALDI-TOF techniques have been proven excellent and simple tools for profiling mixtures of oligosaccharides and can also easily identify sugar modifications such as methylation, acetylation, and sulfation.

The use of powerful mass analyzers with MS/MS (triple quadrupole, TOF/TOF) or even MS^n (ion trap, IT) capabilities in conjunction with reverse-phase, normal phase (NP), porous graphitized carbon, size exclusion, ion exchange, or high-performance anion-exchange, liquid chromatographic (LC) or capillary electrophoretic separation methods has increased the role of mass spectrometry for oligosaccharide structural characterization (Schols et al., 1994; Deery et al., 2001; Kabel et al., 2001; Mazumder et al., 2005; Hilz et al., 2006; Coenen et al., 2008).

The main function of these coupling techniques is reduction of complexity by separation of isobaric structures that are not resolved by MS.

FRAGMENTATION

Oligosaccharides can generate ions via glycosidic bond cleavages and cross-ring fragmentation. Soft fragmentation techniques such as post-source decay (PSD) do usually not produce abundant cross-ring fragments which are observed in high-energy collision induced dissociation (CID) spectra. By a common convention, ions containing the non-reducing end are labeled A_i (cross-ring), B_i, and C_i (glycosidic), and ions containing the reducing end are labeled X_j (cross-ring), Y_j, and Z_j (glycosidic) (Domon and Costello, 1988). The subscript *i* indicates the number of glycosidic bonds cleaved counted from the non-reducing end and *j* refers to the number of interglycosidic bonds counted from the reducing end (**Figure 1A**). The superscript (e.g., $^{0,2}A$) specifies the particular ring bonds cleaved. Ions from side chains are supplemented by a Greek letter (α, β, γ) assigned with decreasing weight of the side chain (**Figure 1B**). The numbering from the backbone continues into the side chain. Depending on the ionization and the oligosaccharide structure, glycosidic bond fragments of one or more series (B, C, Y, or Z) are predominantly or even exclusively formed. Additional fragments (**Figure 1C**) resulting from "rearrangement-elimination" or double cleavages characteristic of 1,2-linked (E) or 1,3-linked (D, F, G) residues and "sugar lactones" (H, W) are diagnostic for the branching pattern (Harvey, 1999; Chai et al., 2001; Spina et al., 2004; Maslen et al., 2007).

Whereas glycosidic bond fragmentation gives information on sequence, cross-ring ions are highly diagnostic for linkage positions and are also indicative for the position of modifications (e.g., acetyl groups). Rules for 1–2, 1–3, 1–4, and 1–6 linkages have been established (Garozzo et al., 1990; Zhou et al., 1990; Hofmeister et al., 1991). For instance, the presence of ions $^{0,2}A_i$ 60 Da (loss of $C_2H_4O_2$) and $^{2,4}A_i$ 120 Da (loss of $C_4H_6O_4$) lower than the molecular peak (or a C_i ion) in combination with the absence of a loss of 90 Da ($C_3O_3H_6$) is typical for a 1–4 linkage to hexoses at the reducing end of the molecule or the respective precursor (C_i).

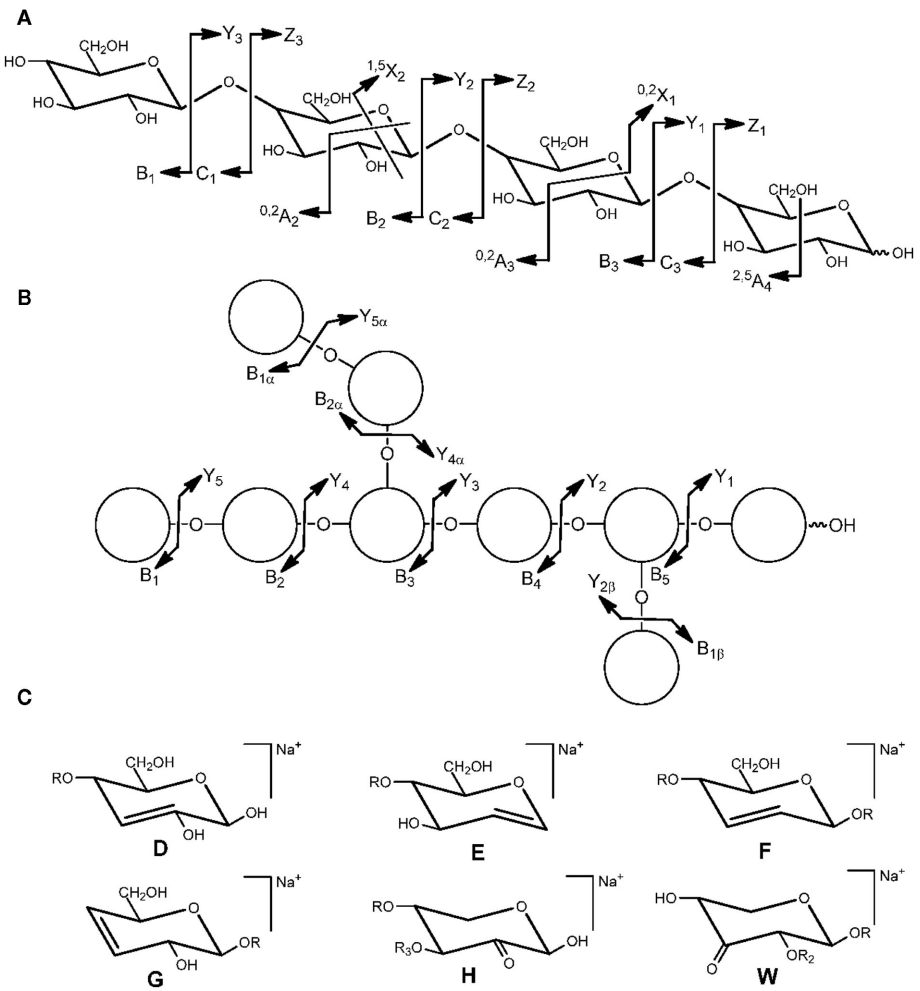


FIGURE 1 | Nomenclature for the cleavages of linear oligosaccharides (A) and branched oligosaccharides (B) and structure of fragment ions (D–H, W) observed via "rearrangement-elimination" and double cleavage (C). R = backbone residue, R_2 = H or side chain, R_3 = H or side chain (adapted from Domon and Costello, 1988; Spina et al., 2004; Maslen et al., 2007).

An O-2-linked acetyl group would be indicated by an $^{0,2}A_i$ ion 102 (60 + 42) Da lower. $^{0,2}X_j$ ions with a loss of 104 Da from C_i ions characteristic for 1–2 linked deoxyhexoses [e.g., rhamnose (Rha)] were observed in pectic rhamnogalacturonan I oligosaccharides (Ralet et al., 2009).

Various algorithms for extracting sequence information from, and assignment of fragment ions in, mass spectra of oligosaccharides are available but are mainly focusing on protein glycans (Gaucher et al., 2000; Lapadula et al., 2005; Tang et al., 2005; Ceroni et al., 2008; Böcker et al., 2011).

DERIVATIZATION

The hydroxyl groups of sugars can be naturally O-methylated in the plant or chemically per-O-methylated through a simple derivatization process (Ciucanu and Kerek, 1984; Ciucanu and Caprita, 2007). Besides a significant increase in signal strength, permethylation is applied in order to locate side chains in isomers (e.g., in arabinoxylans). Since the branch points of backbone residues are not methylated during derivatization they can be distinguished from non-branched residues by a mass difference of 14 ($–CH_3 + H$) per branching point. For oligosaccharides containing natively methylated sugars common in pectins and xylans [e.g., 4-*O*-methyl glucuronic acid (4-O-Me-GlcA)], per-*O*-deuteromethylation is applied (Dell et al., 1989). The mass difference allows the natively methylated sugars to be distinguished from their artificially methylated counterparts.

Hydrolysis, reduction, and O-acetylation of the per-O-methylated oligosaccharides yields partially methylated alditol acetates that are commonly analyzed by gas chromatography(GC)/MS in order to reveal neutral monosaccharide identity and linkage positions, though sequence information is not obtained (Björndal et al., 1967). For acid-containing polysaccharides, reduction of free uronic groups is achieved by activation with a water-soluble carbodiimide (Kim and Carpita, 1992) or reduction of the permethylated oligosaccharides with lithium triethylborodeuteride (Lerouge et al., 1993). A GC/MS method for simultaneous determination of neutral, acidic, deoxy-, methyl- and even acid-labile 2-keto-3-deoxy-sugars and aceric acid (AceA) via their trimethylsilyl methyl glycosides has also been developed (Doco et al., 2001).

The assignment of ions to a particular series is enabled by oligosaccharide labeling. For example, unsaturated linear oligogalacturonides in ESI negative mode mainly exhibit ions corresponding to the C- and Z-series which are isobaric and therefore undistinguishable. By labeling the reducing end with ^{18}O, all ions comprising the reducing end (X, Y, and Z) will show a two mass unit increase (Körner et al., 1999). Xyloglucan oligosaccharides are often reduced to the corresponding alcohols (Vierhuis et al., 2001).

Introduction of a permanent charge (e.g., quartenary ammonium groups) or an ionizable function (amino or carboxy group) not only increases sensitivity and often attaches a chromophore group to the molecule but also fixes the charge state at one side of the molecule (Harvey, 2000; Gouw et al., 2002; Coenen et al., 2008). This greatly enhances the intensities of ions containing the charged label and supports structure confirmation.

RELATIVE QUANTIFICATION

Quantification of oligosaccharides by MS is difficult because ionization efficiencies can differ significantly. Other compounds present in a mixture may compete or interfere with ionization. In order to compensate for these effects, internal standards with properties almost identical to the analytes but differing in their mass are used, in practice fulfilled by isotopologues. Absolute quantification requires pure standards but is constrained by the vast variety of possible oligosaccharides structures. For relative quantification of the levels of individual oligosaccharides, each sample is derivatized by an isotopic tag with a unique mass. An example is the labeling with deuterium (D_4 and D_6) labeled 2-aminopyridine via reductive amination (Yuan et al., 2005). Aliquots of the samples are pooled and analyzed by MS directly or after separation. For chromatography coupling, ^{13}C-isomers are preferred over deuterated ones since the latter can show slight differences in retention time (Zhang et al., 2001). Labeling with commercially available $^{12}C_6$ and $^{13}C_6$ isotopes of aniline was recently introduced (Ridlova et al., 2008).

SELECTED EXAMPLES

The following are a few examples of how various MS techniques have been applied to elucidate structural features in various cell wall polysaccharides.

XYLOGLUCAN

Xyloglucan is a highly branched hemicellulose which is an important component of the primary cell wall of higher plants. The branch pattern and composition is variable and has specific physiological functions in the plant including modulating expansion in growing cells (Fry, 1989). Xyloglucan-specific endoglucanases with known specific activity release typical building blocks from xyloglucan. This enabled the development of high-throughput MALDI-TOF screening methods (Oligosaccharide Mass Profiling, OLIMP) for oligosaccharide fingerprinting of plant cell walls (Lerouxel et al., 2002). The method reveals detailed side chain distribution patterns including acetylation. The procedure can be directly applied to specific leaf tissues (Obel et al., 2009) and has been further expanded to characterize a more comprehensive set of cell wall polysaccharides (Westphal et al., 2010). Although structural isomers such as XXLG and XLXG (for nomenclature see Fry et al., 1993) are not resolved by MS they can be distinguished by MALDI-PSD or -TOF/TOF analysis using their distinct ion intensities (Yamagaki et al., 1998). ESI-MS^2 was found to be superior to MALDI-PSD-TOF for the analysis of XXFG from black currants and revealed that acetyl groups were located on the galactose residue (Hilz et al., 2006). The glycosyl sequence of unusual oligosaccharides from olive fruit with arabinose substitution (XXSG and XLSG) has been confirmed by MALDI-PSD-TOF MS (Vierhuis et al., 2001).

XYLAN

Xylans are hemicelluloses typically found in the secondary cell walls such as those in structural and water conducting tissues. The backbone of α-(1,4)-linked xylosyl (Xyl) residues is decorated with glucuronic acid (GlcA), 4-O-Me-GlcA, and/or arabinose (Ara), depending on the plant species and cell tissue. The biological

function of these side chains is unknown but they have a big influence on the polymer properties and also influence the enzymatic degradation (Ebringerová and Heinze, 2000). MALDI-TOF analysis of glucuronoxylan from *Arabidopsis* after endo-xylanase digestion revealed that the *irx8* mutant is devoid of $[M+Na]^+$ ions corresponding to structures with unmethylated GlcA side chains (Persson et al., 2007). For the *gux1 gux2* double mutant side chains were even completely absent (Mortimer et al., 2010). The distribution patterns of 4-O-Me-GlcA residues of glucuronoxylans oligosaccharides up to *m/z* 6000 from softwood and hardwood were determined by MALDI-TOF-MS analysis after partial acid hydrolysis (Jacobs et al., 2001).

MALDI-TOF/TOF fragmentation produced a series of Y_n and $^{1,5}X$ as well as D, E, F, and G ions that confirmed branching and linkage positions of a short complex oligosaccharide sequence β-Xyl-(1,4)-β-Xyl-(1,3)-α-Rha-(1,2)-α-GalA-(1,4)-Xyl. It is typically found in *Arabidopsis* xylan but is absent in *irx7*, *irx8* and *parvus-3* mutants (Brown et al., 2007).

A study of various underivatized linear xylo-oligosaccharides and nine isomeric mono- and disubstituted arabino-xylo-oligosaccharides [up to degree of polymerization (DP) 9] with known arabinose substitution pattern from enzymatic wheat arabinoxylan was performed using negative ESI-QTOF-MS (Quéméner et al., 2006). Two ions *m/z* 299 ($Y_i\alpha/C_2$) and *m/z* 263 ($Z_i\alpha/C_2$) and their ratio were found to be highly indicative of mono- (ratio ∼3–5) or disubstitution (ratio ∼0.1–0.2) at the penultimate xylose. In addition, cross-ring cleavage ions $^{0,2}A_2$ were only present in linear oligosaccharides but not in the mono-substituted isomers. The absence or presence of a cross-ring fragment $^{0,2}A_3$ also enabled the discrimination between structures with mono-substitution at the penultimate xylose and mono-substitution on either of its two interior neighbors.

Characteristic D, F, G, and W ions for O-3-linked arabinose and E and H ions for O-2-linked arabinose have been observed for NP-LC isolated 2-aminobenzoic acid-labeled wheat arabinoxylan (DP 4–6) oligosaccharides in MALDI-TOF/TOF MS (Maslen et al., 2007). Together with characteristic cross-ring ions $^{1,5}X$, $^{0,2}X$, and $^{3,5}A$ the sequence information and the position of arabinose substitution was obtained.

In another study, endo-xylanase released arabinoxylan oligosaccharides from Switchgrass were analyzed after size-exclusion separation by linkage analysis and ESI-MS^n. The interpretation of B and Y ions of spectra up to MS^4 of the per-O-methylated compounds confirmed structures with either 5, 6, or 8 pentosyl residues and a backbone of β-(1-4)-Xyl with either single α-Ara or α-Ara-(1-2)-α-Ara side chains at O-3 at the second xylose from the non-reducing end (Mazumder and York, 2010).

An impressive example demonstrating the strength of ESI-CID-MS^n using ion trap up to MS^4 is the characterization of complex mixtures of enzymatically released wheat arabinoxylan oligosaccharides without further separation steps (Matamoros Fernandéz et al., 2003). The ion *m/z* 1029.7 (DP 6, $[M+Na]^+$) of the per-O-methylated sample was generated by at least four identified branched isomers with either one (at the 2nd, 3rd, and 4th xylose from the reducing end, respectively) or two arabinose units (at the 3rd xylose).

PECTINS

Pectins are the most complex polysaccharides in plant cell walls. With charged residues, complicated and poorly understood branching, and highly variable modifications, structural analysis is particularly difficult. Determining the type and pattern of substitution and modification is critical to understanding the many functional aspects of pectins ranging from structural adhesive components in the wall to whole plant signaling, self-incompatibility, and defense against pathogens (Ridley et al., 2001). General fragmentation patterns of saturated and unsaturated oligogalacturonides (oligoGalA) by MALDI-PSD-TOF-MS have been determined. In positive mode, saturated and unsaturated oligoGalA gave ions of the B_i-, C_i- and Y_i-series but the C_i-ion series disappeared in saturated methyl-esterified oligoGalA. Unsaturated methyl-esterified oligoGalA additionally showed $^{1,5}X_i$ cross-ring cleavage ions (Van Alebeek et al., 2000). Negative-mode ESI-IT-MS/MS produces mainly C and Z-series ions which enables the location of the methyl-esterification and allows sequencing of partially methyl-esterified oligoGalA up to DP 10 (Körner et al., 1999). A loss of 32 Da from the precursor ion can be attributed to methyl-esterification at the GalA at the reducing end (Quéméner et al., 2003b).

Pectins are often also acetylated. The acetyl groups can be assigned by negative ESI-IT-MS^n through sequential trapping and fragmentation of C-ions (Quéméner et al., 2003a) and precise location of the acetyl group on O-2 or O-3 can be specified by cross-ring fragments $^{0,2}A_i$. The use of anion-exchange chromatography fractionation allows the structural determination of oligoGalA up to DP 5 with multiple methyl and/or acetyl groups at O-2 and/or O-3 (Ralet et al., 2005) and enabled the construction of a hypothetical sugar beet homogalacturonan with a proposed blockwise distribution of acetyl groups (Ralet et al., 2008).

Side-chain determination is particularly complicated in pectins. MALDI-TOF-MS and HPAEC has been used to provide evidence of the complex xylogalacturonan in *Arabidopsis thaliana* (Zandleven et al., 2007). The ESI-IT-MS fragmentation pattern resulting from MS^3 of $GalAXyl_2$ and MS^2 of $GalA_2Xyl_3$ from apple and potato indicated a dimeric xylose side chain (Zandleven et al., 2006).

Mild acid hydrolysis releases the side chains A–D of the complex pectin rhamnogalacturonan II (RG-II) from citrus pectin at their acid-labile branching points (Séveno et al., 2009). Y-type fragmentation patterns in positive ESI-MS/MS provide sequence information and enabled the structural identification while diagnostic ions derived by a loss of 296 and 340 Da resulting from cleavage of the AceA-rhamnose bond and ring-cleavage of AceA were found upon B-chain fragmentation. This acidic fingerprinting methology was applied to the analysis of RG-II from *Arabidopsis mur1* mutant that is deficient in L-fucose (Fuc) synthesis. The $[M+Na]^+$ ion as well as the fragmentation pattern confirmed that the 2-O-Me-Xyl-L-Fuc segment was replaced by L-galactose (Gal) in the A-chain and 2-O-Me-L-Gal instead of 2-O-Me-L-Fuc was incorporated into the B chain.

CONCLUSION AND OUTLOOK

Mass spectrometry has been proven to be a sensitive, fast, and powerful tool for the screening of cell wall changes as well as for the

structural analysis of carbohydrates in complex mixtures providing sequence and branching information including the location of modifications. MS analysis benefits from readily available specific enzymes that selectively release oligosaccharides. Although efforts in this direction have been made (Bauer et al., 2006), discovery of new enzymes with specific activity on specialized linkages in cell wall compounds which cannot be efficiently hydrolyzed or for which enzymes have not been identified (e.g., RG-II) would greatly improve polysaccharide analysis by MS. The complexity of mixtures with many isobaric structures can further be overcome by coupling mass spectrometers to ion mobility (IM) spectrometers that are capable of separating gas-phase ions by their size/charge ratio as well as their interaction with a buffer gas (Kanu et al., 2008). One can envision interesting 3-dimensional separation techniques by using various combinations of chromatography, IM, and MS. Through an expanding repertoire of sensitive techniques, MS will play an ever increasing role in the field of plant cell wall analysis.

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REFERENCES

Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006). Development of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11417–11422.

Björndal, H., Lindberg, B., and Svensson, S. (1967). Mass spectrometry of partially methylated alditol acetates. *Carbohydr. Res.* 5, 433–440.

Böcker, S., Kehr, B., and Rasche, F. (2011). Determination of glycan structure from tandem mass spectra. *Trans. Comput. Biol. Bioinformatics* 8, 976–986.

Brown, D. M., Goubet, F., Wong, V. W., Goodacre, R., Stephens, E., Dupree, P., and Turner, S. R. (2007). Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. *Plant J.* 52, 1154–1168.

Brüll, L., Huisman, M., Schols, H., Voragen, F., Critchley, G., Thomas-Oates, J., and Haverkamp, J. (1998). Rapid molecular mass and structural determination of plant cell wall-derived oligosaccharides using off-line high-performance anion-exchange chromatography/mass spectrometry. *J. Mass. Spectrom.* 33, 713–720.

Ceroni, A., Maass, K., Geyer, H., Geyer, R., Dell, A., and Haslam, S. M. (2008). GlycoWorkbench: A tool for the computer-assisted annotation of mass spectra of glycans. *J. Proteome Res.* 7, 1650–1659.

Chai, W., Piskarev, V., and Lawson, A. M. (2001). Negative electrospray mass spectrometry of neutral underivatized oligosaccharides. *Anal. Chem.* 73, 651–657.

Ciucanu, I., and Caprita, R. (2007). Per-O-methylation of neutral carbohydrates directly from aqueous samples for gas chromatography and mass spectrometry analysis. *Anal. Chim. Acta* 585, 81–85.

Ciucanu, I., and Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* 131, 209–217.

Coenen, G.-J., Kabel, M. A., Schols, H. A., and Voragen, A. G. J. (2008). CE-MSn of complex pectin-derived oligomers. *Electrophoresis* 29, 2101–2111.

Deery, M. J., Stimson, E., and Chappell, C. G. (2001). Size exclusion chromatography/mass spectrometry applied to the analysis of polysaccharides. *Rapid Commun. Mass Spectrom.* 15, 2273–2283.

Dell, A., Khoo, K.-H., Panico, M., McDowell, R. A., Etienne, A., Reason, A. J., and Morris, H. R. (1989). "FAB-MS and ESI-MS of glycoproteins," in *Glycobiology. A Practical Approach*, Vol. 125, eds M. Fukuda and A. Kobata (Oxford: Oxford University Press), 187–222.

Doco, T., O'Neill, M. A., and Pellerin, P. (2001). Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydr. Polym.* 46, 249–259.

Domon, B., and Costello, C. E. (1988). A systematic nomenclature for carbohydrate fragmentation in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate J.* 5, 397–409.

Duus, J. Ø., Gotfredsen, C. H., and Bock, K. (2000). Carbohydrate structural determination NMR spectroscopy: Modern methods and limitations. *Chem. Rev.* 100, 4589–4614.

Ebringerová, A., and Heinze, T. (2000). Xylan and xylan derivatives – biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties. *Macromol. Rapid Commun.* 21, 542–556.

Fry, S. C. (1989). The structure and function of xyloglucan. *J. Exp. Bot.* 40, 1–11.

Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Pérez Lorences, E., Maclachlan, A. J., Grant Reid, J. S., Seitz, H. U., Selvendran, R. R., Voragen, A. G. J., and White, A. R. (1993). An umambiuos nomenclature for xyloglucan-derived oligosaccharides. *Physiol Plant* 89, 1–3.

Garozzo, D., Giuffrida, M., Impallomeni, G., Ballistreri, A., and Montaudo, G. (1990). Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative ion fast atom bombardment mass spectrometry. *Anal. Chem.* 62, 279–286.

Gaucher, S. P., Morrow, J., and Leary, J. A. (2000). STAT: A saccharide topology analysis tool used in combination with tandem mass spectrometry. *Anal. Chem.* 72, 2331–2336.

Gouw, J. W., Burgers, P. C., Trikoupis, M. A., and Terlouw, J. K. (2002). Derivatization of small oligosaccharides prior to analysis by matrix-assisted laser desorption/ionization using glycidyltrimethylammonium chloride and Girard's reagent T. *Rapid Commun. Mass Spectrom.* 16, 905–912.

Harvey, D. J. (1999). Matrix-assisted laser desoption/ionization mass spectrometry of carbohydrates. *Mass Spectrom. Rev.* 18, 349–450.

Harvey, D. J. (2000). Electrospray mass spectrometry and fragmentation of N-linked carbohydrates derivatized at the reducing terminus. *J. Am. Soc. Mass Spectrom.* 11, 900–915.

Hilz, H., de Jong, L. E., Kabel, M. A., Schols, H. A., and Voragen, A. G. J. (2006). A comparison of liquid chromatography, capillary electrophoresis, and mass spectrometry methods to determine xyloglucan structures in black currants. *J. Chromatogr. A* 1133, 275–286.

Hofmeister, G., Zhou, Z., and Leary, J. A. (1991). Linkage position determination in lithium-cationized disaccharides: Tandem mass spectrometry and semiempirical calculations. *J. Am. Chem. Soc.* 113, 5964–5970.

Jacobs, A., Larsson, P. T., and Dahlman, O. (2001). Distribution of uronic acids in xylans from various species of soft- and hardwood as determined by MALDI mass spectrometry. *Biomacromolecules* 9, 979–990.

Kabel, M. A., Schols, H. A., and Voragen, A. G. J. (2001). Mass determination of oligosaccharides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry following HPLC, assisted by on-line desalting and automated sample handling. *Carbohydr. Polym.* 44, 161–165.

Kanu, A. B., Dwivedi, P., Tam, M., Matz, L., and Hill, H. H. Jr. (2008). Ion mobility-mass spectrometry. *J. Mass. Spectrom.* 43, 1–22.

Kim, J. B., and Carpita, N. C. (1992). Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.* 98, 646–653.

Körner, R., Limberg, G., Christensen, T. M. I. E., Mikkelsen, J. D., and Roepstorff, P. (1999). Sequencing of partially methyl-esterified oligogalacturonates by tandem mass spectrometry and its use to determine pectinase specificity. *Anal. Chem.* 71, 1421–1427.

Lapadula, A. J., Hatcher, P. J., Hanneman, A. J., Ashline, D. J., Zhang, H., and Reinhold, V. N. (2005). Congruent strategies for carbohydrate sequencing. 3. OSCAR: an algorithm for assigning oligosaccharide topology from MSn data. *Anal. Chem.* 77, 6271–6279.

Lerouge, P., O'Neill, M. A., Darvill, A. G., and Albersheim, P. (1993). Structural characterization of endoglycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydr. Res.* 243, 359–371.

Lerouxel, O., Choo, T. S., Séveno, M., Usadel, B., Faye, L., Lerouge, P., and Pauly, M. (2002). Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. *Plant Physiol.* 130, 1754–1763.

Maslen, S. L., Goubet, F., Adam, A., Dupree, P., and Stephens, E. (2007). Structure elucidation of arabinoxylan isomers by normal phase HPLC-MALDI-TOF/TOF-MS/MS. *Carbohydr. Res.* 342, 724–735.

Matamoros Fernandéz, L. E., Obel, N., Scheller, H. V., and Roepstorff, P. (2003). Characterization of plant oligosaccharides by matrix-assisted laser desorption/ionization and electrospray mass spectrometry. *J. Mass. Spectrom.* 38, 427–437.

Mazumder, K., and York, W. S. (2010). Structural analysis of arabinoxylans isolated from ball-milled switchgrass biomass. *Carbohydr. Res.* 345, 2183–2193.

Mazumder, S., Lerouge, P., Loutelier-Bourhis, C., Driouich, A., and Ray, B. (2005). Structural characterization of hemicellulosic polysaccharides from Benincasa hispida using specific enzyme hydrolysis, ion exchange chromatography and MALDI-TOF mass spectrometry. *Carbohydr. Polym.* 59, 231–238.

Mortimer, J. C., Miles, G. P., Brown, D. M., Zhang, Z., Segura, M. P., Weimar, T., Yu, X., Seffen, K. A., Stephens, E., Turner, S. R., and Dupree, P. (2010). Absence of branches from xylan in Arabidopsis gux mutants reveals potential for simplification of lignocellulosic biomass. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17409–17414.

Obel, N., Erben, V., Schwarz, T., Kühnel, S., Fodor, A., and Pauly, M. (2009). Microanalysis of plant cell wall polysaccharides. *Mol. Plant* 2, 922–932.

Persson, S., Caffall, K. H., Freshour, G., Hilley, M. T., Bauer, S., Poindexter, P., Hahn, M. G., Mohnen, D., and Somerville, C. (2007). The Arabidopsis irregular xylem8 mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. *Plant Cell* 19, 237–255.

Quéméner, B., Cabrera Pino, J. C., Ralet, M.-C., Bonnin, E., and Thibault, J.-F. (2003a). Assignment of acetyl groups to O-2 and/or O-3 of pectic oligogalacturonides using negative electrospray ionization ion trap mass spectrometry. *J. Mass. Spectrom.* 38, 641–648.

Quéméner, B., Désiré, C., and Lahaye, M. (2003b). Structural characterisation by both positive- and negative-ion electrospray mass spectrometry of partially methyl-esterified oligogalacturonides purified by semi-preparative high-performance anion-exchange chromatography. *Eur. J. Mass. Spectrom* 9, 45–60.

Quéméner, B., Ordaz-Ortiz, J. J., and Saulnier, L. (2006). Structural characterization of underivatized arabino-xylo-oligosaccharides by negative-ion electrospray mass spectrometry. *Carbohydr. Res.* 341, 1834–1847.

Ralet, M.-C., Cabrera, J. C., Bonnin, E., Quéméner, B., Hellín, P., and Thibault, J.-F. (2005). Mapping sugar beet pectin acetylation pattern. *Phytochemistry* 66, 1832–1843.

Ralet, M.-C., Crépeau, M.-J., and Bonnin, E. (2008). Evidence for a blockwise distribution of acetyl groups onto homogalacturonans from commercial sugar beet (Beta vulgaris) pectin. *Phytochemistry* 69, 1903–1909.

Ralet, M.-C., Lerouge, P., and Quéméner, B. (2009). Mass spectrometry for pectin structure analysis. *Carbohydr. Res.* 344, 1798–1807.

Ridley, B. L., O'Neill, M. A., and Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57, 929–967.

Ridlova, G., Mortimer, J. C., Maslen, S. L., Dupree, P., and Stephens, E. (2008). Oligosaccharides relative quantification using isotope tagging and normal-phase liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 2723–2730.

Schols, H. A., Mutter, M., Voragen, A. G. J., Niessen, W. M. A., van der Hoeven, R. A. M., van der Greef, J., and Bruggink, C. (1994). The use of combined high-performance anion-exchange chromatography-thermospray mass spectrometry in the structural analysis of pectic oligosaccharides. *Carbohydr. Res.* 261, 335–342.

Séveno, M., Voxeur, A., Rihouey, C., Wu, A.-M., Ishii, T., Chevalier, C., Ralet, M.-C., Driouich, A., Marchant, A., and Lerouge, P. (2009). Structural characterization of the pectic polysaccharide rhamnogalacturonan II using an acidic fingerprinting methodology. *Planta* 230, 947–957.

Spina, E., Sturiale, L., Romeo, D., Impallomeni, G., Garozzo, D., Waidelich, D., and Glueckmann, M. (2004). New fragmentation mechanisms in matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry of carbohydrates. *Rapid Commun. Mass Spectrom.* 18, 392–398.

Tang, H., Mechref, Y., and Novotny, M. V. (2005). Automated interpretation of MS/MS spectra of oligosaccharides. *Bioinformatics* 21(Suppl. 1), i431–i439.

Van Alebeek, G.-J. W.M., Zabotina, O., Beldman, G., Schols, H. A., and Voragen, A. G. J. (2000). Structural analysis of (methyl-esterified) oligogalacturonides using post-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass. Spectrom.* 35, 831–840.

Vierhuis, E., York, W. S., Kolli, V. S. K., Vincken, J.-P., Schols, H. A., Van Alebeek, G.-J. W. M., and Voragen, A. G. J. (2001). Structural analysis of two arabinose containing oligosaccharides derived from olive fruit xyloglucan: XXSG and XLSG. *Carbohydr. Res.* 332, 285–297.

Westphal, Y., Schols, H. A., Voragen, A. G. J., and Gruppen, H. (2010). MALDI-TOF MS and CE-LIF fingerprinting of plant cell wall polysaccharide digests as a screening tool for Arabidopsis cell wall mutants. *J. Agric. Food Chem.* 58, 4644–4652.

Yamagaki, T., Mitsuishi, Y., and Nakanishi, H. (1998). Determination of structural isomers of xyloglucan octasaccharide using post-source decay fragment analysis in MALDI-TOF mass spectrometry. *Tetrahedron Lett.* 39, 4051–4054.

Yuan, J., Hashii, N., Kawasaki, N., Itoh, S., Kawanishi, T., and Hayakawa, T. (2005). Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography-mass spectrometry. *J. Chromatogr. A* 1067, 145–152.

Zandleven, J., Beldman, G., Bosveld, M., Schols, H. A., and Voragen, A. G. J. (2006). Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase. *Carbohydr. Polym.* 65, 495–503.

Zandleven, J., Oxenbøll Sørensen, S., Harholt, J., Beldman, G., Schols, H. A., Scheller, H. V., and Voragen, A. G. J. (2007). Xylogalacturonan exists in cell walls from various tissues of Arabidopsis thaliana. *Phytochemistry* 68, 1219–1226.

Zhang, R., Sioma, C. S., Wang, S., and Regnier, F. E. (2001). Fractionation of isotopically labeled peptides in quantitative proteomics. *Anal. Chem.* 73, 5142–5149.

Zhou, Z., Ogden, S., and Leary, J. A. (1990). Linkage position determination in oligosaccharides: MS/MS study of lithium-cationized carbohydrates. *J. Org. Chem.* 55, 5444–5446.

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Plant glycosyltransferases beyond CAZy: a perspective on DUF families

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The carbohydrate active enzyme (CAZy) database is an invaluable resource for glycobiology and currently contains 45 glycosyltransferase families that are represented in plants. Glycosyltransferases (GTs) have many functions in plants, but the majority are likely to be involved in biosynthesis of polysaccharides and glycoproteins in the plant cell wall. Bioinformatic approaches and structural modeling suggest that a number of protein families in plants include GTs that have not yet been identified as such and are therefore not included in CAZy. These families include proteins with domain of unknown function (DUF) DUF23, DUF246, and DUF266. The evidence for these proteins being GTs and their possible roles in cell wall biosynthesis is discussed.

Keywords: cell walls, DUF23, DUF246, DUF266, glycosyltransferases

INTRODUCTION

Plant cell walls contain structural polysaccharides such as cellulose, hemicelluloses, and pectins. To assemble these polysaccharides as well as the glycan structures on glycoproteins, the plant needs extensive biosynthetic machinery, and it has been estimated that over 2000 gene products are involved in making and maintaining the wall (Carpita et al., 2001; Dhugga, 2001). The polysaccharides and other glycans are mainly synthesized by glycosyltransferases (GTs; EC 2.4.x.y). Most GTs transfer a sugar residue from an activated nucleotide sugar to a specific acceptor molecule, forming a glycosidic bond, and GTs generally display high specificity for both the sugar donor and the acceptor substrates (Breton et al., 2006). GTs are classified as "retaining" or "inverting" depending on whether glycosylation occurs with retention or inversion of stereochemistry at the anomeric carbon atom of the donor substrate. GTs have been further classified into families on the basis of amino acid sequence similarities in the carbohydrate active enzyme (CAZy) database[1] (Cantarel et al., 2009). CAZy contains proteins with a demonstrated biochemical function as well as orthologous putative GTs. The CAZy database is continually increasing, adding new GTs as they are discovered, and currently contains 91 GT families numbered from GT1 to GT94 (GT36, GT46, and GT86 no longer exist). Only two folds, and variants thereof, have been observed for all structures of nucleotide–sugar-dependent GTs solved to date, termed GT-A and GT-B (Hansen et al., 2010). Whereas many GT-Bs are found to be independent of a metal ion for catalysis, most GT-A enzymes contains a conserved DxD motif that coordinates the phosphate atoms of the nucleotide donors via coordination of a divalent cation, usually Mn^{2+} or Mg^{2+} (Breton et al., 2006). Identifying the precise function of every putative plant GT represents an immense task. For example, about 1.7% of the 27,416 protein coding *Arabidopsis* genes are represented among 42 GT families in the database, but less than 20% of these sequences, have been annotated to date (Caffall and Mohnen, 2009; Scheller and Ulvskov, 2010) and very few of the GTs involved in cell wall biosynthesis have had their biochemical activity unambiguously demonstrated. Some of the CAZy GTs could also have lost their catalytic function and represent proteins with other functions, e.g., carbohydrate binding proteins or non-catalytic members of GT complexes as recently suggested for GAUT7 (Atmodjo et al., 2011). Cell wall biosynthesis involves two classes of GTs: The multi-membrane-spanning GTs of GT2 and GT48 and the more common type II transmembrane proteins consisting of a short cytoplasmic N-terminal tail followed by a single transmembrane helix, a stem region of variable length, and a large globular C-terminal part containing the catalytic domain. GTs that are Type II membrane proteins have been found in the endoplasmic reticulum (ER) and Golgi apparatus, while the multi-membrane-spanning members of GT2 and GT48 are associated with the Golgi apparatus or plasma membrane. The majority of GTs involved in biosynthesis of the complex cell wall polysaccharides are thought to be Golgi localized.

Given the complexity of the plant cell wall, and the fact that new GT families are regularly added to CAZy, it can be expected that some of the cell wall biosynthetic genes have yet to be identified. This could be achieved by for example forward genetics or via orthology to other newly discovered GTs followed by reverse genetics. An example of an activity with no candidates in CAZy is the β-arabinosyltransferase – a retaining GT – that transfers

[1] www.cazy.org

the innermost arabinose to hydroxyproline in extensin, and which must be present in both green algae and plants. However, analysis of retaining CAZy GTs of *Chlamydomonas reinhardtii* and *Arabidopsis* did not reveal any ortologous proteins, which did not have a putative function unrelated to extensin biosynthesis (Harholt, Paiva, Domozych and Ulvskov, unpublished). This strongly suggests that this particular β-arabinosyltransferase is in a family not yet included in CAZy. With the use of various bioinformatic strategies, Hansen et al. (2009) identified plant sequences representing a "Golgi located GT motif" in domain of unknown function (DUF) families DUF246 and DUF266, and proposed that the number of plant GT genes is underestimated. Currently, more than 3000 DUF families are represented in the protein family (Pfam) database, a large collection of protein families, grouped via sequence similarity, and hidden Markov models (HMM; Finn et al., 2010).[2]

The aim of this review is to briefly describe putative GTs that are not currently classified in the CAZy database. DUF246 and DUF266 had previously been identified as putative GTs (Hansen et al., 2009), while DUF23 is related to a novel GT family unknown at the time of the earlier study.

CELL WALL ASSOCIATED DUF266

Bioinformatic studies using HMM and fold recognition identified 14 *Arabidopsis* genes that shared a DUF266 domain and were distantly related to GT14 (Hansen et al., 2009). These putative plant GT sequences were annotated as containing a plant specific DUF266 domain, described in the Pfam database as "likely to be glycosyltransferase related." Fold recognition analysis and hydrophobic cluster analysis demonstrated structural similarities to the Leukocyte core-2 β1, 6*N*-acetylglucosaminyltransferase (C2GnT-L) family protein, which is a member of GT14 (Hansen et al., 2009). Furthermore, invariant amino acid residues were found between C2GnT-L and *Arabidopsis* DUF266 proteins. C2GnT-L is a Golgi localized, inverting GT with a GT-A fold (Pak et al., 2006). Quite uncommon for GTs with a GT-A fold, C2GnT-L lacks the characteristic metal ion binding DxD motif. C2GnT-L is involved in biosynthesis of mucin-type glycoproteins catalyzing the formation of the core-2 branched O-glycan (Galβ1-3[GlcNAcβ1-6]GalNac-*O*-Ser/Thr) from its donor and acceptor substrates, UDP-GlcNAc and the core-1 O-glycan (Galβ1-3GalNAc-*O*-Ser/Thr), respectively (Fukuda et al., 1996; Yeh et al., 1999; Pak et al., 2006). C2GnT-L is the only structure solved in GT14. The structure of C2GnT-L from mice was determined with β-D-galactose and/or *N*-acetyl-D-glucosamine as ligands (Pak et al., 2006). The putative catalytic amino acid residue (glutamic acid) of C2GnT-L is conserved in other inverting GT-A proteins and in the plant DUF266 family (see **Figure 1A**; **Figure A4A** in Appendix; Hansen et al., 2009).

Concurrent with the bioinformatic identification of DUF266 as putative GTs, the rice *brittle culm 10* mutant (*Osbc10*) was characterized. The *Osbc10* mutant displayed brittleness of the plant body and morphological abnormalities including significant decrease in plant height and tiller number (Zhou et al., 2009). The corresponding protein OsBC10 was shown to be a Golgi located type II membrane protein containing a DUF266 domain. The cell wall composition showed reduced content of glucose and increased content of xylose, arabinose, and lignin, and antibody labeling identified a decrease in epitopes associated with arabinogalactan proteins (AGPs). The cellular localization, the type II membrane protein structure and the cell wall phenotypes led the authors to suggest that OsBC10 is involved in cell wall biosynthesis, although the specific enzymatic activity is still unclear. The OsBC10 protein was heterologously expressed in Chinese hamster ovary cells and demonstrated a very low *in vitro* activity in an assay for C2GnT-L. Thus, although OsBC10 is unlikely to have C2GnT-L activity *in vivo* since the core-2 branched O-glycan is almost certainly absent in plants, we conclude that the evidence for OsBC10 being a GT involved in cell wall biosynthesis is strong.

The GT14 family contains 308 sequences from viruses, bacteria, animals, and plants. It includes, beside proteins with unknown function, proteins with β-1,6-*N*-acetylglucosaminyl-transferase and β-xylosyltransferase activity involved in the synthesis of O-glycans in animals (Bierhuizen et al., 1993; Yeh et al., 1999; Hwang et al., 2003). A phylogenetic analysis of plant sequences from family GT14 shows that all clusters into one single subfamily (Aspeborg et al., 2005). To our knowledge, no plant GT14 gene has yet been functionally characterized. The phylogenetic relationship and sequence similarity within the PF02485 domain, between GT14 and DUF266 proteins in plants is illustrated in **Figure A1** in Appendix.

Since the study of Hansen et al. (2009), the DUF266 family has been removed from the Pfam database and merged into the Branch domain (PF02485), which contains core-2 and I-branching enzymes (Bierhuizen et al., 1993; Yeh et al., 1999). The Branch domain (PF02485) contains 484 plant UniProt accessions, shared by 76 GT14, and 86 sequences of the previous DUF266 (**Figure 1D**). Whereas the Branch domain was found among animals, plants, vira, and bacteria, DUF266 was only found in proteins from plants and therefore likely to be involved in biological processes specific to plants.

While the majority of *Arabidopsis* GT14 proteins were predicted to be Golgi located, consistent with a role in cell wall biosynthesis, the predicted localization of DUF266 family proteins was more variable, with most predicted to be in Golgi and plasma membrane (Ye et al., 2011). However, as we have recently discussed, bioinformatic tools for predicting Golgi localization are highly unreliable, and a much more reliable prediction is obtained based on Pfam groupings (Oikawa et al., 2010).

Whereas the DUF266 containing *OsBC10* was mainly expressed in the developing vascular bundle and sclerenchyma cells (Zhou et al., 2009), two plant GT14 members (*PttGT14A* and *PttGT14B*) were identified by expression profiling to be xylem-specific (Aspeborg et al., 2005), indicating their potential role in secondary cell wall biosynthesis. Recently, two DUF266 proteins (At1g11940 and At5g11730) were found in Golgi purified from *Arabidopsis* cell suspension cultures (Parsons et al., 2012) suggesting a role in primary wall biosynthesis. However, the large DUF266 family, with 22 proteins in *Arabidopsis* and 5 in *Selaginella moellendorffii* (**Table 1**) is likely to comprise proteins of quite different biochemical activities, and currently there is insufficient published

[2]http://pfam.sanger.ac.uk/

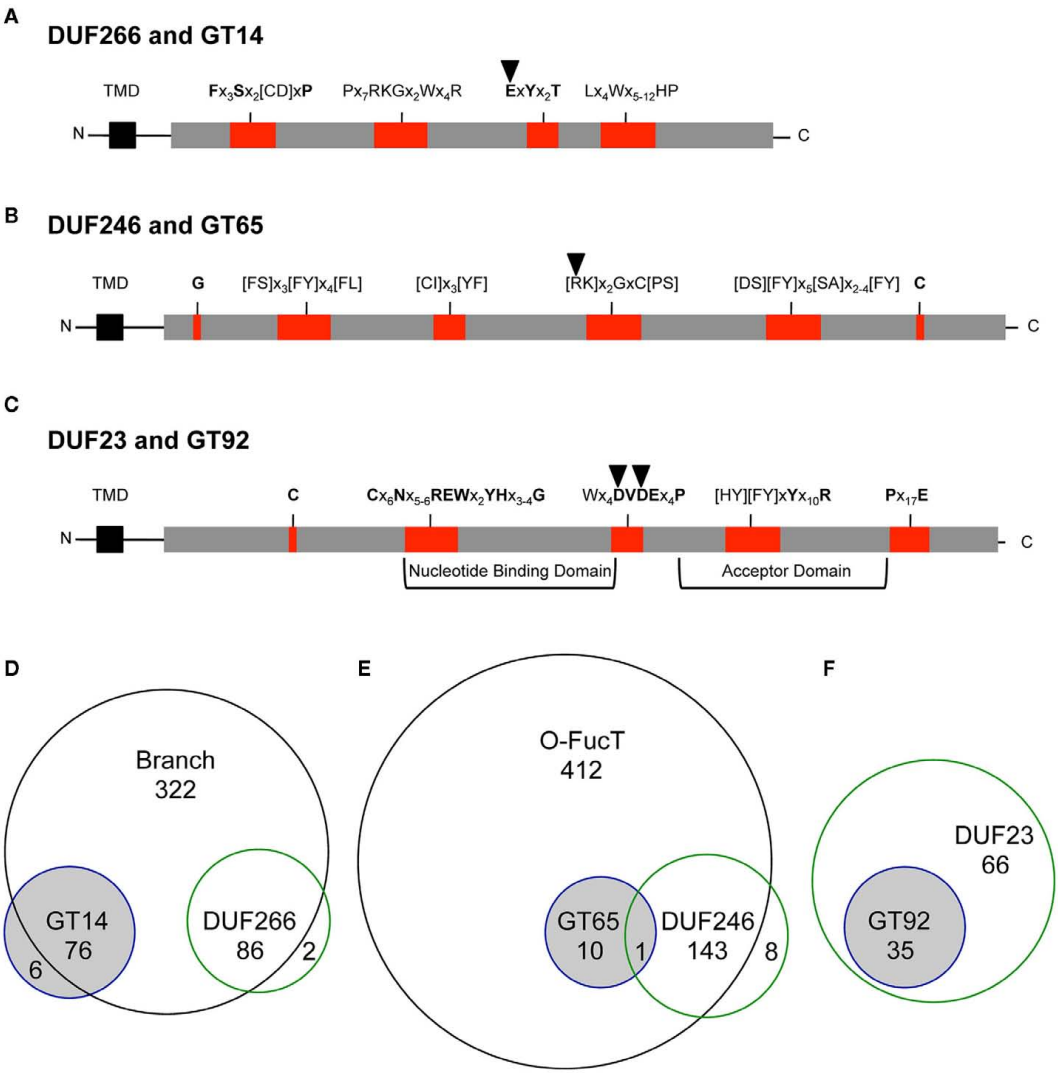


FIGURE 1 | Comparative analysis between DUF families and their related Pfam groups. (A–C) Schematic illustration of conserved motifs for **(A)** DUF266 and GT14, **(B)** DUF246 and GT65, and **(C)** DUF23 and GT92, based on non-redundant *Arabidopsis* sequences with more than 200 amino acid residues. Strickly conserved residues are in bold and residues with only two alternative amino acids are denoted by parentheses. Residues involved in substrate binding and putative catalytic residues are marked with arrowheads. GT-A structure domains (Nucleotide Binding and Acceptor, respectively) are shown as Breton et al., 2006. TMD: transmembrane domain. **(D–F)** Illustration of the total number of plant UniProt sequences retrieved from **(D)** DUF266, GT14 and the Branch domain (PF02485), **(E)** DUF246, GT65, and the *O*-fucosyltransferase domain (*O*-FucT; PF10250), and **(F)** GT92 and DUF23 (PF01697).

Table 1 | Summary of the three putative GT families and the GT families they are related to.

Class	No TMD	1 TMD	>1 TMDs	Total sequences	*Arabidopsis*	Rice	*Selaginella*	*Chlamydomonas*
GT14	22	60	0	82	9	10	2	0
GT65	3	8	0	11	1	1	1	0
GT92	23	12	0	35	3	3	2	0
DUF266	10	77	1	88	22	19	5	0
DUF246	57	91	4	152	34	28	19	2
DUF23	45	54	2	101	7	5	7	4

Columns 2–5 show the total number of plant sequences in UniProt and a prediction for the number of transmembrane domains (TMDs) made using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Columns 6–9 give the number of non-redundant sequences with more than 200 amino acid residues for Arabidopsis, Oryza sativa cv Nipponbare (Rice), S. moellendorfii, and C. reinhardtii.

information to make any qualified guess on what these activities might be.

DUF246 – PLANT SPECIFIC FUCOSYLTRANSFERASES?

In the search for new GT candidates, three sequences (At1g51630, At3g02250, At5g15740) annotated DUF246 (PF03138), were identified, by the use of HMM against GT65 (Hansen et al., 2009). In TAIR[3], 34 *Arabidopsis* sequences were found to contain a DUF246 domain, and a clear GT signature related to GT65 was demonstrated. The DUF246 motif has been found in a variety of plant species, including the evolutionary basal moss *Physcomitrella patens* and spikemoss *S. moellendorffii* (**Table 1**). BLAST searches with DUF246 proteins identified the single representative of *Arabidopsis* in GT65 (At3g05320) as the most similar protein, even though this protein is not included in DUF246 (Hansen et al., 2009).

Carbohydrate active enzyme family GT65 comprises 42 eukaryote sequences from 40 species most of which are annotated as protein-*O*-fucosyltransferase 1 (Pofut1), an inverting enzyme that adds fucose to serine or threonine residues in Epidermal Growth Factor-repeats (Oriol et al., 1999). The crystal structure of Pofut1 from *Caenorhabditis elegans* shows a manganese independent GT-B fold solved with GDP-Fucose attached in the active site (Lira-Navarrete et al., 2011). Phe261 and Phe357 bind the nucleotide sugar and mutagenesis studies showed that Arg240 is important in catalysis and binding. In *C. elegans* Pofut1, Asn43, and Arg240 are important catalytic residues, Asn43 being the flexible amino acid involved in fucose binding (Lira-Navarrete et al., 2011). Strict conservation of any of these amino acids could not be observed in the many plant DUF246 sequences and therefore the substrate utilized by DUF246 might be different from the GDP-Fucose utilized by Pofut1 (see **Figure 1B** and the alignment in **Figure A4B** in Appendix). However, GT65 belongs to the large fucosyltransferase superfamily, and is distantly related to families GT11, GT23, and GT68, which all represent inverting fucosyltransferases.

DUF246 is a large family, with, e.g., 34 members in *Arabidopsis* and 19 in *S. moellendorffii* (**Table 1**). Definitive proof that any of these proteins are in fact GTs has not been presented, but the high similarity to the other members of PF10250 makes the GT assignment very likely. The *O*-fucosyltransferase domain (PF10250), into which DUF246 has been merged, was found among animals, plants, vira, and bacteria, whereas DUF246 was only found in predicted protein sequences from plant species and therefore appeared to be involved in biological processes specific to plants, such as cell wall biosynthesis (**Figure 1E**). α-Fucosyl residues in plants are present in N-glycans, xyloglucan, AGP, and RG-II. Since fucosyltransferases involved in synthesizing xyloglucan and AGP are in GT37 (Perrin et al., 1999; Wu et al., 2010) and fucosyltransferases involved in N-glycosylation are likely in GT10 (Wilson et al., 2001), we suggest that some of the DUF246 proteins are candidates for the RG-II fucosyltransferase. However, the DUF246 family is large and contains conserved, ancient clades (see **Figure A2** in Appendix) so it is unlikely that all the proteins would have the same biochemical function. Several of the DUF246 proteins are co-expressed with GTs involved in secondary cell wall biosynthesis (Oikawa et al., 2010), suggesting another role than RG-II biosynthesis. A number of other DUF246 proteins are abundant in *Arabidopsis* cell suspension cultures, and found in the Golgi fractions, suggesting a role in primary cell wall biosynthesis (Parsons et al., 2012).

DUF23 – A PROTEIN FAMILY RELATED TO GT92

GT92 is the newest family in CAZy that contains plant members. GT92 was created based on characterization of N-glycan core α1,6-fucoside β1,4-galactosyltransferase (GALT-1) from *C. elegans* (Titz et al., 2009). The GT92 proteins all contain a DUF23 motif. GALT-1 encodes a manganese dependent UDP-galactose galactosyltransferase that adds β-galactose to α1,6-linked fucose at the reducing end of GalNAc in N-glycan cores. By search for homologs, the new family GT92 was created, containing eukaryotic sequences from animals and plants. However, it is interesting to note that no β1,4-galactose or α1,6-fucose has been found in plant N-glycans, to date. Three subfamilies emerged after a phylogenetic study, one including GALT-1 and homologs from various animal species, one consisting of homologs exclusively from *C. elegans* and *Caenorhabditis briggsae* and the third comprising plant proteins (Titz et al., 2009). Sequence studies showed the proteins from GT92 to share the putative metal binding DxD motif and to have a predicted type II membrane protein topology (Titz et al., 2009). Although no structure has been solved for GT92 proteins, the DxD motif suggests a GT-A fold.

Another *C. elegans* protein with a DUF23 motif, BAH-1 (Q9XXM0_CAEEL), is expressed in seam cells and required for microbial biofilm attachment (Drace et al., 2009). The BAH-1 protein is not included in GT92 and it is not known if it is a GT. DUF23 is assigned to clan GT-A, which contains carbohydrate interacting proteins as well as multiple nucleotide sugar-dependent GT families (e.g., GT8 and GT43)[4]. The DUF23 domain is found in hypothetical proteins from animals and plants, and unlike GT92, DUF23 is also found in bacteria (Drace et al., 2009; Suzuki and Yamamoto, 2010). The DUF23 embraces all the sequences of GT92 and expands beyond, comprising about three times as many plant proteins as in GT92 (**Table 1**; **Figure 1F**). All the DUF23 proteins contain two conserved cysteine residues and several charged residues, including the DxD motif, involved in substrate binding (**Figure 1C**; **Figure A4C** in Appendix; Drace et al., 2009; Suzuki and Yamamoto, 2010). Since no GT92 protein has had its structure solved it is not currently possible to determine if the essential catalytic residues are conserved between GT92 and the rest of DUF23. However, because of the similarity and the conserved DxD motif we find it highly likely that the DUF23 proteins outside GT92 are also GTs. Many of the DUF23 proteins are predicted to have a single transmembrane domain in the N-terminal part of the protein, consistent with a type II membrane protein topology.

Four of the *Arabidopsis* sequences containing the DUF23 motif were found to be co-expressed with GTs within CAZy either directly (GT47: At5g62220 (GT18) and GT2: CesA6) or indirectly

[3]www.arabidopsis.org

[4]http://pfam.sanger.ac.uk/clan/GT-A

(GT2: CesA1, CesA2, CesA3, CslC5; GT8: GATL2 and AtGolS1; and GT47: At5g62220 (GT18)) by Atted-II[5] (Obayashi et al., 2011).

The putative function of the DUF23 proteins in plants is not clear. GT92 is present in basal plant species, but DUF23 proteins outside of GT92 are present even in *C. reinhardtii*, indicating an ancient function of these proteins (see phylogenetic analysis of plant DUF23 proteins in **Figure A3** in Appendix). Given that *C. reinhardtii* lacks most of the polysaccharides found in plants, a conserved function of the non-GT92 members of DUF23 is more likely to be related, e.g., to glycoprotein biosynthesis than to polysaccharide biosynthesis.

FUTURE PERSPECTIVES

For the three groups of proteins discussed here, comprising proteins currently not in CAZy, we find that there is strong evidence that they are in fact GTs. Thus, they are all good candidates for reverse genetic studies and/or heterologous expression and enzyme activity experiments. These proteins would add 64 *Arabidopsis* proteins to the current 463 *Arabidopsis* GTs in CAZy. An obvious question is how many GTs are there beyond the 529 already present or suggested GTs. In the study of Hansen et al. (2009) other families were suggested, including DUF231 and DUF248, although a clear GT signature could only be demonstrated for DUF246, DUF266, and the single protein At5g28910. DUF231 proteins have recently been shown to be involved in polysaccharide acetylation (Gille et al., 2011) and they are likely to be subunits of acetyltransferase complexes rather than GTs (Anantharaman and Aravind, 2010; Manabe et al., 2011). For DUF248 there is evidence that they may be involved in polysaccharide methylation, but no biochemical evidence has been presented (Mouille et al., 2007). However, it should be noted that GT92 was added to CAZy after the bioinformatic study of Hansen et al. (2009) but GT92 and the related DUF23 were not identified as candidates in that study, which applied stringent filters to prevent erroneous identification of too many proteins that would later turn out not to be GTs. Apparently, even though DUF23 and GT92 belong to the GT-A clan, they are sufficiently diverged from other members of the GT-A clan that they were not identified through structural modeling. It shall be interesting to see to what extent the actual structures of GT92/DUF23 proteins differ from known GT-A structures.

The fact that GT92 was missed suggests that there may well be other families of GTs in plants yet to be discovered. Some GT families were founded following the identification of novel plant GTs, e.g., GT34 and GT37, rather than by GTs from other taxonomic groups. GT37 and GT77 are families that are unique to plants. This illustrates that plants have evolved some divergent GTs that cannot easily be identified through homology with GTs from other organisms. This is not surprising given that plants have many unique properties, e.g., the complex cell wall with a structure and biological role quite different from what is found in any other organisms. Identification of such plant unique families will require isolation of mutants through forward genetics screens or indications, e.g., from gene expression and localization analyses (Manfield et al., 2004; Brown et al., 2005; Persson et al., 2005; Oikawa et al., 2010; Mutwil et al., 2011; Sharma et al., 2011). To estimate the number of such unidentified GTs that might be present is very difficult. However, an indication may be that the last plant GT that was found as an unknown protein outside CAZy was OsBC10 reported in 2009 (Zhou et al., 2009) and it belonged to DUF266, which was already predicted to be putative GTs. In general, forward screens and coexpression studies identify GTs that are already in CAZy. Therefore, apart from the three protein families discussed in this review, we think that the number of unknown plant GTs is small.

[5]http://atted.jp/

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REFERENCES

Anantharaman, V., and Aravind, L. (2010). Novel eukaryotic enzymes modifying cell-surface biopolymers. *Biol. Direct* 5, 1.

Aspeborg, H., Schrader, J., Coutinho, P. M., Stam, M., Kallas, A., Djerbi, S., Nilsson, P., Denman, S., Amini, B., Sterky, F., Master, E., Sandberg, G., Mellerowicz, E., Sundberg, B., Henrissat, B., and Teeri, T. T. (2005). Carbohydrate-active enzymes involved in the secondary cell wall biogenesis in hybrid aspen. *Plant Physiol.* 137, 983–997.

Atmodjo, M. A., Sakuragi, Y., Zhu, X., Burrell, A. J., Mohanty, S. S., Atwood, J. A. III, Orlando, R., Scheller, H. V., and Mohnen, D. (2011). Galacturonosyltransferase (GAUT)1 and GAUT7 are the core of a plant cell wall pectin biosynthetic homogalacturonan:galacturonosyltransferase complex. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20225–20230.

Bierhuizen, M. F., Mattei, M. G., and Fukuda, M. (1993). Expression of the developmental I antigen by a cloned human cDNA encoding a member of a beta-1,6-N-acetylglucosaminyltransferase gene family. *Genes Dev.* 7, 468–478.

Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., and Imberty, A. (2006). Structures and mechanisms of glycosyltransferases. *Glycobiology* 16, 29R–37R.

Brown, D. M., Zeef, L. A., Ellis, J., Goodacre, R., and Turner, S. R. (2005). Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* 17, 2281–2295.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The carbohydrate-active enzymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233–D238.

Carpita, N., Tierney, M., and Campbell, M. (2001). Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. *Plant Mol. Biol.* 47, 1–5.

Dhugga, K. S. (2001). Building the wall: genes and enzyme complexes for polysaccharide synthases. *Curr. Opin. Plant Biol.* 4, 488–493.

Drace, K., Mclaughlin, S., and Darby, C. (2009). *Caenorhabditis elegans* BAH-1 is a DUF23 protein expressed in seam cells and required for microbial biofilm binding to the cuticle. *PLoS ONE* 4, e6741. doi:10.1371/journal.pone.0006741

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., Gavin, O. L., Gunasekaran, P., Ceric, G., Forslund, K., Holm, L., Sonnhammer, E. L., Eddy, S. R., and Bateman, A. (2010). The Pfam protein families database. *Nucleic Acids Res.* 38, D211–D222.

Fukuda, M., Bierhuizen, M. F., and Nakayama, J. (1996). Expression cloning of glycosyltransferases. *Glycobiology* 6, 683–689.

Gille, S., De Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011). O-acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Hansen, S. F., Bettler, E., Rinnan, A., Engelsen, S. B., and Breton, C. (2010). Exploring genomes for glycosyltransferases. *Mol. Biosyst.* 6, 1773–1781.

Hansen, S. F., Bettler, E., Wimmerova, M., Imberty, A., Lerouxel, O., and Breton, C. (2009). Combination of several bioinformatics approaches for the identification of new putative glycosyltransferases in *Arabidopsis*. *J. Proteome Res.* 8, 743–753.

Hwang, H. Y., Olson, S. K., Brown, J. R., Esko, J. D., and Horvitz, H. R. (2003). The *Caenorhabditis elegans* genes sqv-2 and sqv-6, which are required for vulval morphogenesis, encode glycosaminoglycan galactosyltransferase II and xylosyltransferase. *J. Biol. Chem.* 278, 11735–11738.

Lira-Navarrete, E., Valero-Gonzalez, J., Villanueva, R., Martinez-Julvez, M., Tejero, T., Merino, P., Panjikar, S., and Hurtado-Guerrero, R. (2011). Structural insights into the mechanism of protein O-fucosylation. *PLoS ONE* 6, e25365. doi:10.1371/journal.pone.0025365

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., Pauly, M., Knox, J. P., Sakuragi, Y., and Scheller, H. V. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078.

Manfield, I. W., Orfila, C., Mccartney, L., Harholt, J., Bernal, A. J., Scheller, H. V., Gilmartin, P. M., Mikkelsen, J. D., Paul Knox, J., and Willats, W. G. (2004). Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *Plant J.* 40, 260–275.

Mouille, G., Ralet, M. C., Cavelier, C., Eland, C., Effroy, D., Hematy, K., Mccartney, L., Truong, H. N., Gaudon, V., Thibault, J. F., Marchant, A., and Hofte, H. (2007). Homogalacturonan synthesis in *Arabidopsis thaliana* requires a Golgi-localized protein with a putative methyltransferase domain. *Plant J.* 50, 605–614.

Mutwil, M., Klie, S., Tohge, T., Giorgi, F. M., Wilkins, O., Campbell, M. M., Fernie, A. R., Usadel, B., Nikoloski, Z., and Persson, S. (2011). PlaNet: combined sequence and expression comparisons across plant networks derived from seven species. *Plant Cell* 23, 895–910.

Obayashi, T., Nishida, K., Kasahara, K., and Kinoshita, K. (2011). ATTED-II updates: condition-specific gene coexpression to extend coexpression analyses and applications to a broad range of flowering plants. *Plant Cell Physiol.* 52, 213–219.

Oikawa, A., Joshi, H. J., Rennie, E. A., Ebert, B., Manisseri, C., Heazlewood, J. L., and Scheller, H. V. (2010). An integrative approach to the identification of *Arabidopsis* and rice genes involved in xylan and secondary wall development. *PLoS ONE* 5, e15481. doi:10.1371/journal.pone.0015481

Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., and Breton, C. (1999). Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology* 9, 323–334.

Pak, J. E., Arnoux, P., Zhou, S., Sivarajah, P., Satkunarajah, M., Xing, X., and Rini, J. M. (2006). X-ray crystal structure of leukocyte type core 2 beta1,6-N-acetylglucosaminyltransferase. Evidence for a convergence of metal ion-independent glycosyltransferase mechanism. *J. Biol. Chem.* 281, 26693–26701.

Parsons, H. T., Christiansen, K., Knierim, B., Carroll, A., Ito, J., Batth, T. S., Smith-Moritz, A. M., Morrison, S., McInerney, P., Hadi, M. Z., Auer, M., Mukhopadhyay, A., Petzold, C. J., Scheller, H. V., Loqué, D., and Heazlewood, J. L. (2012). Isolation and proteomic characterization of the Arabidopsis Golgi defines functional and novel targets involved in plantcell wall biosynthesis. *Plant Physiol.* (in press).

Perrin, R. M., Derocher, A. E., Bar-Peled, M., Zeng, W. Q., Norambuena, L., Orellana, A., Raikhel, N. V., and Keegstra, K. (1999). Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* 284, 1976–1979.

Persson, S., Wei, H. R., Milne, J., Page, G. P., and Somerville, C. R. (2005). Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8633–8638.

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289.

Sharma, R., Tan, F., Jung, K. H., Sharma, M. K., Peng, Z., and Ronald, P. C. (2011). Transcriptional dynamics during cell wall removal and regeneration reveals key genes involved in cell wall development in rice. *Plant Mol. Biol.* 77, 391–406.

Suzuki, N., and Yamamoto, K. (2010). Molecular cloning of pigeon UDP-galactose:beta-D-galactoside alpha1,4-galactosyltransferase and UDP-galactose:beta-D-galactoside beta1,4-galactosyltransferase, two novel enzymes catalyzing the formation of Gal alpha1-4Gal beta1-4Gal beta1-4GlcNAc sequence. *J. Biol. Chem.* 285, 5178–5187.

Titz, A., Butschi, A., Henrissat, B., Fan, Y. Y., Hennet, T., Razzazi-Fazeli, E., Hengartner, M. O., Wilson, I. B., Kunzler, M., and Aebi, M. (2009). Molecular basis for galactosylation of core fucose residues in invertebrates: identification of *Caenorhabditis elegans* N-glycan core alpha1,6-fucoside beta1,4-galactosyltransferase GALT-1 as a member of a novel glycosyltransferase family. *J. Biol. Chem.* 284, 36223–36233.

Wilson, I. B., Rendic, D., Freilinger, A., Dumic, J., Altmann, F., Mucha, J., Muller, S., and Hauser, M. T. (2001). Cloning and expression of cDNAs encoding alpha1,3-fucosyltransferase homologues from *Arabidopsis thaliana*. *Biochim. Biophys. Acta* 1527, 88–96.

Wu, Y., Williams, M., Bernard, S., Driouich, A., Showalter, A. M., and Faik, A. (2010). Functional identification of two nonredundant *Arabidopsis* alpha(1,2)fucosyltransferases specific to arabinogalactan proteins. *J. Biol. Chem.* 285, 13638–13645.

Ye, C. Y., Li, T., Tuskan, G. A., Tschaplinski, T. J., and Yang, X. (2011). Comparative analysis of GT14/GT14-like gene family in *Arabidopsis*, *Oryza*, *Populus*, *Sorghum* and *Vitis*. *Plant Sci.* 181, 688–695.

Yeh, J. C., Ong, E., and Fukuda, M. (1999). Molecular cloning and expression of a novel beta-1, 6-N-acetylglucosaminyltransferase that forms core 2, core 4, and I branches. *J. Biol. Chem.* 274, 3215–3221.

Zhou, Y., Li, S., Qian, Q., Zeng, D., Zhang, M., Guo, L., Liu, X., Zhang, B., Deng, L., Luo, G., Wang, X., and Li, J. (2009). BC10, a DUF266-containing and Golgi-located type II membrane protein, is required for cell-wall biosynthesis in rice (*Oryza sativa* L.). *Plant J.* 57, 446–462.

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APPENDIX

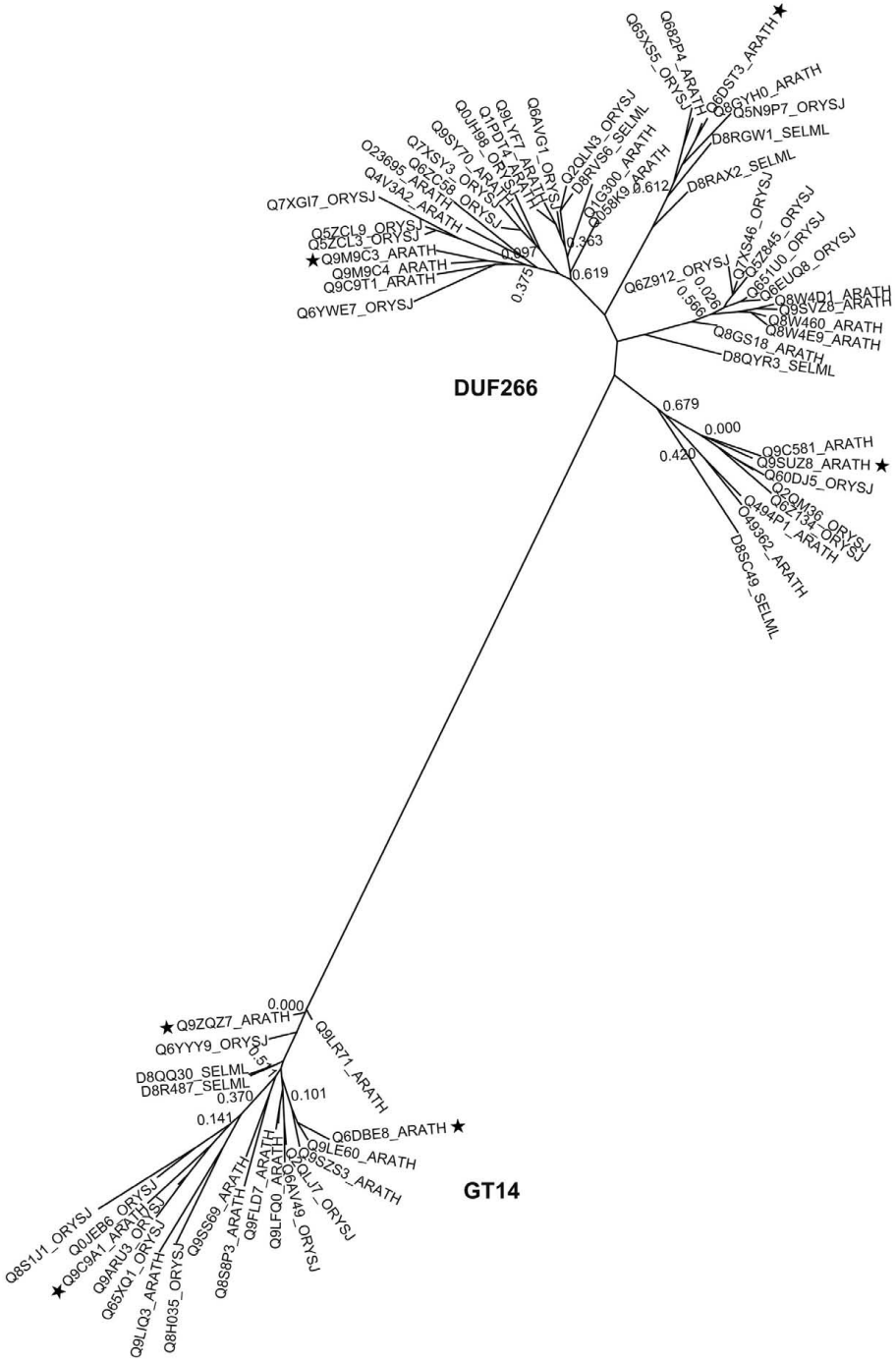


FIGURE A1 | Phylogenetic trees of DUF266/GT14 containing proteins from selected plants. The tree includes sequences from arabidopsis (ARATH), rice (ORYSJ) and *Selaginella moellendorffii* (SELML). After manual curation of sequences the tree was generated using MUSCLE alignment, removal of gaps, phyML, and statistical analysis using SH-like approximate likelihood-ratio test (aLRT). The tree was generated using http://www.phylogeny.fr (Dereeper et al., 2008). Following sequences Q0DT08 and Q0JPU3 were omitted from the tree due to sequence mistakes, mainly intron/exon border malpredictions. Asterisks indicate the selected sequences of the multi alignment displayed in **Figure A4**.

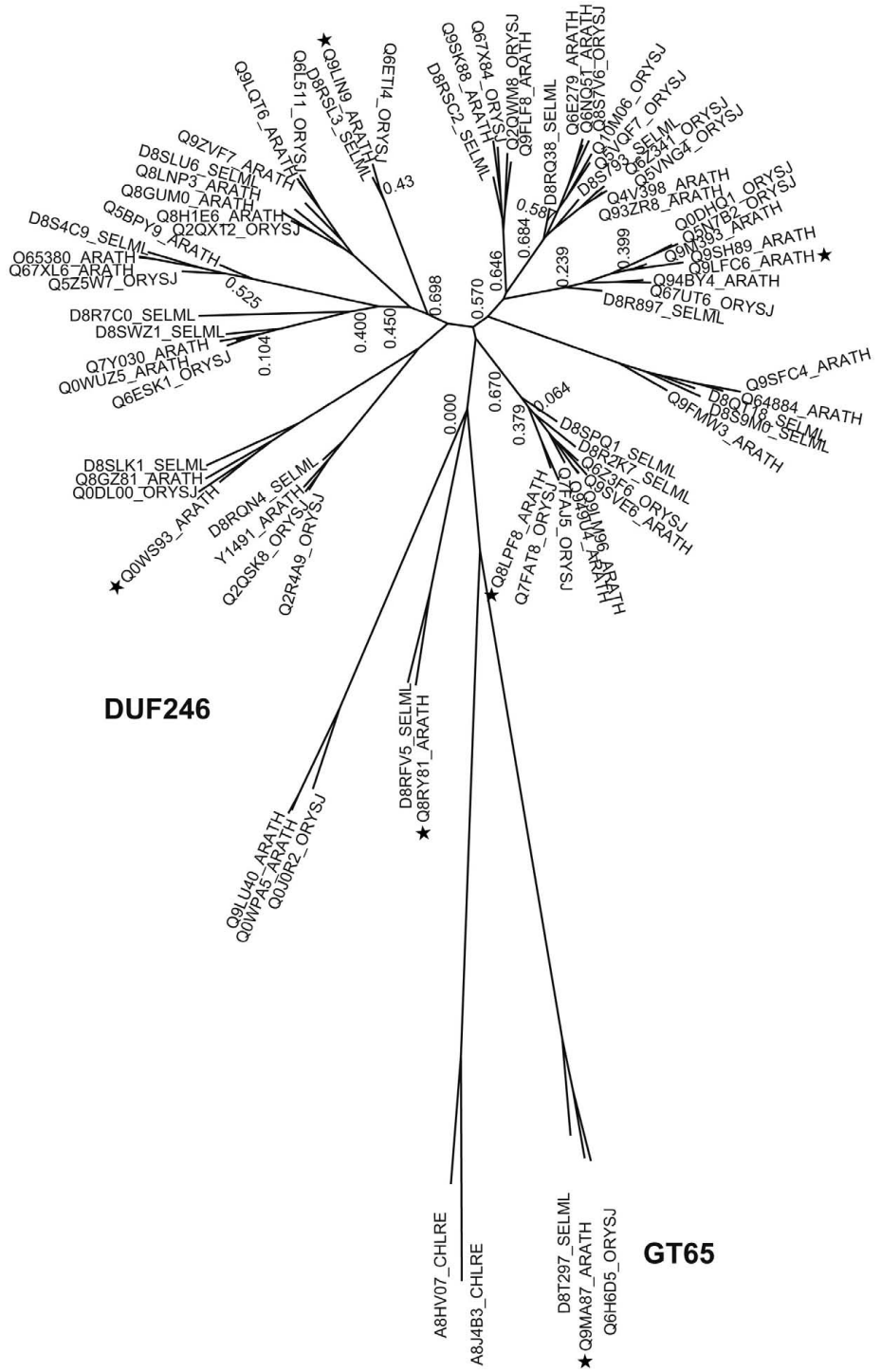


FIGURE A2 | Phylogenetic trees of DUF246/GT65 containing proteins from selected plants. The tree includes sequences from arabidopsis (ARATH), rice (ORYSJ), and *S. moellendorffii* (SELML). After manual curation of sequences the tree was generated using MUSCLE alignment, removal of gaps, phyML, and statistical analysis using SH-like approximate likelihood-ratio test (aLRT). The tree was generated using http://www.phylogeny.fr (Dereeper et al., 2008). Following sequences 8QTM0, D8SPE4, D8TE31, Q0DBZ9, Q53PH8, Q5N9U3, Q651P4, Q653G2, and Q7XAB1 were omitted from the tree due to sequence mistakes, mainly intron/exon border malpredictions. Asterisks indicate the selected sequences of the multi alignment displayed in **Figure A4**.

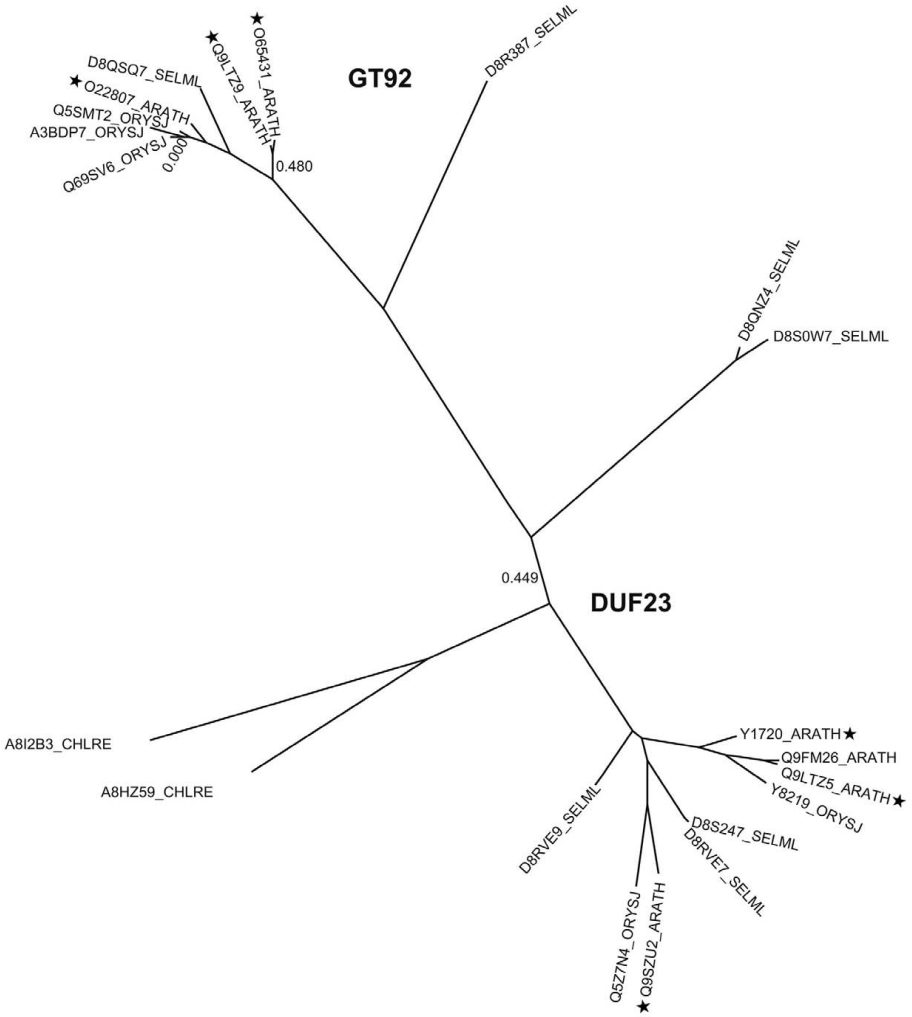


FIGURE A3 | Phylogenetic trees of DUF23 including GT92, containing proteins from selected plants. The tree includes sequences from arabidopsis (ARATH), rice (ORYSJ), and *S. moellendorffii* (SELML). After manual curation of sequences the tree was generated using MUSCLE alignment, removal of gaps, phyML, and statistical analysis using SH-like approximate likelihood-ratio test (aLRT). The tree was generated using http://www.phylogeny.fr (Dereeper et al., 2008). Following sequences A8HZ53 and A8I2B1 were omitted from the tree due to sequence mistakes, mainly intron/exon border malpredictions. Asterisks indicate the selected sequences of the multi alignment displayed in **Figure A4**.

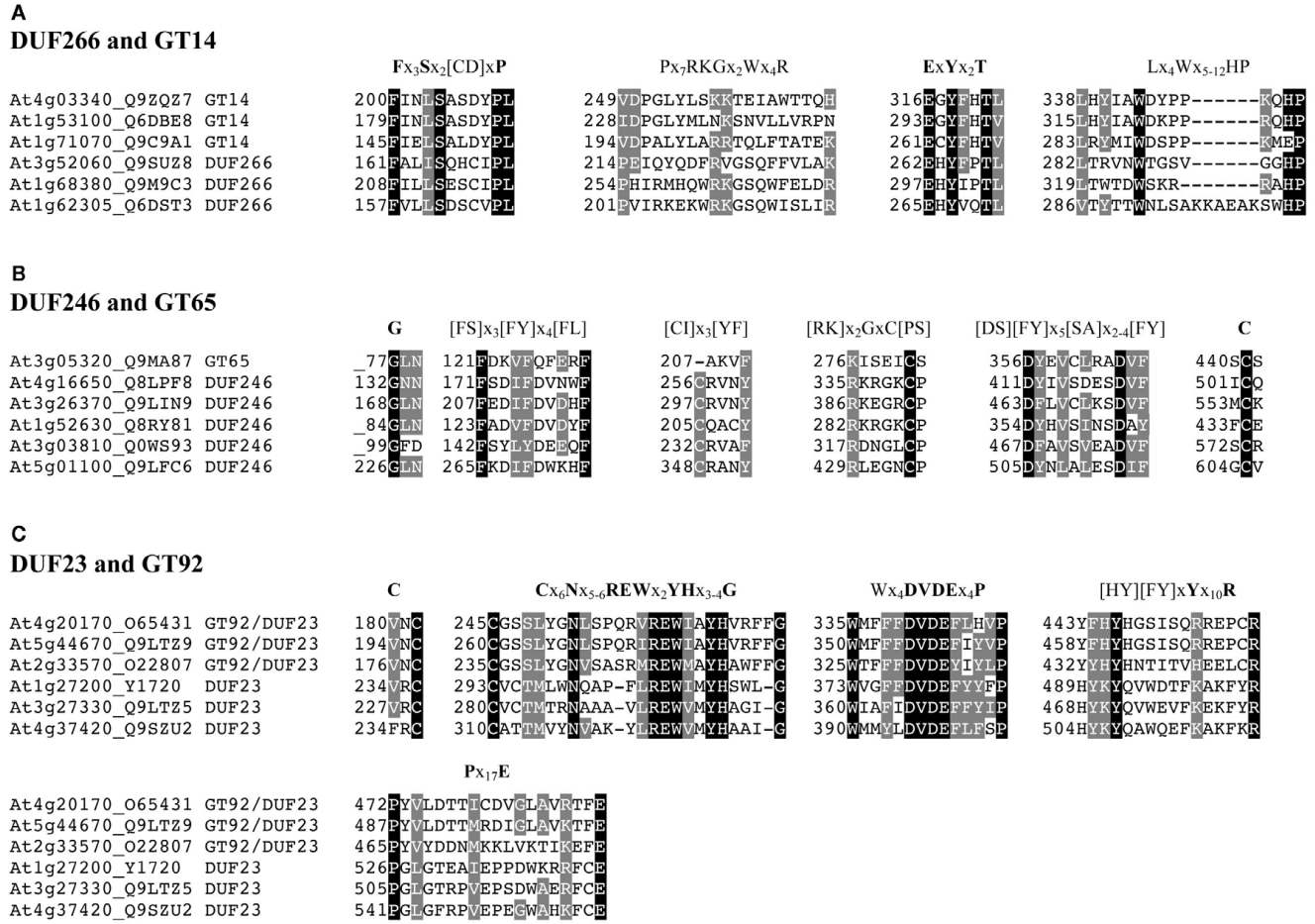


FIGURE A4 | Multiple sequence alignment of the conserved peptide motifs identified in *Arabidopsis*. Representatives of protein sequences from **(A)** DUF266 and GT14, **(B)** DUF246 and GT65, and **(C)** DUF23 (including GT92), from different phylogenetic clades. The selected sequences are marked with asterisks in **Figures A1–3**. The sequence alignments were performed using ClustalW and manually refined. The invariant similar residues are indicated on a black background and other conserved positions are shaded in gray. Dashes indicate gaps. Numbers indicate the amino acid position of the first residue of each motif. Schematic illustration of conserved motifs for **(A)** DUF266 and GT14, **(B)** DUF246 and GT65, and **(C)** DUF23, based on non-redundant *Arabidopsis* sequences are shown above each alignment. Strictly conserved residues are in bold and residues with only two alternative amino acids are denoted by parentheses.

REFERENCE

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., and Gascuel, O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469.

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Arabidopsis seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function

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Arabidopsis seed coat epidermal cells produce a large quantity of mucilage that is extruded upon exposure to water. Chemical analyses and cell biological techniques suggest that this mucilage represents a specialized type of secondary cell wall composed primarily of pectin with lesser amounts of cellulose and xyloglucan. Once extruded, the mucilage capsule has a distinctive structure with an outer non-adherent layer that is easily removed by shaking in water, and an inner adherent layer that can only be removed with strong acid or base. Most of the cellulose in the mucilage is present in the inner layer and is responsible at least in part for its adherence to the seed. There are also differences in the pectin composition between the two layers that could contribute to the difference in adherence. The *Arabidopsis* seed coat epidermis and its mucilage are not essential for seed viability or germination. This dispensability, combined with the fact that the epidermal cells synthesize an accessible pectin-rich cell wall at a specific time in development, makes them well suited as a genetic model for studying cell wall biogenesis, function, and regulation. Mutants defective in seed mucilage identified by both forward and reverse genetic analyses are proving useful in establishing connections between carbohydrate structure and cell wall properties *in vivo*. In the future, genetic engineering of seed coat mucilage carbohydrates should prove useful for testing hypotheses concerning cell wall structure and function.

Keywords: *Arabidopsis*, mucilage, seed coat, cell wall, pectin, cellulose, xyloglucan, genetic model

INTRODUCTION

When wetted, seeds of *Arabidopsis thaliana* (*Arabidopsis*), like those of many angiosperm species, become surrounded by a gelatinous capsule called mucilage (**Figure 1A**).

The mucilage originates from the specialized epidermal cells of the seed coat. During seed coat differentiation following cessation of cell growth, mucilage is synthesized and secreted to a region of the apoplast adjacent to the radial and outer tangential cell walls, forming a donut-shaped pocket between the membrane and the cell wall. Later, a thick cellulose-rich cell wall (columella) is synthesized beneath the mucilage pocket, along the outer tangential and radial sides of the cell that completely displaces the cytoplasm by the end of seed development. At maturity, therefore, the epidermal cells consist of a volcano-shaped cellulosic cell wall surrounded by a donut-shaped mucilage pocket, all of which are contained within the primary cell wall (**Figure 1C**). Upon exposure to water, the expanding hydrophilic mucilage ruptures the primary wall and expands to encapsulate the seed, presumably for dispersal, protection, and/or hydration (reviewed in Arsovski et al., 2010; Western, 2012). Over the past decade, research using chemical analyses and cell biological techniques has revealed that the mucilage has a complex composition and structure and as we argue below, could be considered a specialized pectin-rich secondary cell wall. Because the seed coat is so amenable to molecular–genetic manipulations and the mucilage so accessible, seed coat mucilage provides a unique system for studying many aspects of cell wall structure and function. Below, we review the current literature pertaining to mucilage composition and structure and discuss its potential for enhancing cell wall research.

COMPOSITION OF *ARABIDOPSIS* SEED COAT MUCILAGE

PECTINS

Pectins consist of a heterogeneous group of acidic polysaccharides characterized by the presence of galacturonic acid (GalA). Two key pectins include rhamnogalacturonan I (RG I) and homogalacturonan (HG). RG I has a backbone of alternating $(1 \rightarrow 2)$-α-L-rhamnose (Rha) and $(1 \rightarrow 4)$-β-D-GalA that can be substituted on the Rha residues with side-chains consisting of arabinans, galactans, type I arabinogalactans and terminal galactose (*t*-Gal) residues. HG consists of an unbranched chain of $(1 \rightarrow 4)$-β-D-GalA residues that can be methylesterified or acetylated. Enzymatic removal of the methyl esters can allow Ca^{2+}-based ionic bonding between HG molecules; the degree of gel formation of pectins is largely determined by the extent and pattern of HG de-esterification and disruption of HG Ca^{2+}-bonding by other, branched pectins such as RG I (Harholt et al., 2010).

The pectinaceous nature of *Arabidopsis* seed coat mucilage is evident from staining with ruthenium red and the metachromatic dye toluidine blue O (**Figure 1**; Western et al., 2000). Chemical analysis of extracted mucilage using a variety of

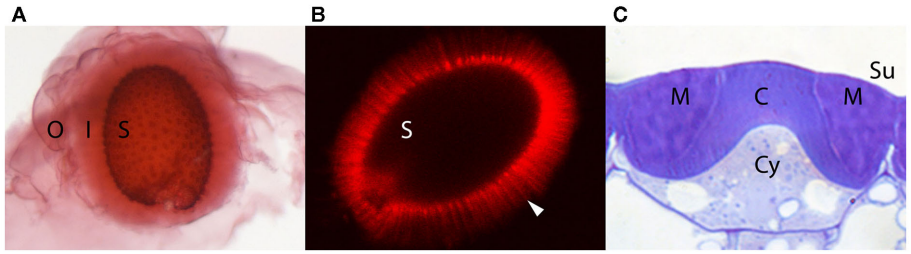


FIGURE 1 | Mature *Arabidopsis* seeds with extruded mucilage and a developing seed coat epidermal cell. (A) Mature seed (S) following exposure to water and staining with ruthenium red without shaking. The extruded mucilage capsule is comprised of an outer, loosely attached, lightly stained layer (O), and an inner adherent layer (I) with faint more darkly staining "rays." **(B)** Mature seed (S) following exposure to water and staining with pontamine fast scarlet. The cellulose network of the extruded mucilage including the more densely stained "rays" (arrowhead) extending outward from the top of each columella can be seen. **(C)** Cross section of a single seed coat epidermal cell on the outer surface (Su) of a seed at 11 days post anthesis stained with toluidine blue O. The mucilage (M) between the columella (C) and the outer primary wall can be seen. At this stage cellulose for the columella is still being synthesized and will continue until the cytoplasm (Cy) is completely displaced.

techniques (GC/MS with and without sugar linkage analysis, HPLC, Fourier transform-infrared spectroscopy) confirms the presence of pectins, primarily unbranched RG I (Goto, 1985; Western et al., 2000; Penfield et al., 2001; Dean et al., 2007; Macquet et al., 2007a). The predominance of RG I as the main component of *Arabidopsis* mucilage is also apparent using antibody staining for unbranched RG I with CCRC-M36 and INRA-RU2 (Young et al., 2008; Arsovski et al., 2009a; Pattathil et al., 2010; Sullivan et al., 2011). A significant reduction of overall mucilage production is also seen in mutants for the UDP-L-Rha synthase MUCILAGE-MODIFIED 4/RHAMNOSE SYNTHASE 2 that is specifically upregulated during mucilage synthesis (Usadel et al., 2004; Western et al., 2004; Oka et al., 2007). Small amounts of typical RG I side-chain polysaccharides, arabinans and galactans, as well as arabinogalactan type I, have been identified in mucilage with whole seed immunofluorescence (LM6, LM5, and JIM13 antibodies, respectively) and confirmed through linkage analysis, which also detects the number of branch points on the RG I backbone (Dean et al., 2007; Macquet et al., 2007a; Arsovski et al., 2009a; HE McFarlane and TL Western, unpublished data). HG of varying degrees of methyl esterification has also been identified using the JIM5 (low–medium esterified HG) and JIM7 (medium–highly esterified HG) antibodies and confirmed with chemical analysis (Willats et al., 2001; Rautengarten et al., 2008; Walker et al., 2011).

CELLULOSE

The presence of cellulose microfibrils has been recognized in many seed coat mucilages and *Arabidopsis* mucilage is no exception. Crystalline cellulose fibrils radiating out from the seed coat mucilage cells have been detected through their birefringent properties in polarized light ("Maltese-cross effect"; Vaughan and Whitehouse, 1971; Sullivan et al., 2011), by staining for dyes that detect β-glucans such as calcofluor white and pontamine fast scarlet S4B, and with fluorescently labeled prokaryotic cellulose binding domains (Willats et al., 2001; Blake et al., 2006; Harpaz-Saad et al., 2011; Mendu et al., 2011). Changes to mucilage structure are also observed in mutants for the cellulose synthase subunit CELLULOSE SYNTHASE 5 (CESA5; previously identified as MUM3; see below Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011).

HEMICELLULOSE

Linkage analysis of *Arabidopsis* mucilage has suggested the presence of small quantities of the cross-linking glycan (hemicellulose) arabinoxylan (Naran et al., 2008; Arsovski et al., 2009a). While xylans have not been detected in mucilage with immunofluorescence with the monoclonal antibody LM10, a polyclonal antibody to another hemicellulose, xyloglucan (XG), detected epitopes throughout the mucilage as well as in the underlying columella (Young et al., 2008). If XG is indeed present, it may associate with the cellulose in the mucilage similar to its proposed role in primary cell walls.

PROTEIN

While there is no direct evidence for a significant amount of protein in *Arabidopsis* mucilage (Macquet et al., 2007a), mutations in three cell wall-associated proteins lead to changes in mucilage structure. *MUM2* encodes a secreted β-galactosidase that is required to remove *t*-Gal and (arabino)galactan side-chains from RG I to enable correct mucilage hydration and release from the seed coat (Dean et al., 2007; Macquet et al., 2007b). The β-xylosidase/α-arabinofuranosidase BETAXYLOSIDASE 1 (BXL1) also affects mucilage release, and is required for trimming of arabinan side-chains (Arsovski et al., 2009a). Conversely, SOS5 is a fasciclin-like arabinogalactan protein (AGP) with a glycophosphatidylinositol (GPI) tail that anchors it to the outer leaflet of the plasma membrane (Shi et al., 2003). The heavily glycosylated AGP domains of SOS5 may interact with cell wall polysaccharides, while the fasciclin repeats may lead to protein interaction; cleavage of the GPI anchor (Shi et al., 2003) may also allow movement of SOS5 into the mucilage. Both linkage analysis and immunofluorescence results demonstrate the presence of type I arabinogalactans in *Arabidopsis* mucilage that may act as side-chains on RG I or represent the glycosylated portions of AGPs such as SOS5 (Dean et al., 2007; Arsovski et al., 2009a; HE McFarlane and TL Western, unpublished data).

STRUCTURE OF *ARABIDOPSIS* SEED COAT MUCILAGE

Ruthenium red staining reveals two domains in *Arabidopsis* mucilage – a cloudy, diffuse outer layer surrounding a dense, inner layer (**Figure 1A**). The inner mucilage layer is marked by

the appearance of darkly staining "rays" that radiate out from the columella of each epidermal cell (Western et al., 2000). Below we discuss the structural characteristics and composition of these two layers, as well as comment on their functions and relationship.

OUTER MUCILAGE

The outer mucilage layer of *Arabidopsis* seeds is seen enveloping the inner layer when seeds are stained without agitation. It is diffuse, with no obvious structure, and poorly adherent such that it is easily extracted by shaking seeds in water or a mild chelator (Western et al., 2000; Macquet et al., 2007a). Tests of water absorption over time demonstrate that it is capable of rapid and significant water absorption (Arsovski et al., 2009a). Linkage analysis and antibody staining of extracted outer mucilage suggests that it is primarily unbranched RG I (>80–90% of sugars in mucilage are Rha and GalA, with an approximately equal molar ratio as would be expected for the RG I backbone) with only a small proportion of branched RG I and arabinoxylan (Dean et al., 2007; Macquet et al., 2007a; Naran et al., 2008; Arsovski et al., 2009a). HG of differing degrees of methylation has also been detected using dot blots with JIM5 and JIM7 (Willats et al., 2001).

INNER MUCILAGE

Unlike the outer mucilage, the inner mucilage layer is strongly adherent to the seed and cannot be removed only by agitation: treatment with harsh chemicals (strong acid and base) or enzymatic digestion is required, suggesting bonding with structures of the seed coat (Western et al., 2000; Macquet et al., 2007a; Huang et al., 2011; Walker et al., 2011). Ruthenium red staining of the inner layer is darker than the outer layer, suggesting that the pectin network is denser (**Figure 1A**). This was confirmed by the inability of large molecules (e.g., the colloidal molecules that make up India ink) to penetrate the inner mucilage layer (Windsor et al., 2000; Western et al., 2001). Willats et al. (2001) demonstrated that, while FITC-labeled 40 kDa dextrans can enter the inner mucilage layer, 150 kDa dextrans cannot. Both Usadel et al. (2004) and Macquet et al. (2007a) showed that there are at least two distinct molecular size fractions in *Arabidopsis* mucilage, one of ∼600 kDa (outer mucilage) and another of ∼50,000 kDa (inner mucilage – molecular sizes from Macquet et al., 2007a). Mucilage extracted with mild chelator followed by increasing concentrations of alkali allowed comparison of the water holding capacity of inner versus outer mucilage, and showed that both the rate and degree of water absorption is reduced for inner mucilage compared with outer mucilage (Arsovski et al., 2009a).

The increased density and altered swelling properties of the inner mucilage layer is explained by its increased chemical complexity as identified through chemical, histochemical, and immunological analyses. While the majority of the inner mucilage is still comprised of unbranched RG I (most of inner layer can be removed by digestion with RG I hydrolase), linkage analysis has suggested that branched RG I, arabinans, galactans, arabinogalactans, and arabinoxylans are present in increased amounts compared with the outer mucilage. Further, when inner mucilage is extracted with sequentially harsher treatments, the proportion of complex/branched pectins increases (Dean et al., 2007; Macquet et al., 2007a; Arsovski et al., 2009a; Huang et al., 2011; Walker et al., 2011). Whole seed immunofluorescence supports the inner mucilage localization of RG I (CCRC-M36; INRA-RU2), arabinans (LM6), galactans (LM5), and arabinogalactans (JIM13), but also suggests the presence of both low and highly methylesterified HG (JIM5 and JIM7), and XG (α-XG; Willats et al., 2001; Macquet et al., 2007a; Young et al., 2008; Arsovski et al., 2009a; Sullivan et al., 2011; HE McFarlane and TL Western, unpublished data). In addition to pectins and hemicellulose, the adherent mucilage contains cellulose arranged in dense "rays" that radiate out from the top of each columella separated by a less dense network of cellulose spanning the inner mucilage layer between the rays (**Figure 1B**; Willats et al., 2001; Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). It has been calculated that ∼12–19% of the adherent mucilage layer is comprised of cellulose (Macquet et al., 2007a). Finally, Macquet et al. (2007a) suggest that the adherent mucilage could be divided into two layers based on chemical composition: a region that contains RG I, HG, cellulose, and galactans that is immediately adjacent to the seed surface, and a zone beyond that that is comprised of RG I, HG, galactans, and arabinans. While there is certainly structure within the inner adherent layer, it is unclear if it can be simply defined into layers within itself – there is some contradictory data as to the exact localization of arabinans and highly methylesterified versus more de-esterified HG within the inner adherent mucilage (Willats et al., 2001; Macquet et al., 2007a; Arsovski et al., 2009a). Further, changes in mucilage staining density with ruthenium red surrounding the cellulosic rays shows structural complexity in multiple dimensions within the adherent layer (Western et al., 2000).

The cohesion of mucilage, and the adhesion to the seed of the inner adherent mucilage layer suggests specific structural organization. A key determinant of this organization is the cellulose that spans the adherent layer. The structuring of the inner adherent layer by cellulose is clearly demonstrated by several mutants affecting cellulose production within mucilage, including the cellulose synthase subunit *cesa5*, the fasciclin-like AGP *sos5*, and the leucine rich receptor-like kinase *fei2*. All three mutants lack or have significantly reduced cellulose, leading to an increase in the amount of diffuse, non-adherent mucilage, and decreased inner adherent layer (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). Suggested mechanisms for cellulose anchoring of the pectins comprising the majority of the mucilage include non-covalent bonding between cellulose and pectins, and molecular entanglement, whereby the large, branched pectins are captured through tangling with each other and with the cellulose microfibrils projecting at right angles to the seed surface (Macquet et al., 2007a; Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). Interactions between HGs through Ca^{2+} bridges are also implicated in maintaining the density of the inner mucilage layer. Treatment of seeds with Ca^{2+}-specific or general heavy metal chelators leads to the apparent expansion of the inner, adherent layer (Willats et al., 2001; Rautengarten et al., 2008).

ROLES AND RELATIONSHIP BETWEEN INNER AND OUTER MUCILAGE

The exact relationship between the outer, non-adherent mucilage layer and inner adherent mucilage is uncertain. It is unclear whether the structural layers exist in mucilage as it is deposited or whether they resolve once the mucilage is released. Due to

differences in swelling, molecular size and likelihood of molecular entanglement, it is possible that the unbranched RG I could simply diffuse away from the seed, while the more complex, branched pectins are trapped within the cellulose-based scaffolding. Whether the polysaccharides comprising mucilage are deposited or organized within the mucilage pocket in a stratified fashion is unknown. However, results from studies of mutants in genes affecting the β-galactosidase MUM2 and the β-xylosidase/α-arabinofuranosidase BXL1 demonstrate that post-deposition trimming of pectin side-chains in mucilage does occur and affects mucilage hydration (Dean et al., 2007; Macquet et al., 2007b; Arsovski et al., 2009a). It has been proposed that a population of RG I molecules that is accessible to these and other enzymes in the mucilage pocket becomes the outer soluble layer as it is both divested of its side-chains and digested into smaller molecules (Walker et al., 2011).

Clearly, there are structural and chemical differences between the two mucilage layers, but whether they are separate functional entities still needs to be elucidated. Differences in their speed and extent of swelling could provide for separate roles. The significant and rapid expansion of the primarily unbranched RG I in the outer mucilage layer could be responsible for breakage of the outer primary cell wall of the mucilage cells to allow mucilage release. This is consistent with results from *mum2* and *bxl1* mutants that have impaired mucilage release due to reduced mucilage swelling when hydrated (Dean et al., 2007; Macquet et al., 2007b; Arsovski et al., 2009a). It also has been suggested that the inner mucilage, being strongly adherent to the seed, is involved in seed hydration and/or adhesion to soil or animals (Macquet et al., 2007a). A role for outer non-adherent mucilage in increasing adhesion to soil substrates also has been proposed for myxospermous seeds in general (Grubert, 1974).

ADVANTAGES OF USING SEED COAT EPIDERMAL CELLS TO STUDY CELL WALL STRUCTURE AND FUNCTION

As described above, *Arabidopsis* seed coat mucilage is composed of pectin, cellulose, hemicellulose, and protein and therefore could be described as a specialized cell wall. Since it is synthesized following cessation of cell growth, mucilage is most accurately described as a specialized, pectin-rich, secondary cell wall even though its composition is distinct from the more typical cellulose-rich secondary cell walls. Despite the fact that its structure and function are unique, differing in many ways from other cell walls, it has potential as a model for investigating the chemical and physical properties of different cell wall components and their interactions *in vivo*, especially with respect to its major component RGI. The value of *Arabidopsis* seed coat mucilage lies in its unique features. First, mucilage is copious and easily accessible, simplifying its extraction. Second, typical cell wall preparations are derived from tissues with multiple cell types, each of which could have a distinct cell wall. Mucilage, on the other hand, is derived from a single cell type and its structure is relatively simple with unbranched RGI and no RGII. Third, the ability of mucilage to expand and adhere to the seed represent important cell wall properties that are easy to observe and measure and may provide insight into common aspects of cell wall biology such as the role of the middle lamella in cell–cell adhesion. Fourth, mucilage synthesis occurs over a short (4 days) period of seed development facilitating the analysis of cellular changes that accompany cell wall synthesis. Finally, seed coat mucilage is amenable to genetic analysis. Genetic manipulation is a powerful approach that can be used to establish causal links between the biochemical function of a gene product, cell wall structure, and its role *in vivo*. However, changing the cell wall composition of many tissues can have severe consequences on plant viability thus limiting genetic analysis. Seed coat epidermal cells are dispensable (Western et al., 2001), thus even genetic manipulations that severely impact the cell can be isolated or engineered. Forward genetic screens for mutants with altered mucilage have already identified a number of genes involved in cell wall biology (reviewed in Arsovski et al., 2010; Western, 2012) and more will be found as screens become more sophisticated (e.g., Arsovski et al., 2009b). Unfortunately, because the seed coat differentiates late in the life cycle of the plant (seed coat is part of the maternal generation) many proteins required for its cell walls will also be important for cell types earlier in development. Mutations in genes encoding such proteins may result in plant lethality or sterility and therefore be missed in forward genetic screens for seed coat defects due to the failure of the mutant plant to produce seeds. In addition, seed coat mutant screens will be subject to the same problems of genetic redundancy that have plagued previous attempts to identify mutant phenotypes for genes encoding carbohydrate active enzymes. An alternate way that mucilage/seed coat epidermal cells could be used to study cell walls is as a bioassay to evaluate the function of carbohydrate active enzymes on the structure and properties of carbohydrates. Genes encoding such enzymes can be expressed in the seed coat epidermis with tissue specific promoters, their protein products targeted to the apoplast or Golgi, and their effect on the structure and properties of mucilage or columella cell wall carbohydrates evaluated without fear of lethal effects. It is conceivable that, with enough knowledge, specific types of cell wall carbohydrates could be engineered and properties tested by introducing sets of enzymes.

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REFERENCES

Arsovski, A. A., Haughn, G. W., and Western, T. L. (2010). Seed coat mucilage cells of *Arabidopsis thaliana* as a model for plant cell wall research. *Plant Signal. Behav.* 5, 1–6.

Arsovski, A. A., Popma, T. M., Haughn, G. W., Carpita, N. C., McCann, M. C., and Western, T. L. (2009a). AtBXL1 encodes a bifunctional beta-D-xylosidase/alpha-L-arabinofuranosidase required for pectic arabinan modification in *Arabidopsis* mucilage secretory cells. *Plant Physiol.* 150, 1219–1234.

Arsovski, A. A., Villota, M. M., Rowland, O., Subramaniam, R., and Western, T. L. (2009b). MUM ENHANCERS are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*. *J. Exp. Bot.* 60, 2601–2612.

Blake, A. W. McCartney L., Flint, J. E., Bolam, D. N., Boraston, A. B., Gilbert, H. J., and Knox, J. P. (2006). Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in Prokaryotic enzymes. *J. Biol. Chem.* 281, 29321–29329.

Dean, G. H., Zheng, H., Tewari, J., Huang, J., Young, D. S., Hwang, Y. T., Western, T. L., Carpita, N. C., Mccann, M. C., Mansfield, S. D., and Haughn, G. W. (2007). The *Arabidopsis* MUM2 gene encodes a beta-galactosidase required for the production of seed coat mucilage with correct hydration properties. *Plant Cell* 19, 4007–4021.

Goto, N. (1985). A mucilage polysaccharide secreted from testa of *Arabidopsis thaliana*. *Arabidopsis Inf. Serv.* 22, 143–145.

Grubert, M. (1974). Studies on the distribution of myxospermy among seeds and fruits of Angiospermae and its ecological importance. *Acta Biol. Venez.* 8, 315–551.

Harholt, J., Suttangkakul, A., and Vibe Scheller, H. V. (2010). Biosynthesis of pectin. *Plant Physiol.* 153, 384–395.

Harpaz-Saad, S., Mcfarlane, H. E., Xu, S., Divi, U. K., Forward, B., Western, T. L., and Kieber, J. J. (2011). Cellulose synthesis via the FEI2 RLK/SOS5 pathway and CELLULOSE SYNTHASE 5 is required for the structure of seed coat mucilage in *Arabidopsis*. *Plant J.* 68, 941–953.

Huang, J., Bowles, D., Esfandiari, E., Dean, G., Carpita, N. C., and Haughn, G. W. (2011). The *Arabidopsis* transcription factor LUH/MUM1 is required for extrusion of seed coat mucilage. *Plant Physiol.* 156, 491–502.

Macquet, A., Ralet, M. C., Kronenberger, J., Marion-Poll, A., and North, H. M. (2007a). In situ, chemical and macromolecular study of the composition of *Arabidopsis thaliana* seed coat mucilage. *Plant Cell Physiol.* 48, 984–999.

Macquet, A., Ralet, M. C., Loudet, O., Kronenberger, J., Mouille, G., Marion-Poll, A., and North, H. M. (2007b). A naturally occurring mutation in an *Arabidopsis* accession affects a beta-D-galactosidase that increases the hydrophilic potential of rhamnogalacturonan I in seed mucilage. *Plant Cell* 19, 3990–4006.

Mendu, V., Griffiths, J., Persson, S., Stork, J., Downie, B., Voiniciuc, C., Haughn, G., and Debolt, S. (2011). Subfunctionalization of cellulose synthases in seed coat epidermal cells mediate secondary radial wall synthesis and mucilage attachment. *Plant Physiol.* 157, 441–453.

Naran, R., Chen, G., and Carpita, N. C. (2008). Novel rhamnogalacturonan I and arabinoxylan polysaccharides of flax seed mucilage. *Plant Physiol.* 148, 132–141.

Oka, T., Nemoto, T., and Jigami, Y. (2007). Functional analysis of *Arabidopsis thaliana* RHM2/MUM4, a multidomain protein involved in UDP-D-glucose to UDP-L-rhamnose conversion. *J. Biol. Chem.* 282, 5389–5403.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., Mcgill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Penfield, S., Meissner, R. C., Shoue, D. A., Carpita, N. C., and Bevan, M. W. (2001). MYB61 is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. *Plant Cell* 13, 2777–2791.

Rautengarten, C., Usadel, B., Neumetzler, L., Hartmann, J., Büssis, D., and Altmann, T. (2008). A subtilisin-like serine protease essential for mucilage release from *Arabidopsis* seed coats. *Plant J.* 54, 466–480.

Shi, H., Kim, Y., Guo, Y., Stevenson, B., and Zhu, J.-K. (2003). The *Arabidopsis* SOS5 locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. *Plant Cell* 15, 19–32.

Sullivan, S., Ralet, M. C., Berger, A., Diatloff, E., Bischoff, V., Gonneau, M., Marion-Poll, A., and North, H. M. (2011). CESA5 is required for the synthesis of cellulose with a role in structuring the adherent mucilage of *Arabidopsis* seeds. *Plant Physiol.* 156, 1725–1739.

Usadel, B., Kuschinsky, A. M., Rosso, M. G., Eckermann, N., and Pauly, M. (2004). RHM2 is involved in mucilage pectin synthesis and is required for the development of the seed coat in *Arabidopsis*. *Plant Physiol.* 134, 286–295.

Vaughan, J. G., and Whitehouse, J. M. (1971). Seed structure and the taxonomy of the Cruciferae. *Bot. J. Linn. Soc.* 64, 383–409.

Walker, M., Tehseen, M., Doblin, M. S., Pettolino, F. A., Wilson, S. M., Bacic, A., and Golz, J. F. (2011). The transcriptional regulator LEUNIG_HOMOLOG regulates mucilage release from the *Arabidopsis* testa. *Plant Physiol.* 156, 46–60.

Western, T. L. (2012). The sticky tale of seed coat mucilages: production, genetics, and role in seed germination and dispersal. *Seed Sci. Res.* 22, 1–25.

Western, T. L., Burn, J., Tan, W. L., Skinner, D. J., Martin-Mccaffrey, L., Moffatt, B. A., and Haughn, G. W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiol.* 127, 998–1011.

Western, T. L., Skinner, D. J., and Haughn, G. W. (2000). Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiol.* 122, 345–355.

Western, T. L., Young, D. S., Dean, G. H., Tan, W. L., Samuels, A. L., and Haughn, G. W. (2004). MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the *Arabidopsis* seed coat. *Plant Physiol.* 134, 296–306.

Willats, W. G. T., Mccartney, L., and Knox, J. P. (2001). In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. *Planta* 213, 37–44.

Windsor, J. B., Symonds, V. V., Mendenhall, J., and Lloyd, A. L. (2000). *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *Plant J.* 22, 483–493.

Young, R. E., Mcfarlane, H. E., Hahn, M. G., Western, T. L., Haughn, G. W., and Samuels, A. L. (2008). Analysis of the Golgi apparatus in *Arabidopsis* seed coat cells during polarized secretion of pectin-rich mucilage. *Plant Cell* 20, 1623–1638.

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Cellulose synthase complexes: composition and regulation

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Live cell imaging has greatly advanced our knowledge on the molecular mechanism by which cellulose is deposited. Both the actin and microtubule cytoskeleton are involved in assuring the proper distribution, organization, and dynamics of cellulose synthase complexes (CSCs). This review is an update on the most recent progress on the characterization of the composition, regulation, and trafficking of CSCs. With the newly identified cellulose synthase interactive protein 1 (CSI1) on hand, we begin to unveil the mystery of an intimate relationship between cellulose microfibrils and microtubules.

Keywords: terminal complexes, cellulose synthase complexes, cellulose microfibrils, microtubules, alignment hypothesis, actin

INTRODUCTION

Cellulose, in its simplest form, is composed of β-1,4-linked glucose. Cellulose synthesis captures a lot of interest not only because cellulose is a major component of plant cell walls, but also due to its potential to become one of the major resources for renewable biofuels. Many excellent reviews have highlighted contribution toward the understanding of cellulose biosynthesis (Delmer, 1999; Williamson et al., 2002; Saxena and Brown, 2005; Somerville, 2006; Crowell et al., 2010). This review will focus on the composition and regulation of cellulose synthase complexes (CSCs).

VISUALIZATION OF CELLULOSE SYNTHASE COMPLEXES

Cellulose synthase complexes, or "granules" as they were referred to at the time, were first observed by electron microscopy in 1972, signifying the origin of the visualization of the cellulose biosynthesis machinery (Robinson et al., 1972). A few years later, Brown et al. observed similar granules in both prokaryotes *Acetobacter xylinum* and eukaryotes *Oocystis* and appropriately named them "terminal complexes" (Brown and Montezinos, 1976; Brown et al., 1976). The name terminal complexes (TCs) reflects the association of complexes at the end of cellulose microfibrils as seen by electron microscopy in freeze fracture replicas. Since then, TCs have been observed in many organisms. It was speculated that TCs were the cellulose-synthesizing units long before the identification of the first cellulose synthase (*CESA*) gene in *A. xylinus* (Saxena et al., 1990) and the immuno-gold labeling of TCs using CESA antibodies (Kimura et al., 1999).

Fluorescent protein tagging has no doubt revolutionized how we visualize protein complex dynamics at the intracellular level. So far, four CESA proteins in *Arabidopsis thaliana* have been visualized using fluorescent protein tagging via confocal microscopy. In epidermal cells from dark grown hypocotyls, both YFP-CESA6 and GFP-CESA3 localize at the plasma membrane as distinct particles and move bidirectionally at an average speed of 270–350 nm/min (Paredez et al., 2006; Desprez et al., 2007). These rates of movement are roughly calculated to correspond to the addition of 300–1000 glucose residues per glucan chain per minute (Paredez et al., 2006). GFP-CESA5 particles move at a similar speed of 270 nm/min when expressed in wild type plants (Bischoff et al., 2011). Using fluorescence loss in photobleaching (FLIP), the velocities of YFP-CESA7 particles in the developing xylem of *Arabidopsis* were determined to be greater than 7 μm/s, more than 1000-fold faster than that of primary CESAs (Wightman et al., 2009). Although secondary wall biosynthesis occurs at a much faster rate than that of primary wall, it cannot account for the drastic difference between the velocities of CESAs in two types of walls. Nevertheless, with many choices of fluorescent proteins in hand and as resolution improves to molecular scales, simultaneous imaging of different CESA isoforms might reveal the composition and positional information of CESA proteins in the rosettes.

CSC MODELS

Primary cell walls are deposited during cell division and cell expansion. Secondary cell walls are deposited between primary walls and plasma membrane after the cessation of cell growth. Cellulose in the primary and secondary cell wall differs in the degree of polymerization and crystallinity. These differences may be due to the different composition and structure of primary and secondary CSCs. Because cellulose microfibrils in vascular plants are estimated to vary from 18 to 36 glucan chains and three isoforms of CESA (CESA4, 7, and 8) are required in the secondary wall formation, a heteromeric model of 18–36 CESA proteins is formulated to support the non-redundant roles of three CESA isoforms in cellulose synthesis of secondary cell walls (Scheible et al., 2001; Doblin et al., 2002; Taylor et al., 2003). For cellulose synthesis of primary cell wall, CSC is composed of CESA1, 3, 6, and 6-like proteins (CESA2, 5, and 9). It is worth noting that these CSC models are based on several assumptions and clarification on the exact composition and stoichiometry of CESAs in both primary and secondary CSCs is needed.

The distinction between primary and secondary CESAs might not be as strict as initially defined. For example, the primary CESA

clades (CESA1, 3, 6, and 6-like) support secondary wall synthesis in *Arabidopsis* trichomes (Betancur et al., 2010). Another example came from the most recent findings that primary CESAs function in the formation of secondary cell walls in the seed coat (Stork et al., 2010; Mendu et al., 2011). Specifically, CESA5 is responsible for mucilage attachment and CESA2, 5, and 9 contribute toward secondary cell wall biosynthesis in the columella cells (Sullivan et al., 2011). These findings may provide insight into how CSCs evolved to fulfill their specific roles in distinct cell types.

CSCs AND MICROTUBULE CYTOSKELETON

The association between cortical microtubules and cellulose microfibril deposition has been well documented in the literature (Hepler and Newcomb, 1964; Mueller and Brown, 1980; Quader et al., 1987; Baskin, 2001; Gardiner et al., 2003). The microtubule–microfibril alignment hypothesis proposes that cortical microtubules, which lie beneath the plasma membrane of elongating cells, provide tracks for CSCs that convert glucose molecules into crystalline cellulose microfibrils (Green, 1962). The alignment hypothesis has been assessed extensively in many organisms including three widely studied green algae *Closterium acerosum*, *Oocystis solitaria*, *Nitella axillaris*, and land plants, e.g., *Coleus blumei*, oat, *Arabidopsis*, maize, cotton (Seagull, 1991; Baskin, 2001). Pharmacological experiments using microtubule-disrupting agents in diffusely growing cells generally support the alignment hypothesis, but discrepancies have also been noted (Seagull, 1991; Baskin, 2001; Wasteneys, 2004).

The alignment hypothesis was recently tested by visualization of microtubules and CSCs simultaneously in living plant cells. The co-alignment of CSCs and microtubules was observed in primary cell walls using a transgenic line co-expressing CFP-TUA1 and YFP-CESA6 (Paredez et al., 2006). YFP-CESA6 localizes at the plasma membrane as distinct particles, tracing linear trajectories that are coincident with the underlying microtubules. Changes in microtubule orientation by blue light excitation resulted in correlated shift in trajectories of YFP-CESA6. Complete removal of microtubules by the microtubule depolymerization agent, oryzalin, resulted in a uniform distribution of CSCs (Crowell et al., 2009; Li et al., 2012). GFP-CESA7 formed thick bands in the developing xylem of *Arabidopsis*, coinciding with bands of microtubules that mark the sites of secondary cell wall deposition (Gardiner et al., 2003). Localization of CSC to sites of secondary wall synthesis is also dependent upon underlying microtubules (Wightman and Turner, 2008). Within 45 min of oryzalin treatment, transverse bands labeled by YFP-CESA7 had disappeared concurrently with the loss of CFP-labeled microtubule bundles.

Two alternative models have been proposed to explain the microfibril–microtubule alignment. The direct hypothesis postulates some types of direct linkage between CESA complexes and microtubules (Heath, 1974). The indirect hypothesis, also known as the bumper model, proposes that the interaction of microtubules with plasma membrane changes membrane fluidity thereby restricting the movement of the CSCs (CESA complexes) and there is no need for direct interaction between CESA complexes and microtubules (Herth, 1980). The indirect hypothesis gained popularity and is now commonly found in textbooks (Alberts et al., 1994) although no direct evidence supports the bumper model.

CSI1: REVISIT THE ALIGNMENT HYPOTHESIS

A yeast two-hybrid screen using the entire central cytosolic domain of CESA was used to identify proteins that interact with CESA and potentially link CSCs to the cytoskeleton. CESA interactive protein 1 (CSI1) is among several dozen putative CESA interactive proteins identified. *csi1* null mutants displayed cell expansion defects in hypocotyls and roots, correlating with a reduction in crystalline cellulose content (Gu and Somerville, 2010; Gu et al., 2010). Red fluorescent protein tagged CSI1 (CSI1-RFP) localizes at the plasma membrane as distinct particles and travels bidirectionally at a similar velocity to GFP-CESA3. Moreover, the linear trajectories of CSI1-RFP coincide with the underlying microtubules labeled by YFP-TUA5. In the absence of CSI1, both the distribution and the motility of CESA complexes are defective and the alignment of CSCs and microtubules is disrupted (Gu et al., 2010; Bringmann et al., 2012; Li et al., 2012). These observations led to the hypothesis that CSI1 directly mediates the interaction between CSCs and microtubules (**Figure 1**).

An *in vitro* microtubule-binding assay supports the hypothesis that CSI1 is a linker protein between CSCs and microtubules. CSI1, like other conventional microtubule-binding proteins, co-sedimented with polymerized tubulin (Li et al., 2012). CSI1 contains multiple tandem copies of Armadillo (ARM) repeats, degenerate protein sequence motifs for protein–protein interaction. Although CSI1 does not share sequence homology with known structural microtubule-associated proteins (MAPs), ARM repeat containing proteins have been shown to be involved in cytoskeletal functions. As a bona fide MAP, CSI1 does associate with microtubules *in vivo*. CSI1 is present in all land plants. It remains to be determined whether CSI1 represents a conserved mechanism for microfibril–microtubule alignment.

Though *csi1* mutants have an expansion defect, these plants are still viable. *Arabidopsis* encodes two CSI1-like proteins, namely CSI2 and CSI3. The triple mutant, *csi1csi2csi3* is viable but has an expansion defect that is greater than that of *csi1* (Bringmann et al., 2012). The viability of the triple mutant might suggest that other proteins may be involved in microfibril–microtubule alignment. Additional work will be required to test this and other possibilities.

CSCs AND ACTIN CYTOSKELETON

In epidermal cells of dark grown *Arabidopsis* hypocotyls, actin is required for the motility and the global distribution of Golgi localized CSC populations (Crowell et al., 2009; Gutierrez et al., 2009). In this cell type, microtubules mark the site for CSC delivery to the plasma membrane for primary cell wall synthesis (Crowell et al., 2009; Gutierrez et al., 2009). Contrastingly, in xylem vessels, which are models for secondary wall synthesis, microtubules are apparently not essential in marking the sites for CSC delivery based on observations that oryzalin treatment does not prevent CSC-containing compartments from pausing at sites of wall synthesis (Wightman and Turner, 2008). In xylem vessels transverse actin bundles are positioned close to the sites of wall synthesis and a disruption of actin by Latrunculin B results in a loss of transverse

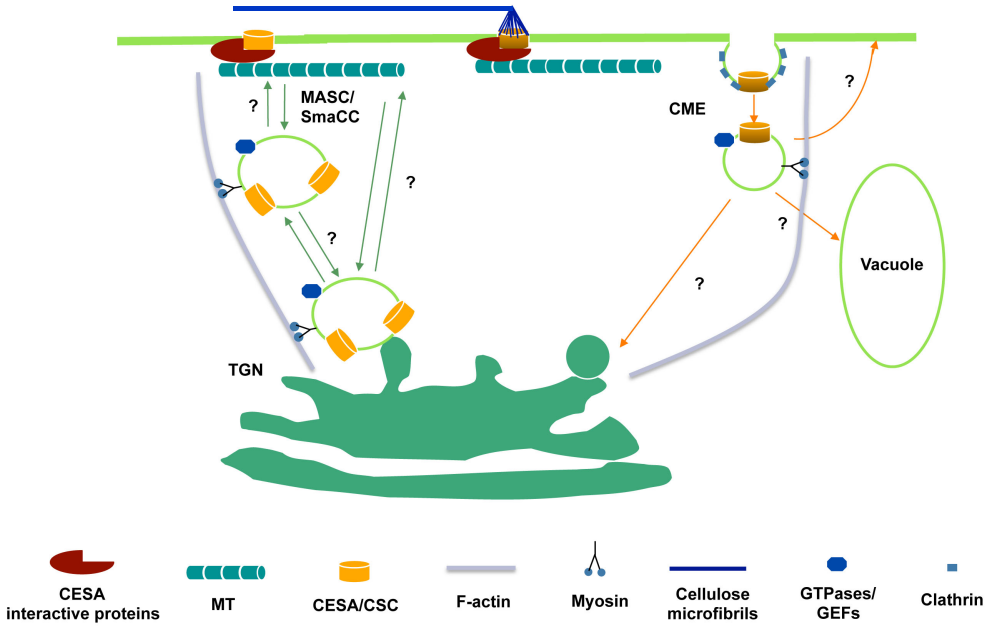


FIGURE 1 | Hypothetical schematic diagram of the trafficking of CSCs to and from the plasma membrane. CSCs are presumably synthesized in ER and delivered to Golgi for assembly. From Golgi to plasma membrane, delivery may occur directly from the trans-Golgi network (TGN) or through an intermediate compartment such as the MASC/SmaCC. MASCs/SmaCCs may also act as an internalization or recycling compartment. Also, CESA proteins may be internalized by clathrin-mediated endocytosis (CME). The association of CESA interactive proteins with CSCs may occur before the insertion event. At the plasma membrane, CESA interactive proteins, e.g., CSI1, bridge between CSCs and microtubules and enforce the co-alignment of newly synthesized cellulose microfibrils and cortical microtubules.

CSC bands. These observations led to a hypothesis that transverse actin bands, rather than microtubules, mark CSC delivery sites at the cell membrane in xylem vessels (Wightman and Turner, 2008). If this hypothesis holds true, then the mechanism for CSC delivery might be different for primary walls vs secondary walls.

During secondary wall synthesis in cotton fibers, actin microfilaments reorient from an axial pattern to a helical pattern, correlating with the secondary wall deposition. Disruption of the actin cytoskeleton by cytochalasins in cotton fibers and *Zinnia* treachery elements resulted in the disorganization of cellulose microfibrils (Seagull, 1990). Microtubules were observed to reorient upon disruption of the actin microfilament array, and it was therefore proposed that the actin cytoskeleton may contribute to cell elongation through the interaction between actin microfilaments and microtubules (Wasteneys and Collings, 2004). The cross-talk between actin and microtubules has been demonstrated mostly by pharmacological studies where stabilizing or disrupting one element affects the other (Collings, 2008). Recently, the dynamic association between actin microfilaments and microtubules was confirmed in interphase plant cells using an F-actin and microtubule dual-labeled line (Sampathkumar et al., 2011). In animal cells, molecular linker proteins such as MAP1 and 2, coronin, ERM proteins, and dynein support the interaction between actin and microtubules. However, plants lack counterparts of any of the mammalian linker proteins. The evidence for functional interactions with both microtubules and microfilaments is still missing for potential candidates such as PLD, LIM, and dynamin-related proteins (Collings, 2008).

CSC TRAFFICKING AND RECYCLING

YFP-CESA6 and GFP-CESA3 label at least three distinct populations. In addition to discrete particles at the focal plane of plasma membrane and donut-shaped Golgi compartments, CESAs also label a small compartment termed SmaCCs (small CESA compartments) or MASCs (microtubule-associated cellulose synthase compartments). The plasma membrane localized particles presumably represent functional complexes that convert glucose molecules into cellulose microfibrils. Other CESA associated compartments have a role in CESA delivery, internalization, and/or recycling event. SmaCCs/MASCs accumulate in the cell cortex upon osmotic stress, protein synthesis inhibition, or cellulose synthesis inhibition (Crowell et al., 2009; Gutierrez et al., 2009). In untreated cells, SmaCCs/MASCs are present in fully elongated cells 10 mm below the apical hook where CSCs are nearly absent (Crowell et al., 2009). Perturbation of CESA activity by either osmotic stress or cellulose synthesis inhibition resulted in prolongation of SmaCCs/MASCs-microtubule interactions, which appeared to be important for directing the plasma membrane delivery of CSCs. However, it remains to be determined whether SmaCCs/MASCs represents an internalization/recycling compartments or delivery compartments or both (Bashline et al., 2011). Disruption of clathrin-mediated endocytosis (CME) may have an effect on the composition of cell walls (Gu, unpublished data). A dynamin-like protein, DRP1A was reported to have a role in CME (Konopka and Bednarek, 2008). The defect in endocytosis in *rsw9*, which contains a mutation in DRP1A, resulted in the cellulose deficiency and cell elongation defect (Collings et al., 2008). CME might play

a role in internalization and recycling of CSC components that are no longer functional (**Figure 1**).

REGULATION OF CELLULOSE SYNTHESIS

The rate of cellulose synthesis is affected by many factors including hormones, light, mechanical stimuli, nutrition, and interactions with the cytoskeleton. These factors may influence the cellulose deposition by affecting the levels of substrate, the abundance of CSCs in the PM, and/or the activity of CSCs. UDP-glucose, produced presumably by sucrose synthase (SUSY), is the substrate of cellulose synthesis. The most popular model, presented by Haigler et al. stated that UDP-glucose is channeled to CSCs in the plasma membrane through SUSY. Consistent with this idea, suppression of SUSY in carrot and potato tubers by expressing an antisense version of SUSY led to a reduction in cellulose formation (Tang and Sturm, 1999; Haigler et al., 2001). However, quadruple mutant plants that lack four of six isoforms of SUSY (*sus1*/*sus2*/*sus3*/*sus4*) are normal in cell wall structure and cellulose content. These mutants lack SUS activity in all cell types except the phloem. The double mutant of the two phloem specific SUSYs (*sus5*/*sus6*) were also normal except for reduced amounts of callose in the sieve plates (Barratt et al., 2009). In contrast to the findings of Barratt et al. recent analysis using optimal pH conditions revealed SUS activity in leaves and stems of *sus1*/*sus2*/*sus3*/*sus4* and *sus5*/*sus6* plants is 85% of that of wild type plants (Baroja-Fernandez et al., 2012). Further analysis of sextuple mutants will be necessary to clarify whether SUS provides the primary route to supply carbon for cellulose and starch biosynthesis in *Arabidopsis*.

Bacterium cellulose synthesis requires the activation of bcsA, the catalytic subunit of cellulose synthase, through c-di-GMP (Ross et al., 1987). It appears to be a specific type of regulation in bacteria since plants does not have c-di-GMP. It has been proposed that phosphorylation might have a potential role in regulating CESA activity in plants (Somerville, 2006). Phosphoproteomics of *Arabidopsis* suspension culture cells revealed that CESA 1, CESA3, and CESA5 were phosphorylated at a number of sites clustered in the N-terminal variable region (Nuhse et al., 2004). The phosphorylation of secondary CESAs (CESA4 and CESA7) was also reported at a similar region (Taylor, 2007). Recently, the role of phosphorylation was tested for CESA1 in *Arabidopsis* (Chen et al., 2010). To modify the phosphorylation status of CESA1, six putative phosphorylation sites containing serine (S) or threonine (T) were substituted with alanine (A) to prevent phosphorylation or glutamine (E) to mimic phosphorylation and the resulting constructs were transformed into *rsw1* mutant background. Abolishing the putative phosphorylation sites for T165, T166, and S167 did not rescue the defect on growth anisotropy (i.e., radial swelling of cells). The other three pairs of mutations (S162, S686, and S688) recovered the growth anisotropic defect in *cesa1*^{rsw1}. The CSC dynamics were examined by introducing YFP-CESA6 into the above transgenic lines. Transgenic lines with reduced cell elongation and loss of cell anisotropy also showed asymmetric motility of CSCs in contrast to bidirectional movement in wild type cells. More interestingly, the asymmetric motility of CSCs in T165E and T165A was recovered by removal of microtubules using depolymerizing agent oryzalin. These observations led to the hypothesis that phosphorylation of CESA differentially affects a polar interaction with microtubules, resulting in velocity differences of CSCs on two sides of a microtubule.

The role of phosphorylation on motility of CSCs was also tested using CESA5. CESA5, presumably redundant with CESA6, did not fully complement the growth defect in *cesa6*$^{prc1\text{-}1}$ when it was expressed under the native promoter of CESA6. When putative phosphorylation sites at Ser122, Ser126, Ser229, and Ser230 were all concomitantly substituted with glutamine, the resulting construct fully complemented the growth defect and reduced motility of CSCs in *cesa6*$^{prc1\text{-}1}$ (Bischoff et al., 2011). Based on these observations, authors propose that phosphorylation is required to render CESA5 functionally equivalent to CESA6. Another CESA6-like protein, CESA2 was previously shown to fully complement the growth defect in *cesa6*$^{prc1\text{-}1}$ using a similar promoter swap strategy (Persson et al., 2007). Apparently, CESA2 may not require phosphorylation to substitute function of CESA6 because CESA2 does not have predicted phosphorylation sites at similar positions as CESA5.

CONCLUSION

Understanding the complex process of cellulose synthesis will be important for optimizing the use of cellulose as a renewable energy source. Although significant progress has been made in understanding the structure and regulation of CSCs, many fundamental aspects remain to be addressed. For example, the exact composition of the CSC is not known, nor do we know about the interaction pattern among isoforms of CESAs. The crystal structure of plant CESAs remains unsolved. *In vitro* synthesis of cellulose with plant CESAs is still at its infancy stage due to various technical difficulties. The exact molecular details of how microtubules guide the deposition of cellulose microfibrils are still missing. Cellulose biosynthesis is an exciting area of study with lots of challenges and opportunities. Advances in biochemical characterization and cell imaging tools in conjunction with the power of plant genetics will one day allow us to reconstitute an intact and fully functional cellulose synthesis machinery.

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REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). "Cell juctions, cell adhesion, and the extracellular matrix," in *Molecular Biology of the Cell*, ed. S. Gibbs (New York: Garland Science), 1124.

Baroja-Fernandez, E., Munoz, F. J., Li, J., Bahaji, A., Almagro, G., Montero, M., Etxeberria, E., Hidalgo, M., Sesma, M. T., and Pozueta-Romero, J. (2012). Sucrose synthase activity in the sus1/sus2/sus3/sus4 *Arabidopsis* mutant is sufficient to support normal cellulose and starch production. *Proc. Natl. Acad. Sci. U.S.A.* 109, 321–326.

Barratt, D. H., Derbyshire, P., Findlay, K., Pike, M., Wellner, N., Lunn, J., Feil, R., Simpson, C., Maule, A. J., and Smith, A. M. (2009). Normal growth of *Arabidopsis* requires cytosolic invertase but not sucrose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13124–13129.

Bashline, L., Du, J., and Gu, Y. (2011). The trafficking and behavior of cellulose synthase and a

glimpse of potential cellulose synthesis regulators. *Front. Biol.* 6:5. doi:10.1007/s11515-011-1161-3

Baskin, T. I. (2001). On the alignment of cellulose microfibrils by cortical microtubules: a review and a model. *Protoplasma* 215, 150–171.

Betancur, L., Singh, B., Rapp, R. A., Wendel, J. F., Marks, M. D., Roberts, A. W., and Haigler, C. H. (2010). Phylogenetically distinct cellulose synthase genes support secondary wall thickening in *Arabidopsis* shoot trichomes and cotton fiber. *J. Integr. Plant Biol.* 52, 205–220.

Bischoff, V., Desprez, T., Mouille, G., Vernhettes, S., Gonneau, M., and Hofte, H. (2011). Phytochrome regulation of cellulose synthesis in *Arabidopsis*. *Curr. Biol.* 21, 1822–1827.

Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M. T., and Persson, S. (2012). POM-POM2/cellulose synthase interacting1 is essential for the functional association of cellulose synthase and microtubules in *Arabidopsis*. *Plant Cell* 24, 163–177.

Brown, R. M. Jr., and Montezinos, D. (1976). Cellulose microfibrils: visualization of biosynthetic and orienting complexes in association with the plasma membrane. *Proc. Natl. Acad. Sci. U.S.A.* 73, 143–147.

Brown, R. M. Jr., Willison, J. H., and Richardson, C. L. (1976). Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the in vivo process. *Proc. Natl. Acad. Sci. U.S.A.* 73, 4565–4569.

Chen, S., Ehrhardt, D. W., and Somerville, C. R. (2010). Mutations of cellulose synthase (CESA1) phosphorylation sites modulate anisotropic cell expansion and bidirectional mobility of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17188–17193.

Collings, D. A. (2008). "Crossed wires: interactions and cross-talk between the microtubule and microfilament networks in plants," in *Plant Cell Monographs: Plant Microtubules, Development, and Flexibility*, ed. P. Nick (Berlin: Springer), 47–82.

Collings, D. A., Gebbie, L. K., Howles, P. A., Hurley, U. A., Birch, R. J., Cork, A. H., Hocart, C. H., Arioli, T., and Williamson, R. E. (2008). *Arabidopsis* dynamin-like protein DRP1A: a null mutant with widespread defects in endocytosis, cellulose synthesis, cytokinesis, and cell expansion. *J. Exp. Bot.* 59, 361–376.

Crowell, E. F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y. D., Schumacher, K., Gonneau, M., Hofte, H., and Vernhettes, S. (2009). Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell* 21, 1141–1154.

Crowell, E. F., Gonneau, M., Stierhof, Y. D., Hofte, H., and Vernhettes, S. (2010). Regulated trafficking of cellulose synthases. *Curr. Opin. Plant Biol.* 13, 700–705.

Delmer, D. P. (1999). Cellulose biosynthesis: exciting times for a difficult field of study. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 245–276.

Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15572–15577.

Doblin, M. S., Kurek, I., Jacob-Wilk, D., and Delmer, D. P. (2002). Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol.* 43, 1407–1420.

Gardiner, J. C., Taylor, N. G., and Turner, S. R. (2003). Control of cellulose synthase complex localization in developing xylem. *Plant Cell* 15, 1740–1748.

Green, P. B. (1962). Mechanism for plant cellular morphogenesis. *Science* 138, 1404–1405.

Gu, Y., Kaplinsky, N., Bringmann, M., Cobb, A., Carroll, A., Sampathkumar, A., Baskin, T. I., Persson, S., and Somerville, C. R. (2010). Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12866–12871.

Gu, Y., and Somerville, C. (2010). Cellulose synthase interacting protein: a new factor in cellulose synthesis. *Plant Signal. Behav.* 5, 1571–1574.

Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M., and Ehrhardt, D. W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nat. Cell Biol.* 11, 797–806.

Haigler, C. H., Ivanova-Datcheva, M., Hogan, P. S., Salnikov, V. V., Hwang, S., Martin, K., and Delmer, D. P. (2001). Carbon partitioning to cellulose synthesis. *Plant Mol. Biol.* 47, 29–51.

Heath, I. B. (1974). A unified hypothesis for the role of membrane bound enzyme complexes and microtubules in plant cell wall synthesis. *J. Theor. Biol.* 48, 445–449.

Hepler, P. K., and Newcomb, E. H. (1964). Microtubules and fibrils in the cytoplasm of coleus cells undergoing secondary wall deposition. *J. Cell Biol.* 20, 529–532.

Herth, W. (1980). Calcofluor white and Congo red inhibit chitin microfibril assembly of *Poterioochromonas*: evidence for a gap between polymerization and microfibril formation. *J. Cell Biol.* 87, 442–450.

Kimura, S., Laosinchai, W., Itoh, T., Cui, X., Linder, C. R., and Brown, R. M. Jr. (1999). Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant vigna angularis. *Plant Cell* 11, 2075–2086.

Konopka, C. A., and Bednarek, S. Y. (2008). Comparison of the dynamics and functional redundancy of the *Arabidopsis* dynamin-related isoforms DRP1A and DRP1C during plant development. *Plant Physiol.* 147, 1590–1602.

Li, S., Lei, L., Somerville, C., and Gu, Y. (2012). Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 185–190.

Mendu, V., Griffiths, J. S., Persson, S., Stork, J., Downie, A. B., Voiniciuc, C., Haughn, G. W., and DeBolt, S. (2011). Subfunctionalization of cellulose synthases in seed coat epidermal cells mediates secondary radial wall synthesis and mucilage attachment. *Plant Physiol.* 157, 441–453.

Mueller, S. C., and Brown, R. M. Jr. (1980). Evidence for an intramembrane component associated with a cellulose microfibril-synthesizing complex in higher plants. *J. Cell Biol.* 84, 315–326.

Nuhse, T. S., Stensballe, A., Jensen, O. N., and Peck, S. C. (2004). Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell* 16, 2394–2405.

Paredez, A. R., Somerville, C. R., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C. R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15566–15571.

Quader, H., Herth, W., Ryser, U., and Schnepf, E. (1987). Cytoskeletal elements in cotton seed hair development in vitro: their possible regulatory role in cell wall organization. *Protoplasma* 137, 56–62.

Robinson, D. G., White, R. K., and Preston, R. D. (1972). Fine structure of swarmers of *Cladophora* and *Chaetomorpha*. III. Wall synthesis and development. *Planta* 107, 131–144.

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G. A., van Boom, J. H., and Benziman, M. (1987). Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature* 325, 279–281.

Sampathkumar, A., Lindeboom, J. J., Debolt, S., Gutierrez, R., Ehrhardt, D. W., Ketelaar, T., and Persson, S. (2011). Live cell imaging reveals structural associations between the actin and microtubule cytoskeleton in *Arabidopsis*. *Plant Cell* 23, 2302–2313.

Saxena, I. M., and Brown, R. M. Jr. (2005). Cellulose biosynthesis: current views and evolving concepts. *Ann. Bot.* 96, 9–21.

Saxena, I. M., Lin, F. C., and Brown, R. M. Jr. (1990). Cloning and sequencing of the cellulose synthase catalytic subunit gene of *Acetobacter xylinum*. *Plant Mol. Biol.* 15, 673–683.

Scheible, W. R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001). Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis* Ixr1 mutants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10079–10084.

Seagull, R. W. (1990). The effects of microtubule and microfilament disrupting agents on cytoskeletal arrays and wall deposition in developing cotton fibers. *Protoplasma* 159, 44–59.

Seagull, R. W. (1991). "Role of the cytoskeletal elements in organized wall microfibril deposition," in *Biosynthesis and Biodegradation of Cellulose*, eds C. H. Haigler and P. J. Weimer (New York: Marcel Dekker), 143–163.

Somerville, C. (2006). Cellulose synthesis in higher plants. *Annu. Rev. Cell Dev. Biol.* 22, 53–78.

Stork, J., Harris, D., Griffiths, J., Williams, B., Beisson, F., Li-Beisson, Y., Mendu, V., Haughn, G., and DeBolt, S. (2010). Cellulose synthase9 serves a nonredundant role in secondary cell wall synthesis in *Arabidopsis* epidermal testa cells. *Plant Physiol.* 153, 580–589.

Sullivan, S., Ralet, M. C., Berger, A., Diatloff, E., Bischoff, V., Gonneau, M., Marion-Poll, A., and North, H.

M. (2011). CESA5 is required for the synthesis of cellulose with a role in structuring the adherent mucilage of *Arabidopsis* seeds. *Plant Physiol.* 156, 1725–1739.

Tang, G. Q., and Sturm, A. (1999). Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. *Plant Mol. Biol.* 41, 465–479.

Taylor, N. G. (2007). Identification of cellulose synthase AtCesA7 (IRX3) in vivo phosphorylation sites – a potential role in regulating protein degradation. *Plant Mol. Biol.* 64, 161–171.

Taylor, N. G., Howells, R. M., Huttly, A. K., Vickers, K., and Turner, S. R. (2003). Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1450–1455.

Wasteneys, G. O. (2004). Progress in understanding the role of microtubules in plant cells. *Curr. Opin. Plant Biol.* 7, 651–660.

Wasteneys, G. O., and Collings, D. A. (2004). "Expanding beyond the great divide: the cytoskeleton and axial growth," in *The Plant Cytoskeleton in Cell Differentiation and Development*, ed. P. J. Hussey (Victoria: Blackwell), 83–115.

Wightman, R., Marshall, R., and Turner, S. R. (2009). A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. *Plant Cell Physiol.* 50, 584–594.

Wightman, R., and Turner, S. R. (2008). The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. *Plant J.* 54, 794–805.

Williamson, R. E., Burn, J. E., and Hocart, C. H. (2002). Towards the mechanism of cellulose synthesis. *Trends Plant Sci.* 7, 461–467.

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Transcriptional regulation of grass secondary cell wall biosynthesis: playing catch-up with *Arabidopsis thaliana*

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Secondary cell wall synthesis occurs in specialized cell types following completion of cell enlargement. By virtue of mechanical strength provided by a wall thickened with cellulose, hemicelluloses, and lignin, these cells can function as water-conducting vessels and provide structural support. Several transcription factor families regulate genes encoding wall synthesis enzymes. Certain NAC and MYB proteins directly bind to the SNBE and AC elements upstream of structural genes and other transcription factors. The most detailed model of this regulatory network is established predominantly for a eudicot, *Arabidopsis thaliana*. In grasses, both the patterning and the composition of secondary cell walls are distinct from that of eudicots. These differences suggest transcriptional regulation is similarly distinct. Putative rice and maize orthologs of several eudicot cell wall regulators genetically complement mutants of *A. thaliana* or result in wall defects when constitutively overexpressed; nevertheless, aside from a maize, ZmMYB31, and a switchgrass protein, PvMYB4, function has not been tested in a grass. Similar to the seminal work conducted in *A. thaliana*, gene expression profiling in maize, rice, and other grasses implicates additional genes as regulators. Characterization of these genes will continue to elucidate the relationship between the transcription regulatory networks of eudicots and grasses.

Keywords: transcription factors, secondary cell wall

INTRODUCTION

Plant cell walls are diverse in their polymer composition, which vary among plant species as well as cell types within a species. The primary cell wall, consisting mostly of cellulose, hemicelluloses, pectin, and proteins demarcates each plant cell. Secondary cell walls, composed mostly of cellulose, hemicelluloses, and lignin, are deposited between the plasma membrane and primary wall in specialized cell types following cessation of cell enlargement. Even though all plant cell walls contain a cell wall matrix made up of cellulose microfibrils, hemicelluloses, and other cell wall polymers, there are significant differences in polymer types and their relative abundance between dictos and monocots (Vogel, 2008). For instance, relatively high amounts of xyloglucan, pectins, and structural proteins are typically present in the primary walls of eudicots, non-commelinoid monocots, and gymnosperms. The commelinoid monocots like grasses contain glucuronoarabinoxylans, small quantities of pectin and structural proteins and high levels of hydroxycinnamates (Vogel, 2008). Apart from wall composition, eudicots such as *Arabidopsis thaliana* and monocots such as rice (*Oryza sativa* L.) exhibit distinct morphological characteristics in addition to their namesake two and single cotyledons. Pinnate or palmate venation is characteristic of eudicots while monocots posses parallel venation. Other morphological and anatomical distinctions exist in their vasculature, tissues highly enriched in secondary cell walls. Stem radial thickening in eudicots is derived from a specialized cell layer called the cambium that differentiates into the phloem and xylem. On the other hand, monocots lack a specialized cambium layer and therefore do not undergo secondary growth (**Figure 1**). The vascular bundles of grasses are often well defined by a single layer of bundle sheath cells surrounding the xylem and phloem and the organization of the bundles is distinct from eudicots. In *A. thaliana*, the vascular bundles are arranged as a ring along the periphery of the stem in a pattern known as eustelic, whereas monocot bundles posses an atactostele arrangement characterized by several circles around the periphery of a stem, as in rice and *Brachypodium distachyon* (**Figure 1**), or scattered throughout the stem as in maize (*Zea mays* L.; Kiesselbach, 1949).

With distinctions and similarities abound, it is decidedly unclear how the transcriptional regulation of secondary cell wall biosynthesis in eudicots and grasses relate. Plant cell wall biosynthesis is regulated at different molecular and cellular levels. Current evidence supports a complex regulatory network consisting of a handful of proteins from only a small portion of over 65 different transcription factor families (**Figure 2A**). Phylogenetic analysis has identified close homologs of *A. thaliana* regulators from both vascular and non-vascular plants and some of those regulators were capable of complementing *A. thaliana* cell wall mutants (Zhong et al., 2011). These findings suggest the evolutionary conservation of the transcriptional regulators in secondary cell wall biosynthesis. On the other hand, due to the pronounced differences between eudicot and monocot secondary wall composition and anatomy, there are likely unique aspects of the regulatory network yet to be resolved.

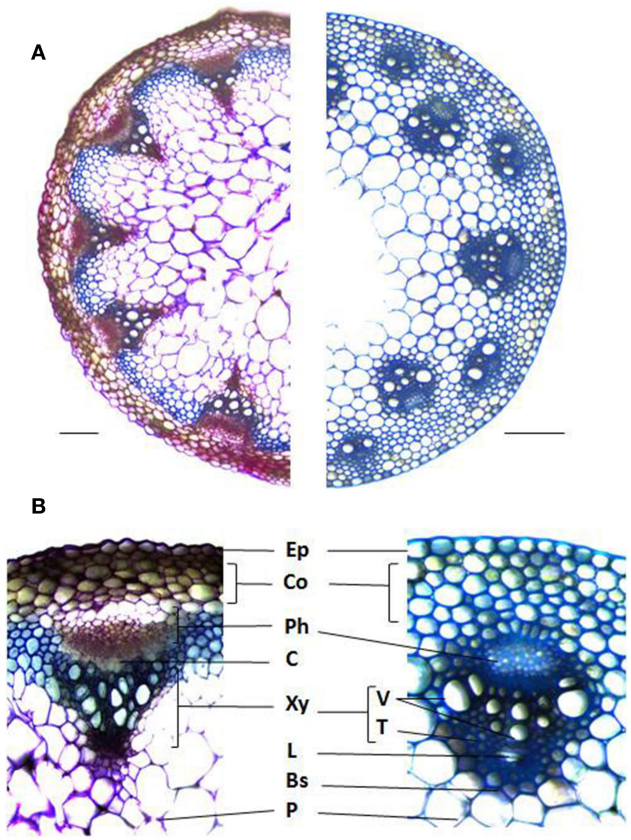


FIGURE 1 | Stem cross sections illustrating the different cell types and arrangements between dicots and monocots. (A) *Arabidopsis thaliana* (left) and *Brachypodium distachyon* (right) stained with Toluidine blue. **(B)** Vascular bundle anatomy of *A. thaliana* (left) and *B. distachyon* (right). Ep, Epidermis; Co, Cortex; Ph, Phloem; C, Cambium; Xy, Xylem; V, Vessels; T, Tracheads; L, Lacuna; Bs, Bundle Sheath; P, Pith. Bars = 0.1 mm.

TRANSCRIPTIONAL REGULATION IN *A. THALIANA*

Transcriptional regulation is one of the most important processes controlling plant cell wall biosynthesis, mediated by the interaction and interplay of *cis*-regulatory DNA elements and the *trans*-acting transcription factor proteins. Recent evidence suggests the involvement of an AP2 family protein *SHINE/WAX INDUCER 1* (*SHN*) as a global level regulator of cell wall biosynthesis (Ambavaram et al., 2011). Constitutive overexpression of *A. thaliana SHN* in rice results in the activation of cellulose and other cell wall-associated genes and the repression of lignin pathway genes. This protein is also capable of activating key *NAC* and *MYB* regulators and electrophoretic mobility shift assays (EMSA) demonstrate the direct binding of SHN protein to promoters of rice cell wall-associated transcription factors (Ambavaram et al., 2011). A WRKY family transcription factor, WRKY12, acts as a global repressor of secondary wall biosynthesis (Wang et al., 2010). Transcripts of this gene are abundant in the cortex and pith cells of *A. thaliana* that lack secondary walls. Loss-of-function *wrky12* mutants exhibit increased expression of transcription factors associated with secondary wall biosynthesis as well as ectopic depositions of lignin, cellulose, and xylan and consequently, and overall increase in plant biomass (Wang et al., 2010). In the presence of WRKY12 protein, pith stem cells are maintained parenchymatous and the deposition of secondary cell walls is repressed. MYB32 similarly acts as a repressor of secondary wall biosynthesis, but in cells where this pathway has been activated; thus, it may provide negative feedback (Preston et al., 2004). Transgenic overexpression of *MYB32* resulted in the repression of *SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1* (*SND1*), a higher order activator of secondary wall biosynthesis (Wang et al., 2011). Interestingly, the SND1 protein directly binds the *MYB32* promoter to activate gene expression (Wang et al., 2011). A group of NAC-domain transcription factors, *NAC SECONDARY WALL THICKENING FACTOR 1* (*NST1*), *NST2, SND1* (also known as *NST3*), *VASCULAR-RELATED NAC-DOMAIN 6* (*VND6*), and *VND7,* collectively known as the secondary wall NACs (SWNs) are implicated as positively acting master regulators in a variety of tissues (Demura and Fukuda, 2007; Zhong and Ye, 2007). Among the NACs, *SND1* functions as a key switch governing the regulation of all secondary wall polymers (Zhong et al., 2006; Mitsuda et al., 2007). Overexpression of *SND1* leads to ectopic deposition and activation of cellulose, hemicellulose, and lignin biosynthesis genes. Conversely, dominant repression of *SND1* results in the absence of secondary wall development in vascular and interfascicular fibers (Zhong et al., 2006; Mitsuda et al., 2007). It also directly activates itself and is repressed by MYB transcription factors under the direct influence of *MYB46* (Wang et al., 2011). The SND1 protein directly binds the *cis*-regulatory regions of *MYB46*, *MYB83*, and *C3H14* genes to activate their expression (Zhong et al., 2008; Ko et al., 2009; McCarthy et al., 2009). It also acts as a direct regulator of *F5H*, a gene encoding a key enzyme involved in lignin biosynthesis (Zhao et al., 2010). Another direct target of *SND1* is the KNOX type Homeodomain transcription factor, *KNAT7*, which interacts with OVATE FAMILY PROTEIN 4 to repress secondary wall biosynthesis (Li et al., 2011). Loss-of-function mutants of *knat7* display an increase in cell wall gene expression, wall thickening in interfascicular fiber cells, and an increase in lignin content (Li et al., 2011). Interestingly, a weak activator of cell wall gene expression, MYB75, was found to physically interact with KNAT7 protein to repress cell wall biosynthesis (Bhargava et al., 2010). These results imply that wall regulators can play multiple roles by interacting with different *trans*-acting factors in different cell types to provide more flexibility and complexity to the regulatory network.

The *SND1* homologs *NST1* and *NST2* play a crucial role in the *A. thaliana* anther endothecium (Mitsuda et al., 2005). In this tissue, these proteins are activated by *MYB26* which is essential for anther dehiscence and proper pollen release (Yang et al., 2007). Other SWNs, in particular *VND6* and *VND7*, regulate metaxylem and protoxylem development and are repressed by the *VND-INTERACTING2 NAC* protein (Kubo et al., 2005; Yamaguchi et al., 2010). The VND proteins VND6 and VND7 driven by *SND1* promoter were capable of complementing the *snd1/nst1* mutant phenotype implying their conserved functionality (Zhong et al., 2007a). They are positively regulated by *ASYMMETRIC*

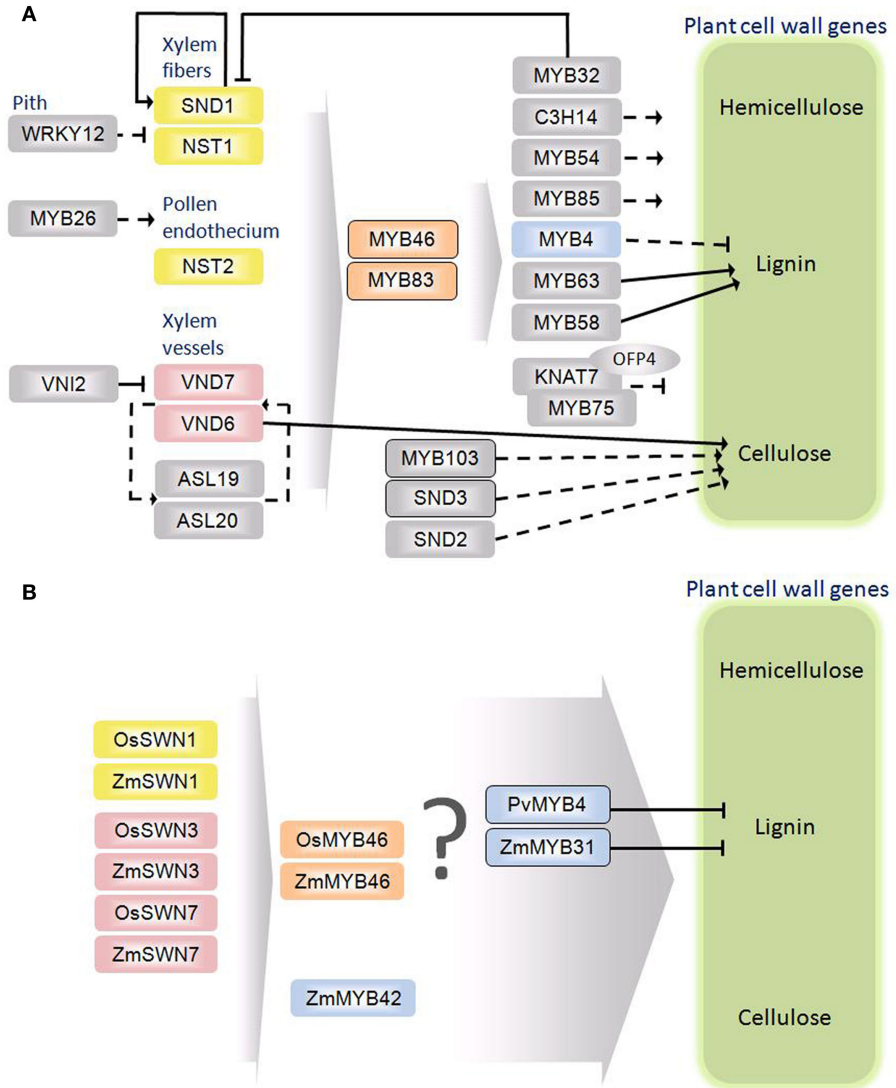


FIGURE 2 | Schematic diagrams of the secondary cell wall regulatory networks in *Arabidopsis thaliana* (A) and monocots (B). Rectangles represent transcription factors. The oval indicates an interacting protein. Solid arrows and bordered rectangles signify evidence for direct interactions. Dashed arrows indicate no evidence for direct interaction. Orthology between *A. thaliana* and grasses is denoted by color.

LEAVES2-LIKE19 (*ASL19*) and *ASL20* (Soyano et al., 2008). Transgenic overexpression of *ASL19* and *ASL20* induces trans-differentiation of non-vascular tissues into treachery elements and an increased cell wall thickening in mutant lines. They are also able to partially recover the dominant negative effect of the VND6 and VND7 repressor lines (Soyano et al., 2008). The SWNs, *SND1*, N*ST1*, *NST2*, *VND6*, and *VND7* activate a cascade of downstream transcription factors such as *SND2*, *SND3*, *MYB103*, *MYB85*, *MYB54*, *MYB46*, *MYB69*, *MYB63*, *MYB83*, and *KNAT7* (Zhong et al., 2008; Ko et al., 2009; McCarthy et al., 2009). Some of the downstream regulators, *SND2*, *SND3*, and *MYB103* exclusively activate cellulose biosynthesis where as the others such as *MYB63*, and *MYB58* regulate lignin biosynthesis (Zhong et al., 2008). Even though direct protein–DNA interactions have been shown for some of the cellulose and lignin specific regulators, further characterization of many downstream regulators is needed.

Discovery of the *trans*-acting transcription factors of cell wall biosynthesis facilitated the opportunity to identify common *cis*-elements shared among the master regulators. The tracheary-element-regulating *cis*-element (TERE) is one such 11-bp motif, CTT/(C)NAAA/(C)GCNA(T), involved in tissue specific cell wall biosynthesis and programmed cell death. First identified in the *Zinnia cysteine protease 4* promoter and is present in numerous cell death and xylem differentiation genes such as *Cysteine protease 1* (*XCP1*), *XCP2*, *Serine protease 1*, and several other genes associated with wall function that include xylanases and acetyltransferases (Pyo et al., 2007). More recent studies demonstrated the physical interaction between VND6 protein and the TERE (Ohashi-Ito et al., 2010). Other SWNs bind a 19-bp imperfect palindromic

sequence (T/A)NN(C/T)(T/C/G)TNNNNNNNA(A/C)GN(A/C/T)(A/T) referred to as the secondary wall NAC binding element (SNBE; Zhong et al., 2010). A synthetic promoter harboring six copies of the SNBE fused to a GUS reporter revealed specific expression in xylem and interfascicular fibers, phenocopying native *MYB46* promoter behavior (Zhong et al., 2007b). Other direct targets of SND1, including *MYB83*, *MYB103*, *SND3*, and *KNAT7* also posses the SNBE element (Zhong et al., 2010). A similar *cis*-element, TACNTTNNNNATGA, was identified recently in the *SND1* promoter and is the target of binding that serves as a target of positive feedback from SND1 itself (Wang et al., 2011).

A series of MYB transcription factor family proteins are also implicated in the cell wall regulatory network, a majority of which act downstream of the SWNs. One such protein, MYB46, acts subsequent the SWNs (**Figure 2A**). Lignin biosynthesis is specifically regulated by MYB63 and MYB58 interacting with the AC/Pal-box promoter sequences. This motif was first identified in the promoter of parsley *PHENYLALANINE AMMONIA-LYASE 1* and subsequently identified as three AC rich elements AC-I (ACCTACC), AC-II (ACCAACC), AC-III (ACCTAAC) involved in lignin gene regulation (Lois et al., 1989; Hatton et al., 1995; Raes et al., 2003). Binding of MYB proteins to the AC elements *trans*-activates the respective promoters thus, activating the genes in a xylem specific manner repressing the expression of the same genes in phloem or the cortical cells (Hatton et al., 1995). The consensus sequence of the AC element was recently expanded to include four more forms interchanging a T with the C at the last position; thus, ACC(T/A)A(A/C)(C/T) (Zhong and Ye, 2012). This 7-bp sequence, the secondary wall MYB responsive element (SMRE), is bound by both *MYB46* and *MYB83* proteins and is sufficient for the activation of a suite of transcription factor and cell wall biosynthetic genes (Zhong and Ye, 2012). The MYB46-responsive *cis*-element (M46RE) is an 8-bp sequence (A/G)(G/T)T(A/T)GGT(A/G) found in the *C3H14* promoter, which is a direct target of MYB46 (Kim et al., 2012). *Trans*-activation assays coupled with EMSA reveled M46RE is required and sufficient for the activation of *C3H14*. The 8-bp core sequence was present in nearly 43% of the genes in the *A. thaliana* genome but was enriched in the downstream genes activated by MYB46 along with secondary cell wall related structural genes (Kim et al., 2012). Apart from the key SWNs and MYBs, a handfull of downstream MYBs, NACs, and transcription factors from other families are involved in this complex cell wall regulatory network. A closely related homolog of *MYB32, MYB4*, functions as a repressor of *CINNAMATE-4-HYDROXYLASE* (C4H; Jin et al., 2000). Loss-of-function mutants of *MYB4* exhibited elevated levels of sinapoyl malate, a component in the lignin pathway, and an increase in *C4H* expression. Collectively, these findings suggest a complex and hierarchical transcription regulatory network for eudicot cell wall biosynthesis (**Figure 2A**). While this review primarily discusses discoveries in *A. thaliana*, it should be noted that a number of regulators have been characterized in other eudicot species such as *Populus trichocarpa, Eucalyptus gunnii, Nicotiana tabacum, Antirrhinum majus, Pinus taeda, Vitis vinifera*, and *Medicago truncatula*.

GRASS CELL WALL REGULATORS

From the time of eudicot and monocot divergence 140–150 million year ago, the transcription factor families have disproportionately expanded and the regulatory networks have likely diverged; thus, the existing eudicot network model for transcription regulation is not wholly generalizable to monocots (Chaw et al., 2004; Shiu et al., 2005). Conversely, recent functional characterization of grass transcription factors implies great commonality in how a similar network could regulate grass cell wall biosynthesis. While many cell wall genes have been characterized in grasses, almost nothing is known about the regulation of walls in monocots. The *A. thaliana* model, which is by far the best developed, is admittedly nascent. This stands in stark contrast to the model for grasses that consists of only a few genes (**Figure 2B**). Maize *CAFFEIC ACID-O-METHYL TRANSFERASE* (*COMT*) is a key lignin pathway gene with an AC-III element recognized by R2R3-MYB transcription factors (Vignols et al., 1995; Fornalé et al., 2010). Group four R2R3-MYB transcription factors are described as repressors and based on sequence homology to the know *A. thaliana* MYB repressors, five maize transcription factors, ZmMYB31, ZmMYB42, ZmMYB2, ZmMYB8, and ZmMYB39 were identified as candidates for direct repression of *ZmCOMT* (Fornalé et al., 2006). When overexpressed in *A. thaliana*, *ZmMYB31* and *ZmMYB42* resulted in down regulation of lignin associated genes and subsequently reduced lignin content (Fornalé et al., 2010). Overexpression of *ZmMYB42* caused a reduction in leaf size, an adaxial curvature indicative of less tertiary vein formation, reduction of the syringyl lignin monomers, and dwarfism in *A. thaliana* (Sonbol et al., 2009). The absence of results in a monocot is likely due to the relative recalcitrance of crop species to genetic study; thus, *A. thaliana* serves as an imperfect heterologous system to study grass gene function. The maize gene, *ZmMYB31* is the first wall specific regulator characterized in a grass (Fornalé et al., 2010). Chromatin immunoprecipitation demonstrated the direct interaction between ZmMYB31 and *ZmCOMT* promoter and an AC element similar to AC-II was identified as the binding motif. The switchgrass (*Panicum virgatum* L.) protein PvMYB4, an ortholog to *A. thaliana* MYB4, is another recently characterized repressor of lignin (Shen et al., 2012). Ectopic expression of *PvMYB4* in switchgrass resulted in a reduction in total lignin and altered lignin monomer ratio (Shen et al., 2012). The AC element is also implicated as the binding site of PvMYB4, which results in repression of lignin pathway genes (Shen et al., 2012). In addition, rice and maize orthologs of *A. thaliana* SWNs and MYB46 were shown to activate secondary wall biosynthesis when overexpressed in *A. thaliana* (Zhong and Ye, 2012). Moreover, OsSWNs and ZmSWNs were able to complement and partially rescue the pendant stem phenotype of the *A. thaliana snd1*/*nst*1 double mutant. Similarly, *OsMYB46* and *ZmMYB46* under the control of *AtMYB46* promoter were able to complement the loss of helical secondary wall thickening in vessels of *A. thaliana myb46/myb83* double mutant. In addition, SNBEs were identified in *OsMYB46* and *ZmMYB46* promoters and were bound and activated by the rice and maize SWNs in *A. thaliana* transient protoplast assays (Zhong and Ye, 2012). While studies in a heterologous system have proven informative, further functional characterization in a grass species is necessary. Contrary to expectations of distinct aspects of monocot

cell wall regulation, the existing model is populated exclusively by homologs of known *A. thaliana* genes.

Gene expression profiling was critical in identifying many of the candidates that formed the foundation of the existing *A. thaliana* cell wall regulatory network (Oh et al., 2003; Ehlting et al., 2005; Kubo et al., 2005; Zhao et al., 2005). Similar tools can be applied to grasses to identify candidates for functional characterization, especially those specific to monocots. Accordingly, a comparison of the transcriptome of maize elongating and non-elongating internodes revealed several transcription factors that are feasibly involved in cell wall regulation (Bosch et al., 2011). Likewise, the expression of three barley (*Hordeum vulgare* L.) NACs, *HvNAC033*, *HvNAC034*, and *HvNAC039*, were significantly greater in stem tissue where extensive secondary cell wall biosynthesis occurs. One of these proteins, HvNAC033, is the closest barley homolog to *A. thaliana* NST1, further supporting the possible role of this protein in grass cell wall regulation (Christiansen et al., 2011). Comparison of expression networks across species similarly reinforces the notion of shared features across eudicots and monocots and revealed potential distinctions. Genes with expression patterns similar to secondary wall *CELLULOSE SYNTHASE A* genes in rice included those most similar to *A. thaliana MYB63*, *MYB103*, *NST1*, *SND2*, and *KNAT7* (Ruprecht et al., 2011). Conversely, several rice transcription factors were co-expressed with structural genes that do not share sequence homology to co-expressed or characterized *A. thaliana* genes. These include MYB and NAC as well as bZip and AP2 family genes (Ruprecht et al., 2011). As with *ZmMYB42* and *PvMYB4*, candidates identified using sequence similarity and expression profiling require further characterization in the native systems in order to solidify both overlap and divergence between eudicot and grass cell wall regulatory networks.

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REFERENCES

Ambavaram, M. M. R., Krishnan, A., Trijatmiko, K. R., and Pereira, A. (2011). Coordinated activation of cellulose and repression of lignin biosynthesis pathways in rice. *Plant Physiol.* 155, 916–931.

Bhargava, A., Mansfield, S. D., Hall, H. C., Douglas, C. J., and Ellis, B. E. (2010). MYB75 functions in regulation of secondary cell wall formation in the *Arabidopsis* inflorescence stem. *Plant Physiol.* 154, 1428–1438.

Bosch, M., Mayer, C.-D., Cookson, A., and Donnison, I. S. (2011). Identification of genes involved in cell wall biogenesis in grasses by differential gene expression profiling of elongating and non-elongating maize internodes. *J. Exp. Bot.* 62, 3545–3561.

Chaw, S.-M., Chang, C.-C., Chen, H.-L., and Li, W.-H. (2004). Dating the monocot–dicot divergence and the origin of core eudicots using whole chloroplast genomes. *J. Mol. Evol.* 58, 424–441.

Christiansen, M., Holm, P., and Gregersen, P. (2011). Characterization of barley (*Hordeum vulgare* L.) NAC transcription factors suggests conserved functions compared to both monocots and dicots. *BMC Res. Notes* 4, 302. doi:10.1186/1756-0500-4-302

Demura, T., and Fukuda, H. (2007). Transcriptional regulation in wood formation. *Trends Plant Sci.* 12, 64–70.

Ehlting, J., Mattheus, N., Aeschliman, D. S., Li, E., Hamberger, B., Cullis, I. F., Zhuang, J., Kaneda, M., Mansfield, S. D., Samuels, L., Ritland, K., Ellis, B. E., Bohlmann, J., and Douglas, C. J. (2005). Global transcript profiling of primary stems from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. *Plant J.* 42, 618–640.

Fornalé, S., Shi, X., Chai, C., Encina, A., Irar, S., Capellades, M., Fuguet, E., Torres, J.-L., Rovira, P., Puigdomènech, P., Rigau, J., Grotewold, E., Gray, J., and Caparrós-Ruiz, D. (2010). ZmMYB31 directly represses maize lignin genes and redirects the phenylpropanoid metabolic flux. *Plant J.* 64, 633–644.

Fornalé, S., Sonbol, F.-M., Maes, T., Capellades, M., Puigdomènech, P., Rigau, J., and Caparrós-Ruiz, D. (2006). Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid O-methyl-transferase genes by two new maize R2R3-MYB transcription factors. *Plant Mol. Biol.* 62, 809–823.

Hatton, D., Sablowski, R., Yung, M.-H., Smith, C., Schuch, W., and Bevan, M. (1995). Two classes of *cis* sequences contribute to tissue-specific expression of a *PAL2* promoter in transgenic tobacco. *Plant J.* 7, 859–876.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., and Martin, C. (2000). Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *EMBO J.* 19, 6150–6161.

Kiesselbach, T. A. (1949). *The Structure and Reporduction of Corn.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Kim, W.-C., Ko, J.-H., and Han, K.-H. (2012). Identification of a *cis*-acting regulatory motif recognized by MYB46, a master transcriptional regulator of secondary wall biosynthesis. *Plant Mol. Biol.* 78, 489–501.

Ko, J.-H., Kim, W.-C., and Han, K.-H. (2009). Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in *Arabidopsis*. *Plant J.* 60, 649–665.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., and Demura, T. (2005). Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev.* 19, 1855–1860.

Li, E., Wang, S., Liu, Y., Chen, J.-G., and Douglas, C. J. (2011). Ovate family protein4 (OFP4) interaction with KNAT7 regulates secondary cell wall formation in *Arabidopsis thaliana*. *Plant J.* 67, 328–341.

Lois, R., Dietrich, A., and Schulz, W. (1989). A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. *EMBO J.* 8, 1641–1648.

McCarthy, R. L., Zhong, R., and Ye, Z.-H. (2009). MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. *Plant Cell Physiol.* 50, 1950–1964.

Mitsuda, N., Iwase, A., Yamamoto, H., Yoshida, M., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2007). NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. *Plant Cell* 19, 270–280.

Mitsuda, N., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2005). The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell* 17, 2993–3006.

Oh, S., Park, S., and Han, K.-H. (2003). Transcriptional regulation of secondary growth in *Arabidopsis thaliana*. *J. Exp. Bot.* 54, 2709–2722.

Ohashi-Ito, K., Oda, Y., and Fukuda, H. (2010). *Arabidopsis* vascular-related NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. *Plant Cell* 22, 3461–3473.

Preston, J., Wheeler, J., Heazlewood, J., Li, S. F., and Parish, R. W. (2004). AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. *Plant J.* 40, 979–995.

Pyo, H., Demura, T., and Fukuda, H. (2007). TERE; a novel *cis*-element responsible for a coordinated expression of genes related to programmed cell death and secondary wall formation during differentiation of tracheary elements. *Plant J.* 51, 955–965.

Raes, J., Rohde, A., Christensen, J., Peer, Y., and Boerjan, W. (2003). Genome-wide characterization of the lignification toolbox in *Arabidopsis*. *Plant Physiol.* 133, 1051–1071.

Ruprecht, C., Mutwil, M., Saxe, F., Eder, M., Nikoloski, Z., and Persson, S. (2011). Large-scale co-expression approach to dissect secondary cell wall formation across plant species. *Front. Plant Sci.* 2:23. doi:10.3389/fpls.2011.00023

Shen, H., He, X., Poovaiah, C. R., Wuddineh, W. A., Ma, J., Mann, D. G. J., Wang, H., Jackson, L., Tang, Y., Neal Stewart, C., Chen, F., and Dixon, R. A. (2012). Functional characterization of the switchgrass (*Panicum virgatum*) R2R3-MYB transcription factor *PvMYB4* for improvement of lignocellulosic feedstocks. *New Phytol.* 193, 121–136.

Shiu, S.-H., Shih, M.-C., and Li, W.-H. (2005). Transcription factor families have much higher expansion rates in plants than in animals. *Plant Physiol.* 139, 18–26.

Sonbol, F.-M., Fornalé, S., Capellades, M., Encina, A., Touriño, S., Torres, J.-L., Rovira, P., Ruel, K., Puigdomènech, P., Rigau, J., and Caparrós-Ruiz, D. (2009). The maize ZmMYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*. *Plant Mol. Biol.* 70, 283–296.

Soyano, T., Thitamadee, S., Machida, Y., and Chua, N.-H. (2008). *ASYMMETRIC LEAVES2-LIKE19/LATERAL ORGAN BOUNDARIES DOMAIN30* and *ASL20/LBD18* regulate tracheary element differentiation in *Arabidopsis*. *Plant Cell* 20, 3359–3373.

Vignols, F., Rigau, J., Torres, M., Capellades, M., and Puigdomenech, P. (1995). The *brown midrib3 (bm3)* mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. *Plant Cell* 7, 407–416.

Vogel, J. (2008). Unique aspects of the grass cell wall. *Curr. Opin. Plant Biol.* 11, 301–307.

Wang, H., Avci, U., Nakashima, J., Hahn, M. G., Chen, F., and Dixon, R. A. (2010). Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22338–22343.

Wang, H., Zhao, Q., Chen, F., Wang, M., and Dixon, R. A. (2011). NAC domain function and transcriptional control of a secondary cell wall master switch. *Plant J.* 68, 1104–1114.

Yamaguchi, M., Ohtani, M., Mitsuda, N., Kubo, M., Ohme-Takagi, M., Fukuda, H., and Demura, T. (2010). VND-INTERACTING2, a NAC domain transcription factor, negatively regulates xylem vessel formation in *Arabidopsis*. *Plant Cell* 22, 1249–1263.

Yang, C., Xu, Z., Song, J., Conner, K., Vizcay Barrena, G., and Wilson, Z. A. (2007). *Arabidopsis* MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. *Plant Cell* 19, 534–548.

Zhao, C., Craig, J. C., Petzold, H. E., Dickerman, A. W., and Beers, E. P. (2005). The xylem and phloem transcriptomes from secondary tissues of the *Arabidopsis* root-hypocotyl. *Plant Physiol.* 138, 803–818.

Zhao, Q., Wang, H., Yin, Y., Xu, Y., Chen, F., and Dixon, R. A. (2010). Syringyl lignin biosynthesis is directly regulated by a secondary cell wall master switch. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14496–14501.

Zhong, R., Demura, T., and Ye, Z.-H. (2006). SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of *Arabidopsis*. *Plant Cell* 18, 3158–3170.

Zhong, R., Lee, C., McCarthy, R. L., Reeves, C. K., Jones, E. G., and Ye, Z.-H. (2011). Transcriptional activation of secondary wall biosynthesis by rice and maize NAC and MYB transcription factors. *Plant Cell Physiol.* 52, 1856–1871.

Zhong, R., Lee, C., and Ye, Z. H. (2010). Global analysis of direct targets of secondary wall NAC master switches in *Arabidopsis*. *Mol. Plant* 3, 1087–1103.

Zhong, R., Lee, C., Zhou, J., McCarthy, R. L., and Ye, Z.-H. (2008). A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. *Plant Cell* 20, 2763–2782.

Zhong, R., Richardson, E., and Ye, Z.-H. (2007a). Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of *Arabidopsis*. *Planta* 225, 1603–1611.

Zhong, R., Richardson, E. A., and Ye, Z. H. (2007b). The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in *Arabidopsis*. *Plant Cell* 19, 2776–2792.

Zhong, R., and Ye, Z.-H. (2007). Regulation of cell wall biosynthesis. *Curr. Opin. Plant Biol.* 10, 564–572.

Zhong, R., and Ye, Z.-H. (2012). MYB46 and MYB83 bind to the SMRE sites and directly activate a suite of transcription factors and secondary wall biosynthetic genes. *Plant Cell Physiol.* 53, 368–380.

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Plant cell wall integrity maintenance as an essential component of biotic stress response mechanisms

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Plant cell walls provide structural support during development and represent together with the cuticle the first line of defense against biotic and abiotic stress. In recent years, evidence has accumulated that a dedicated plant cell wall integrity (CWI) maintenance mechanism exists. This mechanism monitors and maintains functional integrity of the cell wall during different biological processes. The available data suggest that it may represent a component of the stress response mechanisms underlying biotic and abiotic stress responses, which has not been identified previously as a distinct mechanism. Here I will review the available evidence regarding the mode of action of the CWI maintenance mechanism and discuss its role in the context of biotic plant stress response mechanisms.

Keywords: plant cell wall integrity, biotic stress, plant pathogen

INTRODUCTION

Plant cells need to maintain the functional integrity of their walls during cell morphogenesis and exposure to biotic/abiotic stress. The available evidence suggests that a dedicated plant cell wall integrity (CWI) maintenance mechanism exists (Wolf et al., 2011). While our understanding of the mechanisms regulating stress responses and morphogenesis has increased significantly, our knowledge regarding the processes maintaining CWI is still limited. In the last years several reviews have been published on the plant CWI maintenance mechanism illustrating the increased interest in this area (Humphrey et al., 2007; Hematy et al., 2009; Ringli, 2010; Seifert and Blaukopf, 2010). A recently published review focuses on CWI maintenance during plant cell wall morphogenesis (Wolf et al., 2011). Similarities between the yeast and plant CWI maintenance mechanisms have also been reviewed (Hamann and Denness, 2011). Therefore, the available knowledge regarding CWI maintenance during plant development and in yeast will be covered here only briefly to provide a conceptual framework regarding cellular processes involved and to illustrate the degree of functional conservation between species. This review will focus on recent developments regarding the role of CWI maintenance during biotic stress responses. It will discuss how CWI maintenance could have a previously unrecognized role in the perception of and response to biotic stress.

SIMILARITIES AND DIFFERENCES BETWEEN YEAST AND PLANT CELL WALL INTEGRITY MAINTENANCE MECHANISMS

While both plant and yeast cells are enveloped by cell walls, certain important differences exist that affect the biological role and function of the plant CWI maintenance mechanism. Yeast unlike plant cells, do not have to deal with biotic stress. In addition, plant cell walls are structurally and chemically more complex than the yeast cell wall. This means that in plant cells the sheer number of cell wall-related signaling events during development and plant–environment interaction could disguise the activity of a dedicated plant CWI maintenance mechanism.

The yeast CWI monitoring and maintenance network is quite complex, providing an indication of the possible complexity of the plant CWI maintenance network. By combining inputs from a turgor pressure sensor (SLN1), mechano-perception (MID1/CCH1), and dedicated cell wall damage (CWD) sensors (WSC1, 2, 3, MID2, MTL1) the yeast CWI maintenance network generates signals that permit highly specific responses to any challenge that impairs the functional integrity of the yeast cell wall. The available phenotypic and genetic data also implicate turgor pressure, mechano-perception, and CWD detection in plant CWI maintenance (Hamann et al., 2009; Denness et al., 2011). Interestingly, *ARABIDOPSIS HISTIDINE KINASE1* (*AHK1*) and *CYTOKININ RECEPTOR1/ARABIDOPSIS HISTIDINE KINASE4* (*CRE1*/*AHK4*) can at least partially rescue a yeast strain with a loss of function allele in *SLN1* (Urao et al., 1999; Inoue et al., 2001). In addition, expression of the *Arabidopsis thaliana MID1 COMPLEMENTING ACTIVITY 1* and *2* (*MCA1*, *2*) genes rescues a *MID1*-deficient yeast strain suggesting that they could function as stretch-activated calcium channels (Nakagawa et al., 2007; Yamanaka et al., 2010). Up to now no functional homologues for the yeast CWD sensors have been identified in plants. In yeast, the signals generated by the sensors are relayed to the response genes via different signaling cascades involving CALCINEURIN (MID1/CCH1); RHO1/MPK1 (WSC1, MID2), and YPD1 (SLN1) (Levin, 2005). Transcription factors mediating the response are SKN7, RLM1, and SWI4/6 (Levin, 2005). The response can involve activation of genes required for cell wall biosynthetic processes, remodeling of the cytoskeleton, and cell cycle progression. The available data suggest both organizational and functional

similarities between the yeast and plant CWI maintenance mechanism while also highlighting how signals from mechanical and chemical sensors regulate jointly the CWD response.

COMPOSITION AND STRUCTURE OF THE PLANT CELL WALL

The plant cell wall is comparable to an exoskeleton surrounding the plant cell and providing both structural support and protection from biotic as well as abiotic stresses. It consists of cellulose microfibrils, pectin, hemicelluloses, proteins, and in certain cases lignin (Somerville et al., 2004). Plant cell walls are divided into primary (laid down during cell elongation/differentiation) and secondary (formed after cell morphogenesis is concluded) walls. In parallel, dependent on the presence of certain polysaccharides type I and type II cell walls are distinguished (Popper et al., 2011). Cellulose microfibrils are the main load bearing elements, which are cross-linked to hemicelluloses and (*in vitro*) to pectin (Dick-Perez et al., 2011). Hemicelluloses and pectin also form direct links creating a matrix in which the microfibrils are embedded like the steel mesh in a concrete wall. Pectic polysaccharides like homogalacturonan (HG) are connected by calcium bridges between dimethyl-esterified parts of the molecules or through borate ester linkages in the case of rhamnogalacturonan II (RG II). They represent important cell wall components during morphogenesis facilitating cell expansion and plant–pathogen interaction (Hahn et al., 1981; Bellincampi et al., 1996). In the latter situation they are targeted by pathogen-derived cell wall degrading enzymes (polygalacturonases), which generate oligogalacturonides (OGAs) from HG (Hahn et al., 1981; Kars et al., 2005). Biologically active OGAs consist of chains of 9–15 galacturonic acid (GalA) monomers and can function as signaling molecules (see below).

During secondary cell wall formation monolignols (precursors for lignin) are secreted into the cell wall space and randomly cross-linked (Vanholme et al., 2010). The cross-linking is dependent on the availability of reactive oxygen species (ROS) generated by laccases and peroxidases. This process reinforces the wall against pathogen infection, waterproofs it, and increases structural integrity (Tronchet et al., 2010; Vanholme et al., 2010). Reduction of cellulose biosynthesis during primary cell wall formation through genetic or chemical means leads to lignin production (Ellis et al., 2002; Hamann et al., 2009). This highlights the ability of the cell to adapt to changes in cell wall composition and provides evidence for the existence of a CWI maintenance mechanism.

THE PLANT CWI MAINTENANCE MECHANISM AS A COMPONENT OF THE BIOTIC STRESS RESPONSE

Plant cell walls are capable of adjusting their composition and structure in response to pathogen infection (Dong et al., 2008). A mutation in (*CELLULOSE SYNTHASEA3*, *CESA3*) a subunit of the cellulose synthase complex leads to ectopic production of lignin, i.e., replacement of a missing load bearing cell wall component by another one (Cano-Delgado et al., 2000). Follow up studies found that inhibition of cellulose biosynthesis during primary cell wall formation either through mutations like *constitutive expression of VSP1* (*cev1*) and *ectopic lignification1* (*eli1*) or inhibitors such as isoxaben results in transcriptional activation of stress response mechanisms; ectopic production of ethylene (ET), salicylic acid (SA), jasmonic acid (JA), callose, and ROS as well as changes in cell wall composition and structure (Ellis et al., 2002; Cano-Delgado et al., 2003; Manfield et al., 2004; Hamann et al., 2009). The experiments also showed that the response to CWD consists of early and late stages reminiscent of the response to pathogen infection (Denness et al., 2011). During the early stage, ROS- and calcium-based signaling cascades are required for initiating the response to CWD (Denness et al., 2011). Interestingly, 1-aminocyclopropane-1-carboxylic acid (ACC, an ET precursor) and not ET itself seems to be acting as signaling substance during the early response to isoxaben with inhibition of cell expansion being an active process and not simply an automatic consequence of cellulose biosynthesis inhibition (Tsang et al., 2011). During the late stage, responses to CWD-like lignin deposition are initiated and the extent of lignin formation is apparently modulated by a negative feedback loop formed by ROS and JA (Denness et al., 2011). A combination of genetic and phenotypic analysis has implicated the NADPH oxidases *RESPIRATORY BURST OXIDASE HOMOLOGD* and *F* (*RBOHD*, *F*), the serine/threonine kinase *OXIDATIVE SIGNAL INDUCIBLE1* (*OXI1*), *MCA1*, the receptor-like kinase (RLK) *THESEUS1* (*THE1*) as well as the JA biosynthesis genes *ALLENE OXIDE SYNTHASE* (*AOS*) and *JASMONIC ACID RESISTANT1* (*JAR1*) in the signaling mechanism mediating the response to CWD in *Arabidopsis* seedlings. Interestingly, the *cev1* mutation that affects cellulose biosynthesis during primary cell wall formation also causes enhanced resistance to infection by different powdery mildews (*Erysiphe orontii*, *E. cichoracearum*, and *Oidium lycopersicum*; Ellis and Turner, 2001).

A screen for mutants causing resistance to powdery mildew infection provides further evidence of the close relationship between plant cell walls and pathogen resistance (Vogel and Somerville, 2000). Three of the powdery mildew resistance (*PMR*) mutants that have been identified on the molecular level affect genes involved in cell wall biosynthetic processes. *PMR4* encodes a callose synthase, *PMR5* a gene of unknown function required for pectin production and *PMR6* a pectate lyase (Vogel et al., 2002, 2004; Nishimura et al., 2003). *pmr4* resistance seems to be mediated via hyper-activation of SA signaling, whereas *pmr5* and *6* resistance phenotypes are independent of JA, SA, and ET signaling. Mutations in *IRREGULARXYLEM1* (*IRX1*/*CESA8*), *3* (*IRX3*/*CESA7*), and *5* (*IRX5*/*CESA4*) impair cellulose biosynthesis during secondary cell wall formation and cause enhanced resistance to the soil borne bacterium *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina* (Hernandez-Blanco et al., 2007). Mutations affecting cellulose biosynthesis during primary cell wall formation (*cesa1, 3, 6*) or other components of the secondary cell wall (*pmr5*, *pmr6*) did not cause enhanced resistance to the same pathogens. Genetic analysis showed that the enhanced resistance in *cesa4, 7, 8* is independent of JA, SA, and ET-based signaling mechanisms. Results from expression profiling experiments and genetic analysis using different abscisic acid (ABA) mutants (*ABA insensitive 1-1*; *2-1*; *ABA1-6*) suggest that ABA is mediating developmental and pathogen resistance phenotypes caused by the *irx* mutants. However, it remains to be determined if the ABA involvement is direct or a secondary effect due to water stress caused by problems with xylem cell wall

formation in the mutants. To summarize, these results suggest distinct resistance signaling cascades are induced by defects in primary and secondary cell wall formation as well as for different secondary cell wall components. They also highlight the direct impact of changes in cell wall composition/structure on the response to pathogen infection.

SIGNALING MECHANISMS AND SENSORS IMPLICATED IN PLANT CWD PERCEPTION

The mode of action of the plant cell wall maintenance mechanism is not well understood. Based on the knowledge from yeast, chemical and physical signals could act as indicators for the functional integrity of the plant cell wall either individually or jointly. By combining these different types of signal the plant cell would receive precise information regarding the state of its cell wall and the exact type of CWI impairment occurring. Physical signals could be generated by stretching of the plasma membrane due to a weakened cell wall that cannot resist the high turgor pressure levels within a plant cell or a plasma membrane that is displaced relatively to the cell wall. These events could be detected by stretch-activated or mechanosensitive channel proteins that lead to calcium influx into the cytoplasm, indicating CWD. Sensor candidates could be encoded by members of the mechanosensitive channels of small conductance (MscS)-like (*MSL*) gene family like *MSL 9* and *10* affect mechano-perception in protoplasts derived from *Arabidopsis* root cells (Haswell et al., 2008). Another candidate of interest is the putative stretch-activated calcium channel MCA1 is required for CWD-induced lignin deposition. Interestingly, all isoxaben-induced CWD phenotypes can be suppressed by provision of osmotic support suggesting that changes in turgor pressure due to a weakened cell wall could result in signal generation via turgor pressure sensors (Hamann et al., 2009; Denness et al., 2011). While AHK1 and 4/CRE1 can function as osmosensors in yeast and have been implicated in abiotic stress responses, no clear evidence exists implicating them in CWD perception in plants (Urao et al., 1999; Inoue et al., 2001; Tran et al., 2007). In addition, AHK4/CRE1 has been shown to function as a cytokinin receptor (Inoue et al., 2001). Therefore, the question that needs to be resolved at this point is if turgor pressure is a passive element in the process (generating cell wall fragments due to a weakened cell wall) or an active component that is being monitored and provides input into the process.

The plant cell wall contains a large number of components that could generate chemical signals (ligands) indicative of CWD or general danger signals. The term damage associated molecular patterns (DAMPs) has been coined to describe such ligands and the number of possible DAMPs originating in plant cell walls is rather large (Zipfel, 2009). Here I will focus on the best-characterized group of signals, which are probably OGAs. They can be generated through degradation of HG by pathogen-derived enzymes (Kars et al., 2005; Ferrari et al., 2008). OGAs have been shown to induce gene expression changes, stomatal closure, production of ET, and ROS as well as cell wall reinforcement (Denoux et al., 2008; Ferrari et al., 2008). A hybrid kinase consisting of the extra cellular domain of *WALL-ASSOCIATED KINASE1* (*WAK1*) and the intracellular domain of elongation factor Tu receptor (EFR) kinase can bind OGAs and activate defense responses (Brutus et al., 2010). *WAK1* belongs to a family of five WAK genes encoding plasma membrane-localized Ser/Thr kinases that have been implicated in response to pathogen infection and regulation of cell elongation (Kohorn et al., 2011). The effects of the chimeric WAK1 kinase on pathogen resistance suggest that OGAs and WAKs represent an *in vivo* ligand–receptor pair. Results from the analysis of a dominant active WAK2 allele suggest the CWD signals perceived by WAKs could be relayed to downstream response genes through *MAPKINASE6* (*MPK6*) (Kohorn et al., 2009, 2011). Interestingly, WAK2 has also been implicated in regulation of invertase activity and turgor pressure during cell elongation (Kohorn et al., 2006). However, there is currently no confirmation that WAKs are actively involved in CWI maintenance.

In *Arabidopsis*, more than 600 RLKs have been identified and a large number of them have been implicated in developmental and stress response processes (Shiu and Bleecker, 2001). I will focus here on several kinases that have been implicated in CWD perception and/or pathogen response. Most of the RLKs implicated in CWI maintenance [THE1, HERCULES1 (HERK1), FERONIA (FER)] belong to the *Catharanthus roseus* RLK1 (CrRLK1)-like protein family, which has 17 members in *Arabidopsis*. *THE1* was isolated as a suppressor of the cellulose-deficient *cesa6 procuste* (*prc*) mutant, which exhibits a hypocotyl elongation defect (Hematy et al., 2007). Although *the1* suppresses the elongation defect, the cellulose deficiency is not reduced. Subsequently it has been shown that THE1 is required for cellulose biosynthesis inhibition-induced ROS production and lignification in the root elongation zone (Denness et al., 2011). THE1, HERK1, and FER have been implicated in brassinosteroid-induced cell elongation (Guo et al., 2009; Deslauriers and Larsen, 2010). Both FER and NORTIA/MILDEW RESISTANCE LOCUS O 7 (*MLO7*; a seven-transmembrane domain protein involved in powdery mildew resistance) are required for successful fertilization and resistance to infection by *Golovinomyces* (syn. *Erysiphe*) *orontii* (Kessler et al., 2010). Interestingly, ROPGEF (guanine-exchange factors) proteins have been identified as targets of FER activity (Duan et al., 2010). ROPGEFs are required for the activation of Rho GTPases, which in turn activate NADPH oxidases like RBOHD/F. These results suggest the same molecular components could mediate cell–cell interaction during development and plant–pathogen interaction.

Heterotrimeric G-proteins (Gα, Gβ, Gγ) form a highly conserved signaling complex that has been implicated in signal transduction during development and stress responses in mammals, yeast, and plants (Digby et al., 2006; Temple and Jones, 2007). In *Arabidopsis*, five genes GPA1 (Gα), AGB1 (Gβ), AGG1, 2, 3 (Gγ1, 2, 3) encode the subunits of the complex (Thung et al., 2011). Recently, it has been reported that mutations in *AGG1*, *2*, and *AGB1* apparently cause enhanced susceptibility to infection with *P. cucumerina* (Delgado-Cerezo et al., 2011). A combination of metabolomic and microarray-based expression profiling studies of the mutants established that the pathogen phenotype is independent of SA, JA, ABA, and ET signaling cascades. Interestingly, a large number of cell wall biosynthetic/modifying genes are mis-regulated in *agb1* and *agg12* plants. Analysis of cell wall composition/structure in these plants found reduced xylose contents in the mutants compared to wildtype.

To summarize, the available evidence supports the notion that the plant cell wall is an integral component contributing to pathogen response mechanisms and illustrates the influence of cell wall defects on infection. Specific signaling cascades seem to mediate the response to particular cell wall defects, which in turn affect the response to necrotrophic or biotrophic pathogens. More importantly the data presented above allow correlation between certain types of cell wall defects, and not only signaling cascades but also resistance phenotypes. Mutations in *CESA4*, *7*, *8*, *AGG1*, *2*, and *AGB1* affect resistance to necrotrophs and are independent of phytohormone-based signaling cascades. *pmr5*, *6* plants exhibit resistance to biotrophs and also do not rely on phytohormone-based signaling cascades. *pmr4* affects resistance to biotrophs and resistance depends on the integrity of the SA signaling cascade. *cesa3* plants show enhanced JA, ET biosynthesis, and resistance to biotrophs.

The available data allow different explanations for the specific effects on pathogen resistance observed. The cell wall composition/structure changes could prevent pathogen colonization simply because the infection machinery of the pathogen is too specialized to breach the chemically modified cell wall. However, no functional pathogen response mechanisms should be required for this possibility. Another option is that the cell wall mutants cause defects similar to those occurring during infection by particular pathogens, i.e., simulate infection by necrotrophs or biotrophs. This would cause early/constant activation of the CWI maintenance/defense mechanism, which "primes" plant immunity thus making successful infection more difficult. The latter would explain both the specificity of the responses observed and dependence on particular signaling mechanisms. Therefore, studies focusing on the effects of particular cell wall defects on pathogen resistance and the mode of action of the CWI maintenance mechanism could facilitate research into biotic stress response. The reason being, that by removing the potentially multiple effects of the pathogen during infection, they reduce the complexity of the interaction and should therefore allow novel insights into the mechanisms responsible for detection of infection and/or physical damage.

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REFERENCES

Bellincampi, D., Cardarelli, M., Zaghi, D., Serino, G., Salvi, G., Gatz, C., Cervone, F., Altamura, M. M., Costantino, P., and Lorenzo, G. D. (1996). Oligogalacturonides prevent rhizogenesis in rolB-transformed tobacco explants by inhibiting auxin-induced expression of the rolB gene. *Plant Cell* 8, 477–487.

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.

Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* 34, 351–362.

Cano-Delgado, A. I., Metzlaff, K., and Bevan, M. W. (2000). The *eli1* mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. *Development* 127, 3395–3405.

Delgado-Cerezo, M., Sanchez-Rodriguez, C., Escudero, V., Miedes, E., Fernandez, P. V., Jorda, L., Hernandez-Blanco, C., Sanchez-Vallet, A., Bednarek, P., Schulze-Lefert, P., Somerville, S., Estevez, J. M., Persson, S., and Molina, A. (2011). *Arabidopsis* heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi. *Mol. Plant* 5, 98–114.

Denness, L., McKenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., Mansfield, J., Zipfel, C., and Hamann, T. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a ROS- and jasmonic acid dependent process in *Arabidopsis thaliana*. *Plant Physiol.* 156, 1364–1374.

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

Deslauriers, S. D., and Larsen, P. B. (2010). *FERONIA* is a key modulator of brassinosteroid and ethylene responsiveness in *Arabidopsis* hypocotyls. *Mol. Plant* 3, 626–640.

Dick-Perez, M., Zhang, Y., Hayes, J., Salazar, A., Zabotina, O. A., and Hong, M. (2011). Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. *Biochemistry* 50, 989–1000. doi: 10.1021/bi101795q

Digby, G. J., Lober, R. M., Sethi, P. R., and Lambert, N. A. (2006). Some G protein heterotrimers physically dissociate in living cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17789–17794.

Dong, X., Hong, Z., Chatterjee, J., Kim, S., and Verma, D. P. (2008). Expression of callose synthase genes and its connection with *NPR1* signaling pathway during pathogen infection. *Planta* 229, 87–98.

Duan, Q., Kita, D., Li, C., Cheung A. Y., and Wu, H. M. (2010). FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17821–17826.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002). The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14, 1557–1566.

Ellis, C., and Turner, J. G. (2001). The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* 13, 1025–1033.

Ferrari, S., Galletti, R., Pontiggia, D., Manfredini, C., Lionetti, V., Bellincampi, D., Cervone, F., and De Lorenzo, G. (2008). Transgenic expression of a fungal endo-polygalacturonase increases plant resistance to pathogens and reduces auxin sensitivity. *Plant Physiol.* 146, 669–681.

Guo, H., Li, L., Ye, H., Yu, X., Algreen, A., and Yin, Y. (2009). Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7648–7653.

Hahn, M. G., Darvill, A. G., and Albersheim, P. (1981). Host-pathogen interactions: XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. *Plant Physiol.* 68, 1161–1169.

Hamann, T., Bennett, M., Mansfield, J., and Somerville, C. (2009). Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. *Plant J.* 57, 1015–1026.

Hamann, T., and Denness, L. (2011). Cell wall integrity maintenance in plants: lessons to be learned from yeast? *Plant Signal. Behav.* 6, 1–5.

Haswell, E. S., Peyronnet, R., Barbier-Brygoo, H., Meyerowitz, E. M., and Frachisse, J. M. (2008). Two MscS homologs provide mechanosensitive channel activities in the *Arabidopsis* root. *Curr. Biol.* 18, 730–734.

Hematy, K., Cherk, C., and Somerville, S. (2009). Host–pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12, 406–413.

Hematy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J. P., and Hofte, H. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931.

Hernandez-Blanco, C., Feng, D. X., Hu, J., Sanchez-Vallet, A., Deslandes, L., Llorente, F., Berrocal-Lobo, M., Keller, H., Barlet, X., Sanchez-Rodriguez, C., Anderson, L. K., Somerville, S., Marco, Y., and Molina, A. (2007). Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant Cell* 19, 890–903.

Humphrey, T. V., Bonetta, D. T., and Goring, D. R. (2007). Sentinels at the wall: cell wall receptors and sensors. *New Phytol.* 176, 7–21.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409, 1060–1063.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A., and van Kan, J. A. (2005). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* 43, 213–225.

Kessler, S. A., Shimosato-Asano, H., Keinath, N. F., Wuest, S. E., Ingram, G., Panstruga, R., and Grossniklaus, U. (2010). Conserved molecular components for pollen tube reception and fungal invasion. *Science* 330, 968–971.

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., and Obregon, P. (2009). Pectin activation of MAP kinase and gene expression is *WAK2* dependent. *Plant J.* 60, 974–982.

Kohorn, B. D., Kobayashi, M., Johansen, S., Riese, J., Huang, L. F., Koch, K., Fu, S., Dotson, A., and Byers, N. (2006). An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* 46, 307–316.

Kohorn, B. D., Kohorn, S. L., Todorova, T., Baptiste, G., Stansky, K., and McCullough, M. (2011). A dominant allele of *Arabidopsis* pectin-binding wall-associated kinase induces a stress response suppressed by *MPK6* but not *MPK3* mutations. *Mol. Plant.* doi: 10.1093/mp/ssr096

Levin, D. E. (2005). Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 69, 262–291.

Manfield, I. W., Orfila, C., McCartney, L., Harholt, J., Bernal, A. J., Scheller, H. V., Gilmartin, P. M., Mikkelsen, J. D., Paul Knox, J., and Willats, W. G. (2004). Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *Plant J.* 40, 260–275.

Nakagawa, Y., Katagiri, T., Shinozaki, K., Qi, Z., Tatsumi, H., Furuichi, T., Kishigami, A., Sokabe, M., Kojima, I., Sato, S., Kato, T., Tabata, S., Iida, K., Terashima, A., Nakano, M., Ikeda, M., Yamanaka, T., and Iida, H. (2007). *Arabidopsis* plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3639–3644.

Nishimura, M. T., Stein, M., Hou, B. H., Vogel, J. P., Edwards, H., and Somerville, S. C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301, 969–972.

Popper, Z. A., Michel, G., Herve, C., Domozych, D. S., Willats, W. G., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Ann. Rev. Plant Biol.* 62, 567–590.

Ringli, C. (2010). Monitoring the outside: cell wall-sensing mechanisms. *Plant Physiol.* 153, 1445–1452.

Seifert, G. J., and Blaukopf, C. (2010). Irritable walls: the plant extra cellular matrix and signaling. *Plant Physiol.* 153, 467–478.

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.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., and Youngs, H. (2004). Toward a systems approach to understanding plant cell walls. *Science* 306, 2206–2211.

Temple, B. R., and Jones, A. M. (2007). The plant heterotrimeric G-protein complex. *Ann. Rev. Plant Biol.* 58, 249–266.

Thung, L., Trusov, Y., Chakravorty, D., and Botella, J. R. (2011). Gγ1 + Gγ2 + Gγ3 = Gβ: the search for heterotrimeric G-protein gamma subunits in *Arabidopsis* is over. *J. Plant Physiol.* 169, 542–545.

Tran, L. S., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Functional analysis of *AHK1*/*ATHK1* and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 20623–20628.

Tronchet, M., Balague, C., Kroj, T., Jouanin, L., and Roby, D. (2010). Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in *Arabidopsis*. *Mol. Plant Pathol.* 11, 83–92.

Tsang, D. L., Edmond, C., Harrington, J. L., and Nuhse, T. S. (2011). Cell wall integrity controls root elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-independent pathway. *Plant Physiol.* 156, 596–604.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., and Shinozaki, K. (1999). A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* 11, 1743–1754.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., and Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant Physiol.* 153, 895–905.

Vogel, J., and Somerville, S. (2000). Isolation and characterization of powdery mildew-resistant *Arabidopsis* mutants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1897–1902.

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. *Plant Cell* 14, 2095–2106.

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in *PMR5* result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978.

Wolf, S., Hematy, K., and Hofte, H. (2011). Growth control and cell wall signaling in plants. *Ann. Rev. Plant Biol.* doi: 10.1146/annurev-arplant-042811-105449

Yamanaka, T., Nakagawa, Y., Mori, K., Nakano, M., Imamura, T., Kataoka, H., Terashima, A., Iida, K., Kojima, I., Katagiri, T., Shinozaki, K., and Iida, H. (2010). *MCA1* and *MCA2* that mediate Ca^{2+} uptake have distinct and overlapping roles in *Arabidopsis*. *Plant Physiol.* 152, 1284–1296.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* 12, 414–420.

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Co-expression of cell wall-related genes: new tools and insights

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Global transcript analyses based on publicly available microarray dataset have revealed that genes with similar function tend to be transcriptionally coordinated. Indeed, many genes involved in the formation of cellulose, hemicelluloses, and lignin have been identified using co-expression approaches in *Arabidopsis*. To facilitate these transcript analyses, several web-based tools have been developed that allow researchers to investigate co-expression relationships of their gene(s) of interest. In addition, several tools now also provide the possibility of comparative transcriptional analyses across species, which potentially increases the predictive power. In this short review, we describe recent developments and updates of plant-related co-expression tools, and summarize studies that have successfully used expression profiling in cell wall research. Finally, we illustrate the value of comparative co-expression relationships across species using genes involved in lignin biosynthesis.

Keywords: plant, co-expression, cell wall, cellulose, xylan, lignin, comparison across species

INTRODUCTION

In the last decade, the increased use of microarrays for global expression analyses made large-scale transcript analyses possible. Using publicly available microarray datasets, several studies showed that genes with similar function tended to be transcriptionally coordinated (Stuart et al., 2003; Ihmels et al., 2004). Based on this observation, co-expression approaches have been used to assign functions for genes involved in cell wall formation, isoprenoid and glucosinolate biosynthesis, and different metabolic pathways in *Arabidopsis* (Wille et al., 2004; Brown et al., 2005; Persson et al., 2005; Wei et al., 2006; Hirai et al., 2007). Interestingly, certain co-expression relationships appear to also be conserved across different species across the kingdoms of life (Stuart et al., 2003; Bergmann et al., 2004). For example, orthologous genes involved in protein synthesis, cell cycle, and protein degradation formed comparable co-expressed clusters in different species (Stuart et al., 2003). In the above studies, the microarray analyses still had to be quality-controlled and evaluated by the investigators themselves to obtain co-expressed relationships. However, web-based co-expression tools now enable users to easily mine publicly available microarray dataset to investigate their gene(s) of interest. In this review we present several co-expression tools and describe recent developments and updates for them. We also give an overview of how these tools have been used to identify novel cell wall-related genes, and exemplify the use of comparative co-expression analyses across species to infer lignin-related genes.

CO-EXPRESSION TOOLS

Several co-expression tools have been developed for plant biology, including ACT (Manfield et al., 2006), ASIDB (Rawat et al., 2008), ATTED-II (Obayashi et al., 2009), CressExpress (Srinivasasainagendra et al., 2008), CSB.DB (Steinhauser et al., 2004), and GeneCAT (Mutwil et al., 2008), which have been comprehensively described and compared elsewhere (Usadel et al., 2009). In addition, several recent platforms have emerged, such as AraNet, CORNET, GeneMANIA, PlaNet, and RiceArrayNet. AraNet[1] aims at annotation of *Arabidopsis* genes by integrating available large-scale experimental data, e.g., co-expression and protein–protein interaction data, as well as gene associations inferred from other species and literature queried data (Lee et al., 2010). Similarly, GeneMANIA[2] is predicting gene function based on available genomics and proteomics data sets for *Arabidopsis*, yeast, and several animal model species (Warde-Farley et al., 2010). CORNET[3] allows for user-defined selection of microarray experiments and cut-offs for assessing co-regulated genes in *Arabidopsis*. These data may be supplemented with protein–protein interaction, functional annotation, and localization data (De Bodt et al., 2010). PlaNet provides a platform for gene co-expression network analysis for seven plant species and includes information about significant enrichment for functional annotation using MapMan ontology terms[4] (Mutwil et al., 2011). Another interesting co-expression tool is RiceArrayNet, which calculates positive as well as negative correlation of gene expression profiles in rice (Lee et al., 2009). This tool has later been extended to *Brassica* and *Arabidopsis*, referred to as PlantArrayNet[5].

In principle, the platforms above calculate co-expression relationships between two genes of interest by comparing their respective expression profiles. The Pearson's correlation coefficient (PCC) is a commonly used measure to estimate transcriptional co-ordination. Using this measure as a basis, co-expression relationships between many genes can be determined, and can be

[1]http://www.functionalnet.org/aranet/
[2]http://www.genemania.org/
[3]http://bioinformatics.psb.ugent.be/cornet
[4]http://aranet.mpimp-golm.mpg.de/
[5]http://arraynet.mju.ac.kr/arraynet/

visualized as networks in which nodes represent genes and the connection between nodes indicates the transcriptional co-ordination of the genes. Such co-expression networks can be divided into rank-based and value-based networks (Aoki et al., 2007). Previously, the most widely used approach was value-based networks, i.e., edges were established between genes that were co-expressed above a certain PCC-value threshold (Lee et al., 2004; Oldham et al., 2006). One major drawback of this method arises from the fact that some biological processes are tightly transcriptionally co-regulated, while other processes are not. Therefore, when a stringent global PCC-value cut-off is applied many genes involved in weakly transcriptionally coordinated processes that may be biologically relevant become disconnected. In contrast, a lower PCC-threshold will in many instances result in excessively large gene clusters, containing thousands of genes (Mao et al., 2009). To avoid such problems, some tools have introduced the rank-based method, which is based on the ranks of two given genes in their mutual co-expression lists (ATTED-II: Obayashi and Kinoshita, 2009; PlaNet: Mutwil et al., 2010, 2011). Although both the value- and rank-based networks are derived from PCC, rank-based networks appear to lead to a network topology that closer resembles biological networks (Ruan et al., 2010). However, it is important to note that both approaches have their advantages and drawbacks (Usadel et al., 2009).

Most of the co-expression tools have focused on the model plant *Arabidopsis*, and have incorporated the main bulk of publicly available microarray datasets, thus representing condition-independent co-expression relationships (Usadel et al., 2009). However, some biologically relevant transcriptional relationships may be revealed only under specific experimental conditions, or in certain tissues. To find such relationships, tools like CORNET allow for user-defined selection of microarray experiments to calculate co-expression relationships. Another example of condition-dependent databases is the updated version of ATTED-II[6], which enables the user to analyze co-expression relationships of genes under five predefined conditions: tissue and development, abiotic stress, biotic stress, hormone treatment, and different light regimes (Obayashi et al., 2011). Furthermore, SeedNet[7] is a relatively new tissue-specific database, which returns co-expression relationships of genes during seed development (Bassel et al., 2011).

One possible caveat with co-expression analyses is the rate of "false positives," i.e., co-expressed genes that might be co-expressed by chance rather than being functionally related. A useful approach to minimize the rate of such "false positives" may be to investigate whether orthologous genes are also co-expressed in related species. As mentioned above, co-expression relationships are often conserved across species (Stuart et al., 2003). Hence, two co-expressed genes from one species often have orthologs in another species that in turn are also co-expressed. In theory it should therefore be possible to enrich for co-occurring co-expression relationships across species, and hence minimize "false positives." Therefore, across species co-expression analyses might improve the reliability of co-expression-based functional annotation. Consequently, several tools, such as StarNet[8], CoP[9], and ATTED-II, allow pairwise comparison between species (Jupiter et al., 2009; Ogata et al., 2009; Obayashi et al., 2011). Moreover, comparison of several species at the same time was introduced by the NetworkComparer-tool of PlaNet (Mutwil et al., 2011). This tool bins genes into gene families according to their Pfam annotation (Finn et al., 2010), and then finds recurring Pfams in co-expression networks across species (Mutwil et al., 2011).

TRANSCRIPTIONAL CO-ORDINATION OF GENES INVOLVED IN CELL WALL BIOSYNTHESIS

Cellulose is produced by the cellulose synthase (CESA) complex (CSC), which is comprised of CESA1, 3, and 6-related proteins in the primary wall and CESA4, 7, and 8 in the secondary wall (Gardiner et al., 2003; Desprez et al., 2007; Persson et al., 2007). Interestingly, these primary and the secondary *CESAs* display similar expression patterns, respectively (Brown et al., 2005; Persson et al., 2005). In addition, these studies were able to show that many genes involved in xylan and lignin synthesis were co-expressed with the secondary wall *CESAs* (Brown et al., 2005; Persson et al., 2005). Hence, co-expression analyses may be useful to identify new genes involved in secondary cell wall-related synthesis.

Figure 1 shows a truncated node-vicinity network (NVN) of genes co-expressed with the primary wall-related *Arabidopsis CESA1*-gene, which was obtained from PlaNet (Mutwil et al., 2011). Several primary wall *CESA* genes (*CESA2*, *CESA3*, *CESA5*, and *CESA6*) may be found in close vicinity of *CESA1*. In addition, many other genes important for cellulose synthesis, such as *COBRA (COB)*, *CHITINASE-LIKE (CTL)1*, *CELLULOSE SYNTHASE INTERACTING (CSI)1*/*POM-POM2*, and *KORRIGAN (KOR)*, are present in this network (Nicol et al., 1998; Zhong et al., 2002; Roudier et al., 2005; Gu et al., 2010; Bringmann et al., 2012). This result confirms previous findings which were based on a smaller number of microarrays (Persson et al., 2005), and is similar to results obtained from other co-expression tools. For example, the many of the genes in this network are also present in the *AtCESA1*-top 300 list of co-expressed genes in ATTED-II (77 out of 190 genes; see also the genes marked in bold in **Figure 1**).

In addition to cellulose synthesis, co-expression approaches have been used to identify genes involved in the synthesis of hemicelluloses. For example, Cocuron et al. (2007) found that the *Arabidopsis CSLC4* gene, which presumably is involved in glucan backbone synthesis of xyloglucan, was co-expressed with the xylosyltransferase *AtXT1*, which has previously been shown to attach xylose residues to a glucan backbone (Faik et al., 2002). Moreover, expression profiling was used to identify *IRX15* and an *IRX15*-like gene, with corresponding single mutants showing a mild irregular xylem phenotype (Jensen et al., 2010; Brown et al., 2011). Both genes belong to a gene family with a domain of unknown function (DUF) 579 and only the corresponding double mutant showed decreased levels of xylan and altered cell wall morphology in stems (Jensen et al., 2010; Brown et al., 2011). Although their

[6]http://atted.jp/

[7]http://bree.cs.nott.ac.uk/arabidopsis/

[8]http://vanburenlab.medicine.tamhsc.edu/starnet2.html;

[9]http://webs2.kazusa.or.jp/kagiana/cop0911/

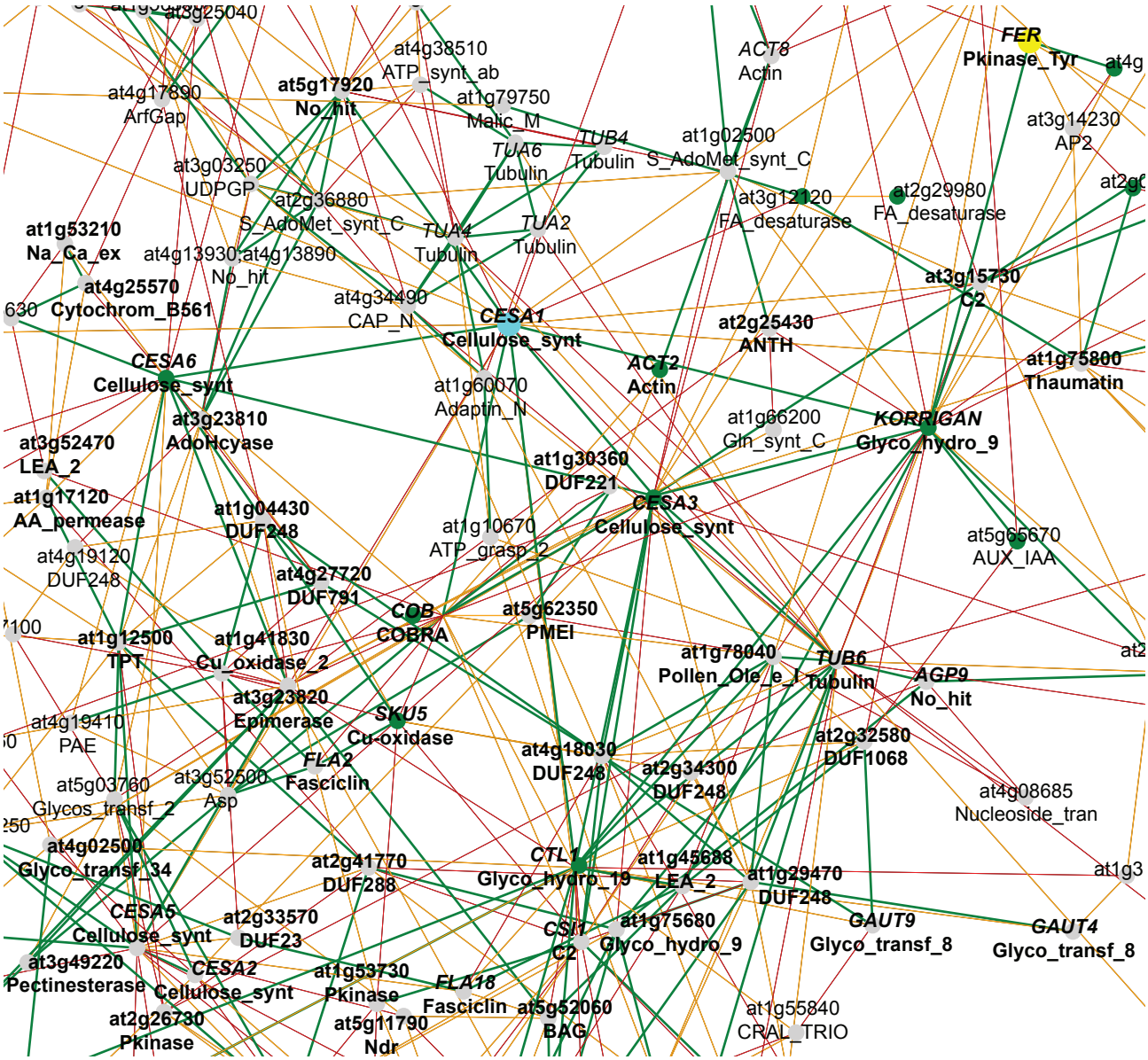


FIGURE 1 | Subset of the co-expression gene vicinity network of *AtCESA1* (turquoise node). Nodes indicate individual genes, and edges indicate whether two genes are co-expressed above a certain mutual rank threshold. Node colors indicate whether mutations in the gene cause gametophytic lethality (yellow), any biological phenotype (green), or if no mutant phenotype information currently is available (gray) according to TAIR. Green, orange, and red edges indicate a mutual rank relationship <10 (green), 10–19 (orange), and 20–29 (red), respectively, for each connected gene pair. This modified network was obtained from PlaNet (http://aranet.mpimp-golm.mpg.de/aranet). Genes marked in bold are also present in the top 300 co-expression list of *AtCESA1* obtained from ATTED-II (http://atted.jp/). The respective gene family according to Pfam annotation is given below the gene names/AGI codes (Finn et al., 2010).

exact function is unknown, these results suggest an important role of this DUF579-gene family in xylan biosynthesis (Jensen et al., 2010; Brown et al., 2011).

Interestingly, also many genes that are important for lignin synthesis are co-expressed. Using microarray data from several developmental stages of *Arabidopsis* stems, Ehlting et al. (2005) showed transcriptional co-ordination of many putative and *bona fide* genes involved in monolignol biosynthesis, transport, and polymerization. This is consistent with results of Persson et al. (2005), and Brown et al. (2005) that found laccase genes, which are probably involved in polymerization of lignin, in the list of genes that are co-expressed with secondary wall-related *CESAs*.

COMPARATIVE EXPRESSION ANALYSES OF CELL WALL-RELATED GENES ACROSS SPECIES

The study of Mitchell et al. (2007) was one of the first studies to compare transcript relationships of cell wall-related genes

across monocots and dicots. Based on EST data, the authors found that members of the GT43-, GT47-, and GT61-family are enriched in monocots and therefore might be involved in biosynthesis of the grass-specific glucuronoarabinoxylan (Mitchell et al., 2007). Indeed, the same group could recently show that several GT61-members are essential for arabinosylation of xylan (Anders et al., 2012). Another recent study compared the co-expression relationships of xylan-related genes from *Arabidopsis* and rice (Oikawa et al., 2010). Using the *Arabidopsis* genes *IRX9*, *IRX10*, and *IRX14*, which have previously been associated with xylan backbone synthesis (Brown et al., 2005; Peña et al., 2007), the tentative rice orthologs were identified based on sequence homology and expression patterns (Oikawa et al., 2010). These genes were then utilized as baits in the ATTED-II tool and revealed many re-occurring homologs in the respective co-expression lists (Oikawa et al., 2010). A similar co-expression approach has been undertaken for cellulose-related genes by comparing the co-expression networks of primary and secondary *CESAs* from seven species using PlaNet (Ruprecht et al., 2011). Interestingly, many gene families are consistently co-expressed with *CESA* genes across species. The function of most of these gene families remains unknown; however, their conserved transcriptional co-ordination suggests that they fulfill important functions during cellulose synthesis (Ruprecht et al., 2011). Based on the results, an *Arabidopsis* mutant corresponding to *PINORESINOL REDUCTASE (PRR)* 1 was identified that displayed distorted xylem vessels (Ruprecht et al., 2011), probably due to decreased lignin or lignan production.

USING A COMPARATIVE CO-EXPRESSION TOOL TO ANALYZE LIGNIN BIOSYNTHESIS

While comparative transcriptional analyses across species have been undertaken for cellulose and hemicelluloses-related genes, conserved co-expression relationships among lignin-related genes have been paid less attention. Similar to previously published results (Ehlting et al., 2005), we found that the co-expression network of *Arabidopsis PHENYLALANINE AMMONIA LYASE (PAL) 1*, which marks the initial step for monolignol synthesis, comprises many genes involved in lignin formation (**Figure 2B**). Using *AtPAL1* as bait in the NetworkComparer-tool of PlaNet, we identified similar co-expression networks for *AtPAL1*-orthologs in barley, Medicago, poplar, rice, soybean, and wheat. The resulting consensus network indicates that most of the gene families needed for monolignol synthesis are conserved across species (**Figure 2**). Surrounding the "*PAL*"-gene family, we found gene families corresponding to the subsequent steps in the lignin pathway such as C4H (which belongs to gene family "p450" according to Pfam annotation, Finn et al., 2010), 4CL ("AMP_binding"), HCT ("Transferase"), C3H ("p450"), CCoAOMT ("Methyltransf_3"), CCR ("Epimerase"), and CAD ("ADH_zinc_N"). In addition, genes functioning upstream of PAL in the phenylalanine synthesis pathway were previously identified as transcriptionally linked with the phenylpropanoid-related genes in *Arabidopsis* (Tohge and Fernie, 2010), which appeared to be also conserved in other species (i.e., gene families "DAHP_synth_2," "DHQ_synthase," "SKI," "EPSP_synthase," "PDT"). Interestingly, a gene family denoted as "UDPGT," corresponding to glycosyltransferases, was present in almost all *PAL1*-derived co-expression networks across the seven species (**Figure 2**). Members of this family have been shown to glucosylate various monolignols (Lim et al., 2001) and we therefore hypothesize that the respective co-expressed genes of this family might have a similar function in the different species. Recently, Miao and Liu (2010) showed that ABC-transporters are likely to mediate transport of monolignols, and their glucoconjugates, across plasma and vacuolar membrane, respectively. However, the exact genes involved in this process have not yet been identified. The *AtPAL1*-network contained three different genes from the ABC-transporter family ("ABC2_membrane") which might mediate this function in *Arabidopsis*. While single knock-out mutants for one of them (*atabcg33*) did not show any obvious phenotypes (Kaneda et al., 2011), it is plausible that the other two members may functionally compensate for the loss of AtABCG33. Interestingly, the consensus network also comprises several highly conserved gene families, whose function in lignin biosynthesis is still elusive, for example, the gene families "Hydrolase," "Abhydrolase_1," and "Abhydrolase_3." One of the co-expressed genes from the "Hydrolase"-family in *Arabidopsis* is *RESPONSIVE-TO-ANTAGONIST (RAN) 1*, which encodes for a copper transporter involved in ethylene signaling (Hirayama et al., 1999). Copper is a co-factor of laccases, which are important for polymerization of monolignols to lignin in the cell wall (Boerjan et al., 2003). We therefore speculate that RAN1 has an additional function in providing copper co-factors for enzymatic lignin formation. Moreover, close homologs of the *Arabidopsis* gene from the "Abhydrolase_1"-family (At3g03990) are methylesterases, suggesting that these genes might function antagonistically to caffeic acid *O*-methyltransferases (COMTs). Another rather unexpected yet interesting example is the highly conserved "Peroxidase"-family, which one might associate to monolignol polymerization in the cell wall. However, the respective co-expressed gene in *Arabidopsis* is *ASCORBATE PEROXIDASE (APX)1*, a cytosolic enzyme that has an important role in scavenging hydrogen peroxide (Davletova et al., 2005). APX1 might therefore function in preventing premature radical coupling of monolignols already in the cytosol. Thus, exemplified by the lignin-related analysis, we propose that comparative co-expression analyses might be useful in the future to reveal novel players for different aspects of cell wall biosynthesis, but also in highlighting conserved and divergent elements in different biological processes.

CONCLUSION AND PERSPECTIVE

Co-expression approaches have been especially valuable for identifying new genes involved in secondary cell wall synthesis (Brown et al., 2005, 2011; Persson et al., 2005; Jensen et al., 2010; Ruprecht et al., 2011). A comparison of the candidate genes from several different studies showed that similar results were obtained regardless of the underlying microarray datasets (i.e., condition-independent or stem-specific samples) and the bait genes (xylan or cellulose synthesis-related) used (Oikawa et al., 2010). This is probably due to the fact that secondary cell wall formation is a highly coordinated process that is mainly restricted to certain tissue and cell types.

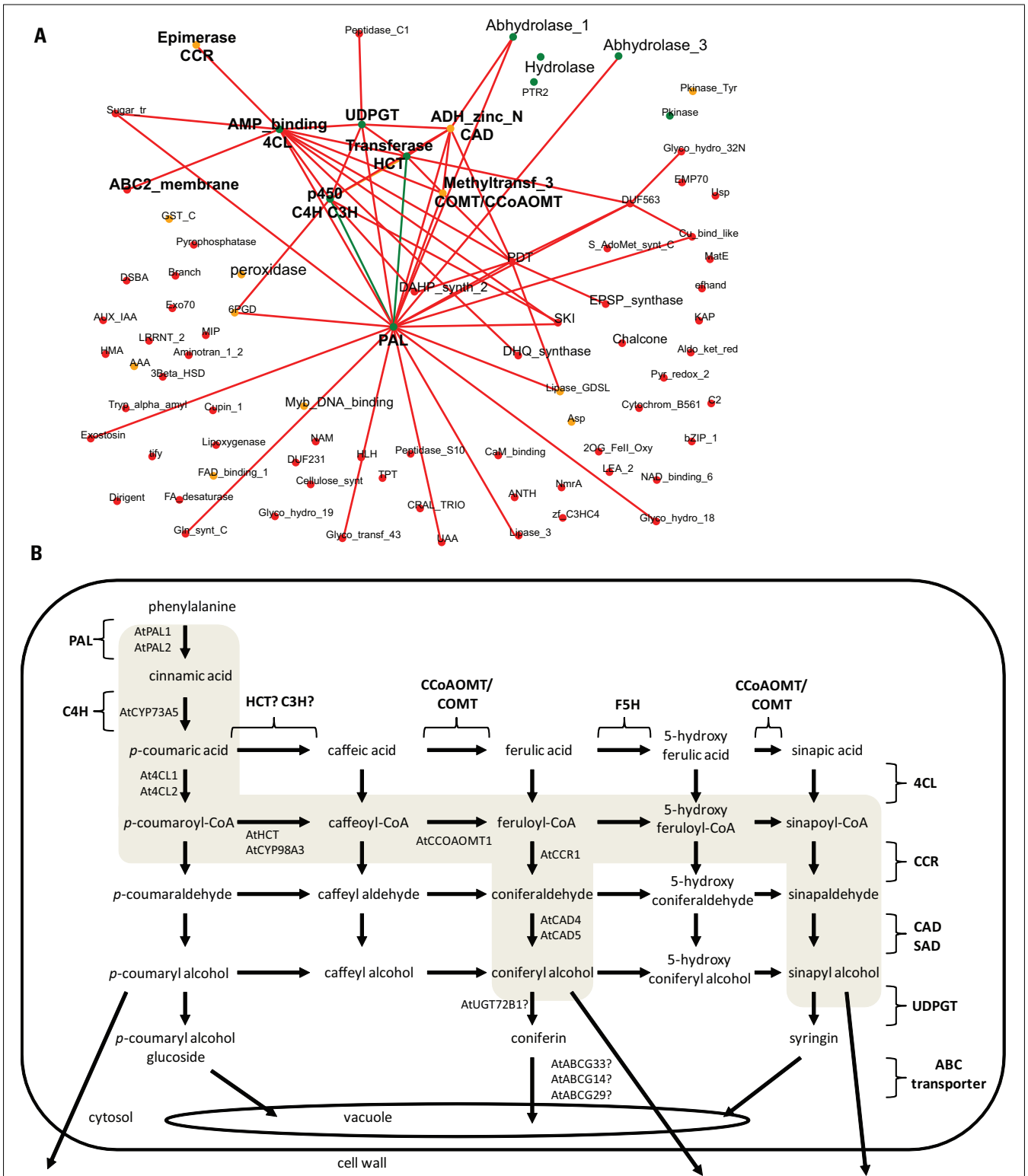


FIGURE 2 | Co-expression of genes involved in lignin formation across species. (A) Consensus co-expression network of lignin formation across seven plant species obtained from the NetworkComparer-tool from PlaNet (http://aranet.mpimp-golm.mpg.de/aranet/NetworkComparer). Co-expression network of following genes/probeset IDs were used: *Arabidopsis*, At2g37040; rice, LOC_Os02g41650; Medicago, Medtr1g076720; barley, Contig1805_s_at; poplar, PtpAffx.1672.3.A1_a_at; wheat, TaAffx.45277.1.S1_x_at. **(B)** Monolignol biosynthesis pathway (adopted from Boerjan et al., 2003). *Arabidopsis* genes present in the co-expression network of *AtPAL1* were mapped on the pathway. Question marks indicate that the function of the respective genes has not been shown yet. The main routes for monolignol synthesis in dicots are highlighted in gray.

However, many genes that are transcriptionally associated with secondary cell wall formation have already been investigated, and mutant analyses targeting only one gene are likely to only yield mild or no phenotypes (Jensen et al., 2010; Brown et al., 2011; Ruprecht et al., 2011). One likely reason for this is genetic redundancy, and hence, mutant combinations or knock-down approaches that target several homologous genes might be needed in the future to generate informative phenotypes. In addition, detailed comparative transcriptional studies across and within species might be needed to obtain more reliable candidate genes related to cell wall synthesis.

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REFERENCES

Anders, N., Wilkinson, M. D., Lovegrove, A., Freeman, J., Tryfona, T., Pellny, T. K., Weimar, T., Mortimer, J. C., Stott, K., Baker, J. M., Defoin-Platel, M., Shewry, P. R., Dupree, P., and Mitchell, R. A. C. (2012). Glycosyl transferases in family 61 mediate arabinofuranosyl transfer onto xylan in grasses. *Proc. Natl. Acad. Sci. U.S.A.* 109, 989–993.

Aoki, K., Ogata, Y., and Shibata, D. (2007). Approaches for extracting practical information from gene co-expression networks in plant biology. *Plant Cell Physiol.* 48, 381–390.

Bassel, G. W., Lan, H., Glaab, E., Gibbs, D. J., Gerjets, T., Krasnogor, N., Bonner, A. J., Holdsworth, M. J., and Provart, N. J. (2011). Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9709–9714.

Bergmann, S., Ihmels, J., and Barkai, N. (2004). Similarities and differences in genome-wide expression data of six organisms. *PLoS Biol.* 2, e9. doi: 10.1371/journal.pbio.0020009

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54, 519–546.

Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M., and Persson, S. (2012). POM-POM2/CELLULOSE SYNTHASE INTERACTING1 Is essential for the functional association of cellulose synthase and microtubules in *Arabidopsis*. *Plant Cell* 24, 163–177.

Brown, D., Wightman, R., Zhang, Z., Gomez, L. D., Atanassov, I., Bukowski, J., Tryfona, T., McQueen-Mason, S. J., Dupree, P., and Turner, S. (2011). *Arabidopsis* genes IRREGULAR XYLEM (IRX15) and IRX15L encode DUF579-containing proteins that are essential for normal xylan deposition in the secondary cell wall. *Plant J.* 66, 401–413.

Brown, D. M., Zeef, L. A. H., Ellis, J., Goodacre, R., and Turner, S. R. (2005). Identification of novel genes in *Arabidopsis* involved in secondary cell wall formation using expression profiling and reverse genetics. *Plant Cell* 17, 2281–2295.

Cocuron, J., Lerouxel, O., Drakakaki, G., Alonso, A. P., Liepman, A. H., Keegstra, K., Raikhel, N., and Wilkerson, C. G. (2007). A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8550–8555.

Davletova, S., Rizhsky, L., Liang, H., Shengqiang, Z., Oliver, D. J., Coutu, J., Shulaev, V., Schlauch, K., and Mittler, R. (2005). Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. *Plant Cell* 17, 268–281.

De Bodt, S., Carvajal, D., Hollunder, J., Van den Cruyce, J., Movahedi, S., and Inzé, D. (2010). CORNET: a user-friendly tool for data mining and integration. *Plant Physiol.* 152, 1167–1179.

Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochylova, Z., Parcy, F., Höfte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15572–15577.

Ehlting, J., Mattheus, N., Aeschliman, D. S., Li, E., Hamberger, B., Cullis, I. F., Zhuang, J., Kaneda, M., Mansfield, S. D., Samuels, L., Ritland, K., Ellis, B. E., Bohlmann, J., and Douglas, C. J. (2005). Global transcript profiling of primary stems from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. *Plant J.* 42, 618–640.

Faik, A., Price, N. J., Raikhel, N. V., and Keegstra, K. (2002). An *Arabidopsis* gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7797–7802.

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., Gavin, O. L., Gunasekaran, P., Ceric, G., Forslund, K., Holm, L., Sonnhammer, E. L. L., Eddy, S. R., and Bateman, A. (2010). The Pfam protein families database. *Nucleic Acids Res.* 38, D211–D222.

Gardiner, J. C., Taylor, N. G., and Turner, S. R. (2003). Control of cellulose synthase complex localization in developing xylem. *Plant Cell* 15, 1740–1748.

Gu, Y., Kaplinsky, N., Bringmann, M., Cobb, A., Carroll, A., Sampathkumar, A., Baskin, T. I., Persson, S., and Somerville, C. R. (2010). Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12866–12871.

Hirai, M. Y., Sugiyama, K., Sawada, Y., Tohge, T., Obayashi, T., Suzuki, A., Araki, R., Sakurai, N., Suzuki, H., Aoki, K., Goda, H., Nishizawa, O. I., Shibata, D., and Saito, K. (2007). Omics-based identification of *Arabidopsis* Myb transcription factors regulating aliphatic glucosinolate biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6478–6483.

Hirayama, T., Kieber, J. J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J. M., Dailey, W. P., Dancis, A., and Ecker, J. R. (1999). RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. *Cell* 97, 383–393.

Ihmels, J., Levy, R., and Barkai, N. (2004). Principles of transcriptional control in the metabolic network of *Saccharomyces cerevisiae*. *Nat. Biotechnol.* 22, 86–92.

Jensen, J. K., Kim, H., Cocuron, J., Orler, R., Ralph, J., and Wilkerson, C. G. (2010). The DUF579 domain containing proteins IRX15 and IRX15-L affect xylan synthesis in *Arabidopsis*. *Plant J.* 66, 387–400.

Jupiter, D., Chen, H., and VanBuren, V. (2009). STARNET 2: a web-based tool for accelerating discovery of gene regulatory networks using microarray co-expression data. *BMC Bioinformatics* 10, 332. doi: 10.1186/1471-2105-10-332

Kaneda, M., Schuetz, M., Lin, B. S. P., Chanis, C., Hamberger, B., Western, T. L., Ehlting, J., and Samuels, A. L. (2011). ABC transporters coordinately expressed during lignification of *Arabidopsis* stems include a set of ABCBs associated with auxin transport. *J. Exp. Bot.* 62, 2063–2077.

Lee, H. K., Hsu, A. K., Sajdak, J., Qin, J., and Pavlidis, P. (2004). Coexpression analysis of human genes across many microarray data sets. *Genome Res.* 14, 1085–1094.

Lee, I., Ambaru, B., Thakkar, P., Marcotte, E. M., and Rhee, S. Y. (2010). Rational association of genes with traits using a genome-scale gene network for *Arabidopsis thaliana*. *Nat. Biotechnol.* 28, 149–156.

Lee, T., Kim, Y., Pham, T. T. M., Song, S. I., Kim, J., Kang, K. Y., An, G., Jung, K., Galbraith, D. W., Kim, M., Yoon, U., and Nahm, B. H. (2009). RiceArrayNet: a database for correlating gene expression from transcriptome profiling, and its application to the analysis of coexpressed genes in rice. *Plant Physiol.* 151, 16–33.

Lim, E. K., Li, Y., Parr, A., Jackson, R., Ashford, D. A., and Bowles, D. J. (2001). Identification of glucosyltransferase genes involved in sinapate metabolism and lignin synthesis in *Arabidopsis*. *J. Biol. Chem.* 276, 4344–4349.

Manfield, I. W., Jen, C., Pinney, J. W., Michalopoulos, I., Bradford, J. R., Gilmartin, P. M., and Westhead, D. R. (2006). *Arabidopsis* Co-expression Tool (ACT): web server tools for microarray-based gene expression analysis. *Nucleic Acids Res.* 34, W504–W509.

Mao, L., Van Hemert, J. L., Dash, S., and Dickerson, J. A. (2009). *Arabidopsis* gene co-expression network and its functional modules. *BMC Bioinformatics* 10, 346. doi: 10.1186/1471-2105-10-346

Miao, Y., and Liu, C. (2010). ATP-binding cassette-like transporters are involved in the transport of lignin precursors across plasma and vacuolar membranes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22728–22733.

Mitchell, R. A. C., Dupree, P., and Shewry, P. R. (2007). A novel bioinformatics approach identifies candidate genes for the synthesis and feruloylation of arabinoxylan. *Plant Physiol.* 144, 43–53.

Mutwil, M., Klie, S., Tohge, T., Giorgi, F. M., Wilkins, O., Campbell, M. M., Fernie, A. R., Usadel, B., Nikoloski, Z., and Persson, S. (2011). PlaNet: combined sequence and expression comparisons across plant networks derived from seven species. *Plant Cell* 23, 895–910.

Mutwil, M., Obro, J., Willats, W. G. T., and Persson, S. (2008). GeneCAT – novel webtools that combine BLAST and co-expression analyses. *Nucleic Acids Res.* 36, W320–W326.

Mutwil, M., Usadel, B., Schütte, M., Loraine, A., Ebenhöh, O., and Persson, S. (2010). Assembly of an interactive correlation network for the *Arabidopsis* genome using a novel heuristic clustering algorithm. *Plant Physiol.* 152, 29–43.

Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H., and Höfte, H. (1998). A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J.* 17, 5563–5576.

Obayashi, T., Hayashi, S., Saeki, M., Ohta, H., and Kinoshita, K. (2009). ATTED-II provides coexpressed gene networks for *Arabidopsis*. *Nucleic Acids Res.* 37, D987–D991.

Obayashi, T., and Kinoshita, K. (2009). Rank of correlation coefficient as a comparable measure for biological significance of gene coexpression. *DNA Res.* 16, 249–260.

Obayashi, T., Nishida, K., Kasahara, K., and Kinoshita, K. (2011). ATTED-II updates: condition-specific gene coexpression to extend coexpression analyses and applications to a broad range of flowering plants. *Plant Cell Physiol.* 52, 213–219.

Ogata, Y., Sakurai, N., Suzuki, H., Aoki, K., Saito, K., and Shibata, D. (2009). The prediction of local modular structures in a co-expression network based on gene expression datasets. *Genome Inform.* 23, 117–127.

Oikawa, A., Joshi, H. J., Rennie, E. A., Ebert, B., Manisseri, C., Heazlewood, J. L., and Scheller, H. V. (2010). An integrative approach to the identification of *Arabidopsis* and rice genes involved in xylan and secondary wall development. *PLoS ONE* 5, e15481. doi: 10.1371/journal.pone.0015481

Oldham, M. C., Horvath, S., and Geschwind, D. H. (2006). Conservation and evolution of gene coexpression networks in human and chimpanzee brains. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17973–17978.

Peña, M. J., Zhong, R., Zhou, G., Richardson, E. A., O'Neill, M. A., Darvill, A. G., York, W. S., and Ye, Z. (2007). *Arabidopsis* irregular xylem8 and irregular xylem9: implications for the complexity of glucuronoxylan biosynthesis. *Plant Cell* 19, 549–563.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C. R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15566–15571.

Persson, S., Wei, H., Milne, J., Page, G. P., and Somerville, C. R. (2005). Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8633–8638.

Rawat, A., Seifert, G. J., and Deng, Y. (2008). Novel implementation of conditional co-regulation by graph theory to derive co-expressed genes from microarray data. *BMC Bioinformatics* 9(Suppl. 9), S7. doi: 10.1186/1471-2105-9-S9-S7

Roudier, F., Fernandez, A. G., Fujita, M., Himmelspach, R., Borner, G. H. H., Schindelman, G., Song, S., Baskin, T. I., Dupree, P., Wasteneys, G. O., and Benfey, P. N. (2005). COBRA, an *Arabidopsis* extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. *Plant Cell* 17, 1749–1763.

Ruan, J., Dean, A. K., and Zhang, W. (2010). A general co-expression network-based approach to gene expression analysis: comparison and applications. *BMC Syst. Biol.* 4, 8. doi: 10.1186/1752-0509-4-8

Ruprecht, C., Mutwil, M., Saxe, F., Eder, M., Nikoloski, Z., and Persson, S. (2011). Large-scale co-expression approach to dissect secondary cell wall formation across plant species. *Front. Plant Sci.* 2:23. doi: 10.3389/fpls.2011.00023

Srinivasasainagendra, V., Page, G. P., Mehta, T., Coulibaly, I., and Loraine, A. E. (2008). CressExpress: a tool for large-scale mining of expression data from *Arabidopsis*. *Plant Physiol.* 147, 1004–1016.

Steinhauser, D., Usadel, B., Luedemann, A., Thimm, O., and Kopka, J. (2004). CSB.DB: a comprehensive systems-biology database. *Bioinformatics* 20, 3647–3651.

Stuart, J. M., Segal, E., Koller, D., and Kim, S. K. (2003). A gene-coexpression network for global discovery of conserved genetic modules. *Science* 302, 249–255.

Tohge, T., and Fernie, A. R. (2010). Combining genetic diversity, informatics and metabolomics to facilitate annotation of plant gene function. *Nat. Protoc.* 5, 1210–1227.

Usadel, B., Obayashi, T., Mutwil, M., Giorgi, F. M., Bassel, G. W., Tanimoto, M., Chow, A., Steinhauser, D., Persson, S., and Provart, N. J. (2009). Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. *Plant Cell Environ.* 32, 1633–1651.

Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., Franz, M., Grouios, C., Kazi, F., Lopes, C. T., Maitland, A., Mostafavi, S., Montojo, J., Shao, Q., Wright, G., Bader, G. D., and Morris, Q. (2010). The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res.* 38, W214–W220.

Wei, H., Persson, S., Mehta, T., Srinivasasainagendra, V., Chen, L., Page, G. P., Somerville, C., and Loraine, A. (2006). Transcriptional coordination of the metabolic network in *Arabidopsis*. *Plant Physiol.* 142, 762–774.

Wille, A., Zimmermann, P., Vranová, E., Fürholz, A., Laule, O., Bleuler, S., Hennig, L., Prelic, A., von Rohr, P., Thiele, L., Zitzler, E., Gruissem, W., and Bühlmann, P. (2004). Sparse graphical Gaussian modeling of the isoprenoid gene network in *Arabidopsis thaliana*. *Genome Biol.* 5, R92.

Zhong, R., Kays, S. J., Schroeder, B. P., and Ye, Z. (2002). Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *Plant Cell* 14, 165–179.

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Deciphering the molecular functions of sterols in cellulose biosynthesis

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Sterols play vital roles in plant growth and development, as components of membranes and as precursors to steroid hormones. Analysis of *Arabidopsis* mutants indicates that sterol composition is crucial for cellulose biosynthesis. Sterols are widespread in the plasma membrane (PM), suggesting a possible link between sterols and the multimeric cellulose synthase complex. In one possible scenario, molecular interactions in sterol-rich PM microdomains or another form of sterol-dependent membrane scaffolding may be critical for maintaining the correct subcellular localization, structural integrity and/or activity of the cellulose synthase machinery. Another possible link may be through steryl glucosides, which could act as primers for the attachment of glucose monomers during the synthesis of β−(1 → 4) glucan chains that form the cellulose microfibrils. This mini-review examines genetic and biochemical data supporting the link between sterols and cellulose biosynthesis in cell wall formation and explores potential approaches to elucidate the mechanism of this association.

Keywords: sterols, steryl glucosides, cellulose, plasma membrane microdomains, lipid rafts, cell wall

INTRODUCTION

In plants, cellulose functions as a major component of the cell wall to provide mechanical support and structural integrity to various tissues. Cellulose consists of glucose molecules arranged in parallel hydrogen-bonded β-(1 → 4) glucan chains that form microfibrils of 2–4 nm in diameter and up to several micrometer in length depending on their origin (Mutwil et al., 2008; Fernandes et al., 2011). Cellulose microfibrils function in scaffolding other cell wall polymers such as hemicelluloses and pectins.

Cellulose is synthesized at the plasma membrane (PM) by terminal complexes organized as six-lobed rosettes in higher plants (Kimura et al., 1999). Current models suggest that the rosettes are organized as tetramers (Endler and Persson, 2011) or hexamers (Fernandes et al., 2011) consisting of multiple cellulose synthase catalytic subunits (CESA) arranged with a sixfold symmetry. Each CESA subunit has a predicted topology of eight transmembrane helices for anchorage in the PM (Delmer, 1999). Although much progress has been made in the identification of proteins involved in cellulose formation, our understanding of the biosynthetic process is far from complete (Guerriero et al., 2010). One major difficulty is to determine the precise composition of the cellulose synthase complex (CSC) and to solve its structure. This has proven extremely challenging using biochemical approaches because of the instability of the complex, and molecular genetics have provided only partial and essentially indirect answers to some of the most fundamental questions related to cellulose formation.

Several pieces of evidence suggest that the lipid environment of the CSC is crucial for its proper structural organization and function at the PM. In metazoans, sterols act as components of membranes, molecular ligands, or as precursors of steroid hormones. However, in plants, much less is known about the roles of sterols. They predominantly occur in the PM (Grebe et al., 2003) and are abundantly synthesized during the early stages of seed development, coincident with intense cell division and expansion (Schrick et al., 2011). Below we describe the genetic and biochemical evidence that support a link between sterols and cellulose biosynthesis, and we discuss experimental approaches to decipher the molecular mechanisms that underlie this connection.

THE ROLES OF STEROLS IN HIGHER PLANTS

Compared to vertebrates, in which mainly cholesterol synthesis occurs, higher plants synthesize a complex mixture of sterols, commonly referred to as phytosterols (Benveniste, 2004). In *Arabidopsis* seedlings, sitosterol is the major sterol, followed by campesterol, stigmasterol, and over 20 minor sterols, many of which are biosynthetic intermediates (Schrick et al., 2000, 2002). Sitosterol and stigmasterol play major roles as PM components and are critical for membrane fluidity and permeability (Schuler et al., 1991; Grandmougin-Ferjani et al., 1997). Campesterol is a precursor of the brassinosteroids which stimulate stem elongation and cell division, and are the only plant steroids known to act as hormones (Clouse, 2011).

The first genetic evidence for the role of sterols in plant growth and development came from the identification of three *Arabidopsis* sterol biosynthesis mutants: *fackel* (*fk*; Jang et al., 2000; Schrick et al., 2000), *cephalopod/sterol methyl transferase 1* (*cph/smt1*; Diener et al., 2000; Schrick et al., 2002), and *hydra1* (*hyd1*; Schrick et al., 2002; Souter et al., 2002). These mutants exhibit cell division and expansion defects, as well as embryonic patterning defects, and correspond to sterol C-14 reductase,

C-24 sterol methyltransferase, and sterol C-8,7 isomerase, respectively. The discovery that all three affect enzymes upstream of brassinosteroid DWARF (DWF) steps, coupled with the inability to rescue the mutants by brassinosteroids, led to the hypothesis that sterols participate in novel signaling pathways (Lindsey et al., 2003). Although such pathways have not been revealed to date, this idea is further supported by the characterization of *smt2/cvp1* and *smt3* double mutants of C-24 sterol methyltransferases that exhibit homeotic floral transformations and other developmental defects (Carland et al., 2010).

IMPACT OF MUTATIONS IN STEROL BIOSYNTHETIC ENZYMES ON CELLULOSE FORMATION

In addition to embryonic defects, *fk*, *cph/smt1*, and *hyd1* mutants display a striking deficiency in cellulose content, a phenotype that can be mimicked by the sterol biosynthesis inhibitors fenpropimorph and 15-azasterol (Schrick et al., 2004). As is typical for cellulose deficiency, the mutants exhibit characteristic symptoms such as cell wall gaps, multiple nuclei, and aberrant cell wall thickenings with ectopic deposition of callose and lignin. Other cell wall components such as pectins are not reduced, arguing against a general defect in cell wall biogenesis. The decrease in cellulose content in the *fk*, *cph/smt1*, and *hyd1* mutants is comparable to that observed in several cellulose biosynthesis mutants (**Table 1**). In contrast, brassinosteroid mutants exhibit mild or no cellulose deficiency (**Table 1**) although CESA genes are transcriptionally up-regulated by brassinolides (Xie et al., 2011). It is intriguing that *dwf1/dim* mutants which display normal cellulose content are deficient in campesterol, the precursor to brassinosteroids, while abnormally accumulating 24-methylenecholesterol, a membrane sterol (Klahre et al., 1998; Choe et al., 1999). Recently, a tomato mutant with a hyper-cracking fruit phenotype coupled with pericarp cell division and expansion defects and reduced cellulose levels was shown to correspond to a 3-β-hydroxysteroid dehydrogenase/C-4 decarboxylase (3-betaHSD/D) (Jocelyn Rose, personal communication). A multienzyme complex containing 3-betaHSD/D is required for removal of two methyl groups at C-4, rendering sterols functional as membrane constituents (Rahier et al., 2006). Taken together, these observations suggest that membrane sterols, and not brassinosteroids, are critical for cellulose accumulation.

It is striking that sterol composition differs considerably between the *fk*, *cph/smt1*, and *hyd1* mutants despite their similar cellulose deficiencies. In particular, *fk* mutants accumulate $\Delta^{8,14}$ sterols and exhibit a reduction in both sitosterol and campesterol (Schrick et al., 2000), while *hyd1* mutants are similarly reduced in these sterols, but they accumulate stigmasta-monoen-3β-ol (Schrick et al., 2002). In contrast, *cph/smt1* mutants abnormally accumulate both cycloartenol and cholesterol, and are reduced in sitosterol but not in campesterol (Schrick et al., 2002). Consistent with the possibility that accumulation of abnormal sterols, such as biosynthetic intermediates, contributes to cellulose deficiency, *fk* mutants are not rescued by exogenous application of sterol end-products (Schrick et al., 2000). Even subtle changes in membrane sterol composition may disrupt the functional requirements for cellulose biosynthesis, since simple structural variations in the acyl chain alter membrane protein function *in vitro* (Litman and Mitchell, 1996). In mouse, the accumulation of cholesterol precursors results in embryonic defects although the cholesterol content is normal, suggesting that the build-up of precursors interferes with sterol function (Engelking et al., 2006). In this system, the drug lovastatin, which inhibits an early step leading to sterol biosynthesis (HMG-CoA reductase), was used to effectively block the accumulation of sterol intermediates and restore function. Therefore, it may be informative to prevent the accumulation of abnormal sterols in *fk*, *hyd1*, or *cph/smt1* by application of lovastatin, and then to observe whether the cellulose defects can be complemented by application of sterol end-products.

OTHER EFFECTS OF STEROL BIOSYNTHESIS MUTATIONS IN PLANTS

In addition to effects on cellulose synthesis, abnormal sterol composition is associated with other physiological and cellular processes related to membranes, such as plastid biogenesis

Table 1 | Summary of cellulose contents for cellulose and steroid mutants of *Arabidopsis*.

Mutant	Description of corresponding protein and/or predicted function	Tissue analyzed	% Cellulose reduction from WT	Reference
rsw1	CESA1 (temperature sensitive allele)	Shoot of seedling	56 (5)	Arioli et al. (1998)
rsw1-2	CESA1 (strong allele)	Embryo	77 (1)	Gillmor et al. (2002)
asw1	KORRIGAN (temperature sensitive allele), β-(1 → 4) glucanase	Shoot	60 (3)	Sato et al. (2001)
kob1	KOBITO1, plasma membrane protein	Seedling	37 (6)	Pagant et al. (2002)
cob	COBRA, glycophosphatidylinositol (GPI)-anchored protein	Root of seedling	33 (6)	Schindelman et al. (2001)
fk	FACKEL, sterol C-14 reductase	Seedling	47 (11)	Schrick et al. (2004)
hyd1	HYDRA1, sterol C-8,7 isomerase	Seedling	38 (11)	Schrick et al. (2004)
cph/smt1	CEPHALOPOD/SMT1, C-24 sterol methyl transferase	Seedling	28 (6)	Schrick et al. (2004)
dwf1	DWARF1, sterol C-24 reductase	Seedling	0 (6)	Schrick et al. (2004)
bri1	BRI1, brassinolide receptor	Stem	8 (2)	Xie et al. (2011)
det2	DEETIOLATED2, steroid 5α-reductase	Stem	12 (1)	Xie et al. (2011)

Average percent cellulose reduction from a wild-type (WT) control is indicated with SD in parentheses. While sterol biosynthesis mutants (fk, hyd1, and cph/smt1) exhibit cellulose deficiencies that are similar to other characterized cellulose mutants (rsw1, asw1, kob1, and cob) brassinosteroid mutants (dwf1, bri1, and det2) exhibit mild or no cellulose deficiency.

(Babiychuk et al., 2008) and the regulation of reactive oxygen species (Pose et al., 2009). Sterol biosynthesis takes place at the endoplasmic reticulum (Benveniste, 2004). At steady state, sterols are found in the Golgi membranes and endocytic compartments, although they accumulate mostly at the PM (Grebe et al., 2003). Several studies incorporating sterol biosynthesis mutants have shown that sterols play critical roles in cell polarity through their requirement for clathrin-mediated endocytosis of PIN proteins, which are presumptive auxin efflux carriers (Boutte and Grebe, 2009). The molecular mechanism is not clear nor is it known how specific this effect is, since the cytokinesis-specific syntaxin KNOLLE was shown to also require sterol-dependent endocytosis for proper localization (Boutte et al., 2010). More recent work suggests that isoprenoids and sterols are crucial for microRNA processing in providing the correct membrane composition for ARGONAUTE1 membrane association and function (Brodersen et al., 2012). Collectively, these findings indicate that sterol biosynthesis mutations can perturb membrane structure and protein trafficking.

STEROL-RICH PM MICRODOMAINS MAY BE CRITICAL FOR MAINTAINING THE STRUCTURAL INTEGRITY AND ACTIVITY OF CELLULOSE SYNTHASE

As for many integral membrane complexes, biochemical analysis of cellulose synthase has proven to be a major challenge. The enzyme complex is highly unstable, and PM extractions typically result in loss of cellulose synthase activity (Delmer, 1999; Bessueille and Bulone, 2008). *In vitro* synthesis of cellulose from plant cell-free extracts is sensitive to detergents that facilitate the isolation of intact complexes (Lai-Kee-Him et al., 2002; Colombani et al., 2004; Cifuentes et al., 2010), suggesting that cellulose synthase requires a specific lipid environment. Consistent with this idea, its activity has been identified in detergent-resistant membranes (DRMs) that exhibit biochemical properties similar to sterol-rich PM microdomains (Bessueille et al., 2009).

In the past 15 years, a model for the organization of the PM has emerged in which sterol and sphingolipid rich microdomains also known as lipid rafts coexist with more fluid domains containing phospholipids and unsaturated hydrocarbon chains (Simons and Ikonen, 1997). Lipid rafts, to which specific classes of proteins are associated, play a role in many biological processes, including cell polarity, protein trafficking, and signal transduction. While lipid rafts are defined by their composition and distribution *in vivo*, DRMs are biochemically characterized by their insolubility in the non-ionic detergent Triton X-100 at 4°C (Mongrand et al., 2004; Borner et al., 2005). The relationship between DRMs and lipid rafts is debated since the experimental conditions used for DRM isolation may artificially induce their formation (Lichtenberg et al., 2005; Tanner et al., 2011). Despite this consideration, extractions of DRMs could reflect differential affinities of resident membrane proteins to various lipid environments.

Plant lipid rafts are thought to exhibit unique structural features, owing to the greater molecular diversity of plant sterols and sphingolipids compared to animal or yeast counterparts. Plant-derived DRMs contain multiple sterol molecules such as 24-methylcholesterol, sitosterol, and stigmasterol (Mongrand et al., 2004), instead of primarily cholesterol as in mammals, and two distinct classes of sphingolipids, inositol phosphorylceramides, and glucosylceramides (Markham et al., 2006). Methylcyclodextrin, a chaotropic agent that extracts sterols from membranes, can serve as a tool to purify DRM sub-populations that rely on sterol function (Kierszniowska et al., 2009). In oomycetes, β-(1 → 3)-glucan synthase is released from DRMs by methylcyclodextrin, while chitin synthase is not (Briolay et al., 2009). Analogously, methylcyclodextrin might be applied to study the dependency of cellulose synthase activity on sterol-rich membrane environments in DRM preparations.

It is intriguing that lipid rafts and the CSC are formed in the same compartment before they emerge at the PM. In yeast and mammalian cells, it was shown that sterols and sphingolipids are enriched at the trans-Golgi network from where they are sorted via Golgi-derived vesicles to the PM (Klemm et al., 2009). Although rosettes have been visualized in the Golgi apparatus (Haigler and Brown, 1986), they appear to be non-functional in this compartment. Rosettes could assemble on the Golgi stacks followed by transport to the PM on Golgi-derived vesicles.

STEROLS MIGHT PROVIDE DIRECT STRUCTURAL SCAFFOLDING FOR CELLULOSE SYNTHASE

Alternatively or in addition to their function in lipid rafts, sterols may interact directly with cellulose synthase by providing a scaffold to assist in proper structural conformation of the enzyme or to stabilize the complex (**Figure 1**). According to the original fluid-mosaic model (Singer and Nicolson, 1972), a small fraction of lipids may interact specifically with membrane proteins. It was recently shown that specific structural lipids are bound to the intact integral membrane complex ATPase within the membrane rotors (Zhou et al., 2011). Sterols could similarly directly bind the CESA subunits and/or other proteins associated with the CSC, thus enabling and/or stabilizing the function of the complex.

PROBING THE FUNCTION OF STEROLS IN CELLULOSE BIOSYNTHESIS

The cellulose-deficient sterol biosynthesis mutants and/or pharmacological inhibitors may serve as tools to investigate the role of sterols and plasma PM microdomains in subcellular targeting, structural integrity and/or activity of the CSC. In leek seedlings treated with the sterol biosynthesis inhibitor fenpropimorph, recovery of DRMs from the Golgi, but not from the PM, has been reported (Laloi et al., 2007). Moreover, raft protein contents are altered in mammalian cells by the sterol biosynthesis inhibitor AY9944 (Keller et al., 2004). These observations suggest that sterol composition is critical for the transport and organization of membrane microdomains.

Live imaging of fluorescently tagged CESA subunits in sterol biosynthesis mutants may be used to determine whether normal sterol composition is needed for correct localization of the CSC. In wild-type *Arabidopsis*, subcellular localization of CESA6 and CESA3 subunits reveals their presence at the Golgi and in the PM (Paradez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009). In sterol biosynthesis mutants, this localization might be shifted toward the Golgi if the translocation of DRMs to the PM is altered. If it is found that the CSC is properly targeted to the PM, the next step will be to investigate whether the DRMs and/or lipid

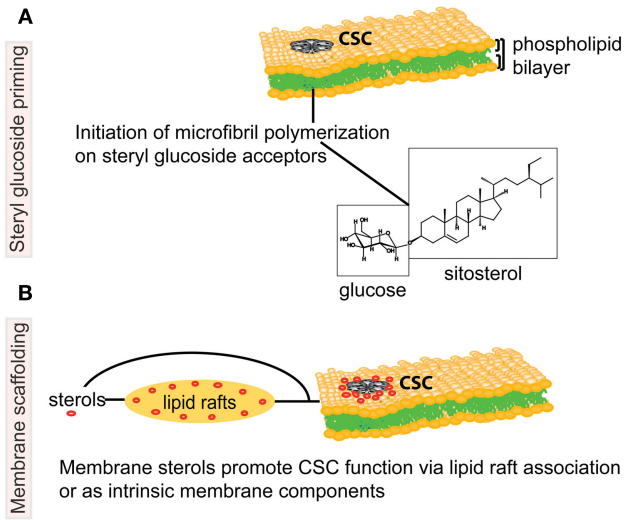


FIGURE 1 | Schematic representation of potential associations between sterols and the cellulose biosynthetic machinery in higher plants. The cellulose synthase complex (CSC) is embedded in the phospholipid bilayer of the plasma membrane (PM), and catalyzes production of a cellulose microfibril, a major constituent of the plant cell wall. In the upper model **(A)**, steryl glucosides (SGs) comprised of sitosterol (molecule shown in boxes) prime the synthesis of glucan chains as proposed by Peng et al. (2002). The lower model **(B)** depicts molecular interactions whereby sterols (in red) are necessary for the correct targeting, structural integrity and/or catalytic activity of the CSC. The proposed molecular interactions between sterols and the CSC may occur in PM microdomains, also known as lipid rafts, or via another form of molecular scaffolding. Note that the proposed models are not mutually exclusive. Figure is not drawn to scale.

rafts of sterol biosynthesis mutants lack cellulose synthase activity. Such experiments could resolve the possible mechanism by which sterols and/or sterol-rich PM microdomains or another type of sterol-dependent membrane scaffolding affects CSC function.

THE ROLES OF STEROL GLYCOSIDES IN PLANT CELLS

In probing the potential functions of sterols in cellulose biosynthesis, the role of conjugated sterols should not be overlooked. An abundant portion of sterols in plant membranes form steryl glycoside (SG) conjugates and the most commonly observed SG contains glucose as a sugar moiety (Grille et al., 2010). It is not clear why some sterols are glucosylated *in vivo* while others remain as free sterols, except that glucosylation requires end-product sterols and does not occur on intermediates. SGs have been found to be prevalent in DRM preparations from *Arabidopsis* and leek, suggesting their presence in lipid rafts (Laloi et al., 2007).

Steryl glycosides are synthesized by UDP-glucose:sterol glucosyltransferases (UGTs) that catalyze the glycosylation of the C3-hydroxyl of the sterol (Warnecke et al., 1997, 1999). In *Arabidopsis*, two genes code for the related UGT80 enzymes, UGT80A2 and UGT80B1 (DeBolt et al., 2009). Consistent with their predicted UGT functions, *ugt80A2,B1* T-DNA insertion double mutants exhibit a significant reduction in SGs in various plant tissues examined (DeBolt et al., 2009) including seeds (Schrick et al., 2012). While *ugt80A2* mutants display only minor effects on growth, *ugt80B1* mutants exhibit an array of phenotypes in the seed, such as transparent testa, defects in flavonoid deposition, loss of the cuticle, and a decrease in aliphatic suberin and cutin-like polymers (DeBolt et al., 2009). The findings suggest a role for SGs in trafficking lipid polyester precursors in seeds.

A PROPOSED FUNCTION FOR SITOSTERYL GLUCOSIDE IN CELLULOSE BIOSYNTHESIS

Sitosteryl glucoside (SSG) has been proposed to act as a primer for cellulose biosynthesis based on the evidence that SSGs can be used by cellulose synthase as a glucose acceptor *in vitro* (**Figure 1**; Peng et al., 2002). Using crude membranes from cotton fibers, radioactive sterol cellodextrins were synthesized upon incubation of sitosterol-[^{14}C]glucoside and non-radioactive UDP-glucose. Membranes from yeast heterologously expressing a cotton CESA subunit were shown to catalyze the same reaction. These data provide evidence that SSGs can be used by cellulose synthase as an acceptor *in vitro*, but whether SSGs are primers for cellulose biosynthesis *in vivo* remains an open question. The idea that in higher plants polymerization of cellulose chains may be initiated on lipid acceptors by cellulose synthase is consistent with models proposed for bacterial systems in which polyisoprenes and their phosphorylated forms are acceptor molecules for a transglycosylase enzyme (Matthysse et al., 1995).

To test the hypothesis that SGs play a critical role in cellulose biosynthesis *in vivo*, cellulose content was examined in the *Arabidopsis ugt80* mutants (DeBolt et al., 2009). Although the *ugt80A2,B1* double mutants exhibit a slow growth phenotype coupled with elongation defects in embryos, no cellulose deficiency was observed (DeBolt et al., 2009). It is possible that the low levels of SGs in the *ugt80A2,B1* mutants can fulfill a role in priming cellulose biosynthesis. In *ugt80A2,B1*, residual SG levels indicate that additional plant enzymes are able to catalyze steryl glucosylation. One candidate for this function is glucosylceramide synthase (GCS), encoded by At2g19880 in *Arabidopsis*, which shares a low level of sequence similarity with UGT80 enzymes. A predicted GCS enzyme from cotton was shown to produce SGs in yeast (Hillig et al., 2003), consistent with the possibility that it catalyzes steryl glucosylation *in planta*. BLAST searches with UGT80 protein sequences reveal another candidate enzyme encoded by At5g24750, which has thus far not been characterized. Investigations of mutants corresponding to these and other candidate enzymes are expected to reveal the putative functions of SGs in cellulose biosynthesis.

CONCLUDING REMARKS

Several studies point toward an involvement of sterols in cellulose biosynthesis of higher plants, but investigations on this specific fundamental question are still at their infancy. In particular, the mode of interaction of sterols with the cellulose synthase machinery, either direct or indirect, remains to be determined (**Figure 1**). The in-depth analysis of cellulose formation in mutants affected in sterol biosynthesis represents a promising strategy to address this basic question. Multiple experimental approaches need to be developed to (i) determine the impact of mutations in sterol biosynthesis enzymes, or other manipulations of sterol content,

on the catalytic activity of cellulose synthase, both *in vitro* and *in vivo*; (ii) analyze the impact of these perturbations on cellulose synthase subcellular localization, lipid and protein composition of membrane microdomains, and stability of the cellulose synthase machinery; (iii) quantify the effect of sterol depletion on the structural properties of cellulose microfibrils; and (iv) evaluate the importance of SGs in priming cellulose biosynthesis or in any other step of cellulose formation. This emerging topic of research in the plant cell wall field is poised to advance our mechanistic understanding of cellulose biosynthesis, and may lead to novel insights in the dynamic interplay between sterols and proteins in membranes systems.

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REFERENCES

Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Hofte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998). Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* 279, 717–720.

Babiychuk, E., Bouvier-Nave, P., Compagnon, V., Suzuki, M., Muranaka, T., Van, M. M., Kushnir, S., and Schaller, H. (2008). Allelic mutant series reveal distinct functions for *Arabidopsis* cycloartenol synthase 1 in cell viability and plastid biogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3163–3168.

Benveniste, P. (2004). Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* 55, 429–457.

Bessueille, L., and Bulone, V. (2008). A survey of cellulose biosynthesis in higher plants. *Plant Biotechnol.* 25, 315–322.

Bessueille, L., Sindt, N., Guichardant, M., Djerbi, S., Teeri, T. T., and Bulone, V. (2009). Plasma membrane microdomains from hybrid aspen cells are involved in cell wall polysaccharide biosynthesis. *Biochem. J.* 420, 93–103.

Borner, G. H., Sherrier, D. J., Weimar, T., Michaelson, L. V., Hawkins, N. D., Macaskill, A., Napier, J. A., Beale, M. H., Lilley, K. S., and Dupree, P. (2005). Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. *Plant Physiol.* 137, 104–116.

Boutte, Y., Frescatada-Rosa, M., Men, S., Chow, C. M., Ebine, K., Gustavsson, A., Johansson, L., Ueda, T., Moore, I., Jürgens, G., and Grebe, M. (2010). Endocytosis restricts *Arabidopsis* KNOLLE syntaxin to the cell division plane during late cytokinesis. *EMBO J.* 29, 546–558.

Boutte, Y., and Grebe, M. (2009). Cellular processes relying on sterol function in plants. *Curr. Opin. Plant Biol.* 12, 705–713.

Briolay, A., Bouzenzana, J., Guichardant, M., Deshayes, C., Sindt, N., Bessueille, L., and Bulone, V. (2009). Cell wall polysaccharide synthases are located in detergent-resistant membrane microdomains in oomycetes. *Appl. Environ. Microbiol.* 75, 1938–1949.

Brodersen, P., Sakvarelidze-Achard, L., Schaller, H., Khafif, M., Schott, G., Bendahmane, A., and Voinnet, O. (2012). Isoprenoid biosynthesis is required for miRNA function and affects membrane association of ARGONAUTE 1 in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1778–1783.

Carland, F., Fujioka, S., and Nelson, T. (2010). The sterol methyltransferases SMT1, SMT2, and SMT3 influence *Arabidopsis* development through nonbrassinosteroid products. *Plant Physiol.* 153, 741–756.

Choe, S., Dilkes, B. P., Gregory, B. D., Ross, A. S., Yuan, H., Noguchi, T., Fujioka, S., Takatsuto, S., Tanaka, A., Yoshida, S., Tax, F. E., and Feldmann, K. (1999). The *Arabidopsis* dwarf1 mutant is defective in the conversion of 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis. *Plant Physiol.* 119, 897–907.

Cifuentes, C., Bulone, V., and Emons, A. M. (2010). Biosynthesis of callose and cellulose by detergent extracts of tobacco cell membranes and quantification of the polymers synthesized in vitro. *J. Integr. Plant Biol.* 52, 221–233.

Clouse, S. D. (2011). "Brassinosteroids," in *The Arabidopsis Book 9*, eds N. Winchester and D. McCauley (Rockville, MD: American Society of Plant Biologists), e0151.

Colombani, A., Djerbi, S., Bessueille, L., Blomqvist, K., Ohlsson, A., Berglund, T., Teeri, T. T., and Bulone, V. (2004). In vitro synthesis of (1(3)-beta-D-glucan (callose) and cellulose by detergent extracts of membranes from cell suspension cultures of hybrid aspen. *Cellulose* 11, 313–327.

Crowell, E. F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y. D., Schumacher, K., Gonneau, M., Hofte, H., and Vernhettes, S. (2009). Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell* 21, 1141–1154.

DeBolt, S., Scheible, W.-R., Schrick, K., Auer, M., Beisson, F., Bischoff, V., Bouvier-Navé, P., Carroll, A., Hematy, K., Li, Y., Milne, J., Nair, M., Schaller, H., Zemla, M., and Somerville, C. (2009). Mutations in UDP-glucose:sterol glucosyltransferase in *Arabidopsis* cause transparent testa phenotype and suberization defect in seeds. *Plant Physiol.* 151, 78–87.

Delmer, D. P. (1999). Cellulose biosynthesis: exciting times for a difficult field of study. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 245–276.

Diener, A. C., Li, H., Zhou, W., Whoriskey, W. J., Nes, W. D., and Fink, G. R. (2000). Sterol methyltransferase 1 controls the level of cholesterol in plants. *Plant Cell* 12, 853–870.

Endler, A., and Persson, S. (2011). Cellulose synthases and synthesis in *Arabidopsis*. *Mol. Plant* 4, 199–211.

Engelking, L. J., Evers, B. M., Richardson, J. A., Goldstein, J. L., Brown, M. S., and Liang, G. (2006). Severe facial clefting in Insig-deficient mouse embryos caused by sterol accumulation and reversed by lovastatin. *J. Clin. Invest.* 116, 2356–2365.

Fernandes, A. N., Thomas, L. H., Altaner, C. M., Callow, P., Forsyth, V. T., Apperley, D. C., Kennedy, C. J., and Jarvis, M. C. (2011). Nanostructure of cellulose microfibrils in spruce wood. *Proc. Natl. Acad. Sci. U.S.A.* 108, E1195–E1203.

Gillmor, C. S., Poindexter, P., Lorieau, J., Palcic, M. M., and Somerville, C. (2002). Alpha-glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis*. *J. Cell Biol.* 156, 1003–1013.

Grandmougin-Ferjani, A., Schuler-Muller, I., and Hartmann, M. A. (1997). Sterol modulation of the plasma membrane H+-ATPase activity from corn roots reconstituted into soybean lipids. *Plant Physiol.* 113, 163–174.

Grebe, M., Xu, J., Mobius, W., Ueda, T., Nakano, A., Geuze, H. J., Rook, M. B., and Scheres, B. (2003). *Arabidopsis* sterol endocytosis involves actin-mediated trafficking via ARA6-positive early endosomes. *Curr. Biol.* 13, 1378–1387.

Grille, S., Zaslawski, A., Thiele, S., Plat, J., and Warnecke, D. (2010). The functions of steryl glycosides come to those who wait: recent advances in plants, fungi, bacteria and animals. *Prog. Lipid Res.* 49, 262–288.

Guerriero, G., Fugelstad, J., and Bulone, V. (2010). What do we really know about cellulose biosynthesis in higher plants? *J. Integr. Plant Biol.* 52, 161–175.

Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M., and Ehrhardt, D. W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nat. Cell Biol.* 11, 797–806.

Haigler, C. H., and Brown, R. M. (1986). Transport of rosettes from the Golgi apparatus to the plasma membrane in isolated mesophyll cells of *Zinnia elegans* during differentiation to tracheary elements in suspension culture. *Protoplasma* 134, 111–120.

Hillig, I., Leipelt, M., Ott, C., Zahringer, U., Warnecke, D., and Heinz, E. (2003). Formation of glucosylceramide and sterol glucoside by a UDP-glucose-dependent glucosylceramide synthase from cotton expressed in *Pichia pastoris*. *FEBS Lett.* 553, 365–369.

Jang, J. C., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S., and Sheen, J. (2000). A critical role of sterols in embryonic patterning and meristem programming revealed by the fackel mutants of *Arabidopsis thaliana*. *Genes Dev.* 14, 1485–1497.

Keller, R. K., Arnold, T. P., and Fliesler, S. J. (2004). Formation of 7-dehydrocholesterol-containing membrane rafts in vitro and in vivo, with relevance to the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* 45, 347–355.

Kierszniowska, S., Seiwert, B., and Schulze, W. X. (2009). Definition of *Arabidopsis* sterol-rich membrane microdomains by differential treatment with methyl-beta-cyclodextrin and quantitative proteomics. *Mol. Cell. Proteomics* 8, 612–623.

Kimura, S., Laosinchai, W., Itoh, T., Cui, X., Linder, C. R., and Brown, R. M. Jr. (1999). Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis*. *Plant Cell* 11, 2075–2086.

Klahre, U., Noguchi, T., Fujioka, S., Takatsuto, S., Yokota, T., Nomura, T., Yoshida, S., and Chua, N. H. (1998). The *Arabidopsis* DIMINUTO/DWARF1 gene encodes a protein involved in steroid synthesis. *Plant Cell* 10, 1677–1690.

Klemm, R. W., Ejsing, C. S., Surma, M. A., Kaiser, H. J., Gerl, M. J., Sampaio, J. L., de Robillard, Q., Ferguson, C., Proszynski, T. J., Shevchenko, A., and Simons, K. (2009). Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. *J. Cell Biol.* 185, 601–612.

Lai-Kee-Him, J., Chanzy, H., Muller, M., Putaux, J. L., Imai, T., and Bulone, V. (2002). In vitro versus in vivo cellulose microfibrils from plant primary wall synthases: structural differences. *J. Biol. Chem.* 277, 36931–36939.

Laloi, M., Perret, A. M., Chatre, L., Melser, S., Cantrel, C., Vaultier, M. N., Zachowski, A., Bathany, K., Schmitter, J. M., Vallet, M., Lessire, R., Hartmann, M. A., and Moreau, P. (2007). Insights into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. *Plant Physiol.* 143, 461–472.

Lichtenberg, D., Goni, F. M., and Heerklotz, H. (2005). Detergent-resistant membranes should not be identified with membrane rafts. *Trends Biochem. Sci.* 30, 430–436.

Lindsey, K., Pullen, M. L., and Topping, J. F. (2003). Importance of plant sterols in pattern formation and hormone signalling. *Trends Plant Sci.* 8, 521–525.

Litman, B. J., and Mitchell, D. C. (1996). A role for phospholipid polyunsaturation in modulating membrane protein function. *Lipids* 31(Suppl.) S193–S197.

Markham, J. E., Li, J., Cahoon, E. B., and Jaworski, J. G. (2006). Separation and identification of major plant sphingolipid classes from leaves. *J. Biol. Chem.* 281, 22684–22694.

Matthysse, A. G., Thomas, D. L., and White, A. R. (1995). Mechanism of cellulose synthesis in *Agrobacterium tumefaciens*. *J. Bacteriol.* 177, 1076–1081.

Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J. P., Hartmann, M. A., Bonneu, M., Simon-Plas, F., Lessire, R., and Bessoule, J. J. (2004). Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* 279, 36277–36286.

Mutwil, M., Debolt, S., and Persson, S. (2008). Cellulose synthesis: a complex complex. *Curr. Opin. Plant Biol.* 11, 252–257.

Pagant, S., Bichet, A., Sugimoto, K., Lerouxel, O., Desprez, T., McCann, M., Lerouge, P., Vernhettes, S., and Hofte, H. (2002). KOBITO1 encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in *Arabidopsis*. *Plant Cell* 14, 2001–2013.

Paradez, A., Wright, A., and Ehrhardt, D. W. (2006). Microtubule cortical array organization and plant cell morphogenesis. *Curr. Opin. Plant Biol.* 9, 571–578.

Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002). Sitosterol-beta-glucoside as primer for cellulose synthesis in plants. *Science* 295, 147–150.

Pose, D., Castanedo, I., Borsani, O., Nieto, B., Rosado, A., Taconnat, L., Ferrer, A., Dolan, L., Valpuesta, V., and Botella, M. A. (2009). Identification of the *Arabidopsis* dry2/sqe1-5 mutant reveals a central role for sterols in drought tolerance and regulation of reactive oxygen species. *Plant J.* 59, 63–76.

Rahier, A., Darnet, S., Bouvier, F., Camara, B., and Bard, M. (2006). Molecular and enzymatic characterizations of novel bifunctional 3beta-hydroxysteroid dehydrogenases/C-4 decarboxylases from *Arabidopsis thaliana*. *J. Biol. Chem.* 281, 27264–27277.

Sato, S., Kato, T., Kakegawa, K., Ishii, T., Liu, Y. G., Awano, T., Takabe, K., Nishiyama, Y., Kuga, S., Nakamura, Y., Tabata, S., and Shibata, D. (2001). Role of the putative membrane-bound endo-1,4-beta-glucanase KORRIGAN in cell elongation and cellulose synthesis in *Arabidopsis thaliana*. *Plant Cell Physiol.* 42, 251–263.

Schindelman, G., Morikami, A., Jung, J., Baskin, T. I., Carpita, N. C., Derbyshire, P., McCann, M. C., and Benfey, P. N. (2001). COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in *Arabidopsis*. *Genes Dev.* 15, 1115–1127.

Schrick, K., Cordova, C., Li, G., Murray, L., and Fujioka, S. (2011). A dynamic role for sterols in embryogenesis of *Pisum sativum*. *Phytochemistry* 72, 465–475.

Schrick, K., Fujioka, S., Takatsuto, S., Stierhof, Y. D., Stransky, H., Yoshida, S., and Jürgens, G. (2004). A link between sterol biosynthesis, the cell wall, and cellulose in *Arabidopsis*. *Plant J.* 38, 227–243.

Schrick, K., Mayer, U., Horrichs, A., Kuhnt, C., Bellini, C., Dangl, J., Schmidt, J., and Jürgens, G. (2000). FACKEL is a sterol C-14 reductase required for organized cell division and expansion in *Arabidopsis* embryogenesis. *Genes Dev.* 14, 1471–1484.

Schrick, K., Mayer, U., Martin, G., Bellini, C., Kuhnt, C., Schmidt, J., and Jürgens, G. (2002). Interactions between sterol biosynthesis genes in embryonic development of *Arabidopsis*. *Plant J.* 31, 61–73.

Schrick, K., Shiva, S., Arpin, J. C., Delimont, N., Isaac, G., Tamura, P., and Welti, R. (2012). Steryl glucoside and acyl steryl glucoside analysis of *Arabidopsis* seeds by electrospray ionization tandem mass spectrometry. *Lipids* 47, 185–193.

Schuler, I., Milon, A., Nakatani, Y., Ourisson, G., Albrecht, A. M., Benveniste, P., and Hartman, M. A. (1991). Differential effects of plant sterols on water permeability and on acyl chain ordering of soybean phosphatidylcholine bilayers. *Proc. Natl. Acad. Sci. U.S.A.* 88, 6926–6930.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* 387, 569–572.

Singer, S. J., and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* 175, 720–731.

Souter, M., Topping, J., Pullen, M., Friml, J., Palme, K., Hackett, R., Grierson, D., and Lindsey, K. (2002). Hydra mutants of *Arabidopsis* are defective in sterol profiles and auxin and ethylene signaling. *Plant Cell* 14, 1017–1031.

Tanner, W., Malinsky, J., and Opekarova, M. (2011). In plant and animal cells, detergent-resistant membranes do not define functional membrane rafts. *Plant Cell* 23, 1191–1193.

Warnecke, D., Erdmann, R., Fahl, A., Hube, B., Muller, F., Zank, T., Zahringer, U., and Heinz, E. (1999). Cloning and functional expression of UGT genes encoding sterol glucosyltransferases from *Saccharomyces cerevisiae*, *Candida albicans*, *Pichia pastoris*, and *Dictyostelium discoideum*. *J. Biol. Chem.* 274, 13048–13059.

Warnecke, D. C., Baltrusch, M., Buck, F., Wolter, F. P., and Heinz, E. (1997). UDP-glucose:sterol glucosyltransferase: cloning and functional expression in *Escherichia coli*. *Plant Mol. Biol.* 35, 597–603.

Xie, L., Yang, C., and Wang, X. (2011). Brassinosteroids can regulate cellulose biosynthesis by controlling the expression of CESA genes in *Arabidopsis*. *J. Exp. Bot.* 62, 4495–4506.

Zhou, M., Morgner, N., Barrera, N. P., Politis, A., Isaacson, S. C., Matak-Vinkovic, D., Murata, T., Bernal, R. A., Stock, D., and Robinson, C. V. (2011). Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding. *Science* 334, 380–385.

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The plant cell wall: a dynamic barrier against pathogen invasion

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Prospective plant pathogens must overcome the physical barrier presented by the plant cell wall. In addition to being a preformed, passive barrier limiting access of pathogens to plant cells, the cell wall is actively remodeled and reinforced specifically at discrete sites of interaction with potentially pathogenic microbes. Active reinforcement of the cell wall through the deposition of cell wall appositions, referred to as papillae, is an early response to perception of numerous categories of pathogens including fungi and bacteria. Rapid deposition of papillae is generally correlated with resistance to fungal pathogens that attempt to penetrate plant cell walls for the establishment of feeding structures. Despite the ubiquity and apparent importance of this early defense response, relatively little is known about the underlying molecular mechanisms and cellular processes involved in the targeting and assembly of papillae. This review summarizes recent advances in our understanding of cell wall-associated defenses induced by pathogen perception as well as the impact of changes in cell wall polymers on interactions with pathogens and highlights significant unanswered questions driving future research in the area.

Keywords: papilla, cell wall apposition, disease resistance, pathogen, immunity, cell wall adhesion, callose

INTRODUCTION

Plants have evolved a multi-layered system of defenses to contend with the threat of infection by microbial pathogens. The current view of the plant immune system is that it can be conceptually divided into two modes of response. These modes are distinguished by the signals perceived to initiate the response and the nature or severity of the defense response exhibited, in particular, the presence or absence of the programmed cell death referred to as the hypersensitive response (HR). The first line of inducible defenses are initiated upon perception of conserved, microbe-derived elicitor molecules referred to as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) or by host-derived elicitor molecules, referred to as damage-associated molecular patterns (DAMPs), which result from attempted infection by pathogens. This mode of plant immune response is referred to as pattern-triggered immunity (PTI; Jones and Dangl, 2006). The full spectrum of immune responses associated with PTI in plants is not currently understood, but discretely localized reinforcement of the cell wall through deposition of papillae at sites of pathogen detection appears to be a common component of the PTI response (Nicaise et al., 2009).

All prospective microbial phytopathogens must interact with the cell wall in some manner, and the nature of this interaction is typically determined by the lifestyle of the pathogen. Necrotrophic pathogens, which kill cells and feed on dead tissues, typically macerate plant tissues by secreting abundant hydrolytic enzymes that degrade cell wall polymers (Laluk and Mengiste, 2010). In contrast, biotrophic and hemibiotrophic pathogens must interact with living plant cells for all or part of their life cycles and typically employ more subtle strategies for interacting with the cell wall. Haustorium-forming pathogens, such as various fungal and oomycete mildews, must penetrate cell walls to establish haustorial feeding structures in close contact with the underlying host cell (Szabo and Bushnell, 2001). Bacterial phytopathogens typically manipulate plant cells by delivering effector proteins to host cells via secretion pili that must traverse the cell wall to access the underlying cell membrane and cytosol (Büttner and He, 2009). Deposition of papillae at sites of pathogen detection is thought to act as a physical barrier to limit access of pathogens to the underlying protoplast. Additionally, papillae are sites of accumulation of antimicrobial secondary metabolites (Bednarek et al., 2009; Clay et al., 2009). Rapid formation of papillae is correlated with enhanced resistance to fungal penetration, whereas, delayed papilla formation correlates with increased fungal penetration success (Bayles et al., 1990; Collins et al., 2003). Successful cell wall-associated defenses can halt invading pathogens at an early stage, before the establishment of disease, and can eliminate the need for more costly defense responses such as HR cell death. Therefore, it is of fundamental importance to understand the mechanisms through which cell wall-associated defenses are elaborated and to understand why these defenses fail to prevent infection by adapted pathogens. Here, we will focus on recent progress in our understanding of the role of the cell wall in plant–microbe interactions, concentrating primarily on interactions with biotrophic and hemibiotrophic pathogens.

CELL WALL APPOSITIONS

Observations of cell wall-associated defense responses to attempted pathogen invasion were first made over a century ago (Smith, 1900). In the intervening years, numerous studies have focused on characterization of papillae through ultrastructural observations or histochemical and immunohistochemical

analyses. These studies have revealed significant details about the structure and composition of papillae and related structures such as collars at the neck regions of fungal haustoria and haustorial encasements (**Figure 1**). The specific biochemical constituents of papillae seem to vary somewhat between plant species, but classes of compounds commonly found associated with papillae include: callose; phenolics including lignin; and phenolic conjugates such as phenolic–polyamines; reactive oxygen species (ROS); peroxidases; cell wall structural proteins such as arabinogalactan proteins and hydroxyproline-rich glycoproteins; and cell wall polymers including pectin and xyloglucans (Aist, 1976; Zeyen et al., 2002).

The β-1,3-glucan polymer callose is an abundant and ubiquitous component of cell wall appositions. However, the role of callose in papillae is not clear. The *Arabidopsis thaliana* callose synthase responsible for wound- and pathogen-induced callose deposition was identified as being encoded by *PMR4/GSL5* (Jacobs et al., 2003; Nishimura et al., 2003). Loss of function *pmr4* mutants retained the ability to deposit papillae at sites of attempted powdery mildew penetration and only a minor increase in penetration frequency by the barley powdery mildew *Blumeria graminis* f.sp. *hordei* was observed, suggesting that callose does not play a dramatic role as a structural barrier in papillae, at least in the context of powdery mildew penetration. In contrast, RNAi knockdown of a callose synthase in lemon (*Citrus limon*) leaves resulted in enhanced susceptibility to the bacterial pathogen *Xanthomonas citri* (Enrique et al., 2011). *X. campestris* pv *campestris* has been shown to block callose deposition in *Nicotiana benthamiana* and *Arabidopsis* through the production of the exopolysaccharide xanthan (Yun et al., 2006). These results suggest that callose deposition in papillae may play a role in limiting access of *Xanthomonas* to host cells. However, whether callose acts as a direct physical barrier is still not clear as it is not known whether the absence of callose has additional effects on papilla structure or other defenses

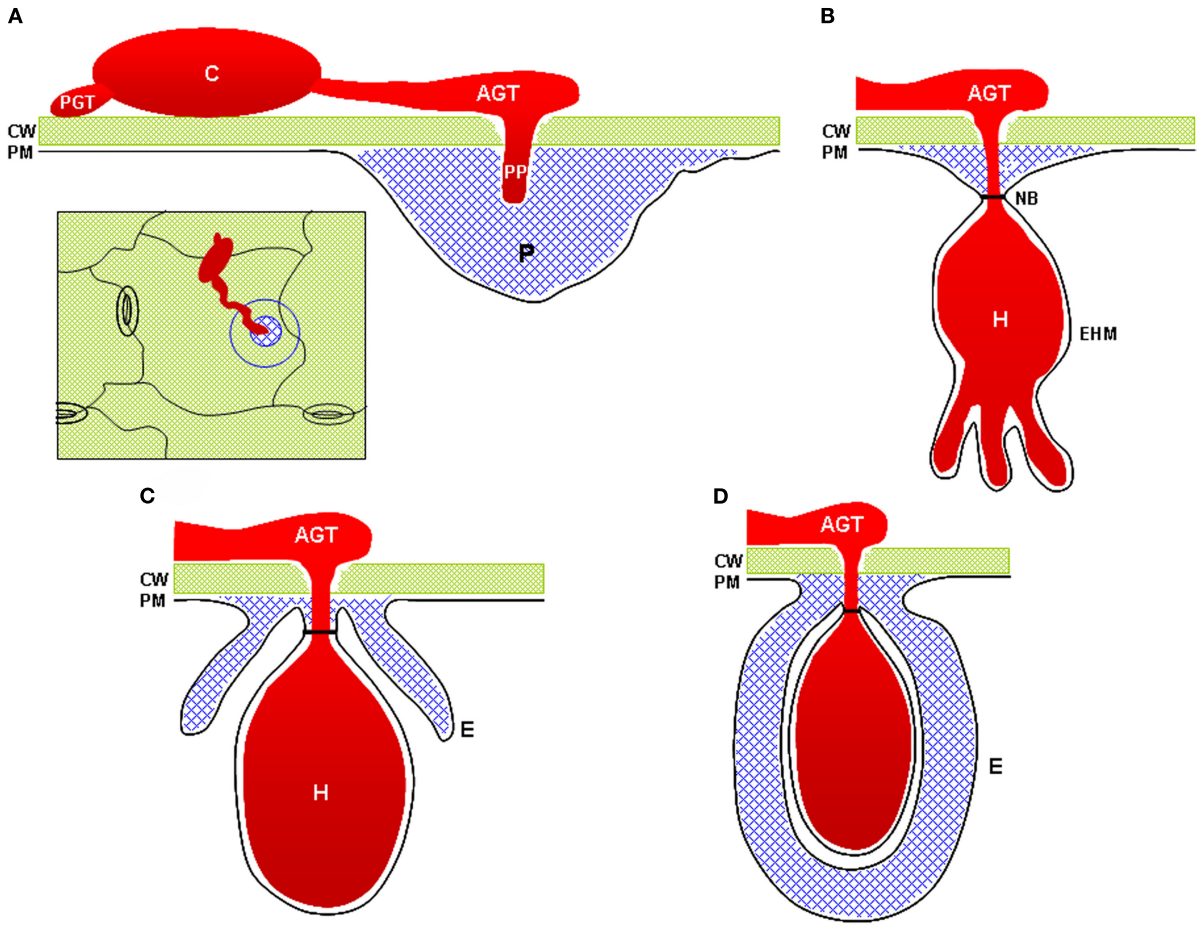


FIGURE 1 | Cell wall-associated structures commonly observed at sites of interaction with powdery mildews and other fungal pathogens. (A) A fungal penetration attempt halted by deposition of a cell wall apposition (blue). Inset image illustrates a top-down view of the penetration site as typically visualized by light microscopy. **(B)** A successful penetration event in which the fungus has formed a haustorial feeding structure. The cell wall apposition materials form a neck-band or collar around the neck of the haustorium. **(C)** A haustorium partially surrounded by a haustorial encasement. Encasements contain materials similar to those found in cell wall appositions. **(D)** A fully encased haustorium. CW, cell wall; PM, plasma membrane; C, conidiospore; PGT, primary germ tube (note that not all powdery mildew species develop PGTs); AGT, appressorial germ tube; PP, penetration peg; H, haustorium; EHM, extra-haustorial membrane; NB, haustorial neck-band; P, papilla (e.g., cell wall apposition); E, haustorial encasement.

in lemon leaves. Unexpectedly, *pmr4* mutants displayed enhanced resistance to normally virulent biotrophic pathogens including *Golovinomyces cichoracearum*, *G. orontii*, and *Hyaloperonospora arabidopsidis* (Vogel and Somerville, 2000; Jacobs et al., 2003). Enhanced resistance was dependent on an intact salicylic acid (SA) defense signaling pathway and common SA-induced genes were found to be upregulated in *pmr4* and hyperinduced in *pmr4* upon powdery mildew inoculation. These results imply that callose or the PMR4 protein itself negatively regulates SA synthesis or signaling. One possible interpretation of these unexpected results is that callose serves as a protective containment barrier to shield the plant cell from toxic metabolites that accumulate in papillae and haustorial encasements. Such a hypothesis is supported by the observations that callose-containing cell wall appositions occur at sites of plasmodesmata in cells neighboring those undergoing HR cell death and that cells that have undergone HR typically become encased by callose (Jacobs et al., 2003; An et al., 2006). Alternatively, callose deposition in papillae and haustorial encasements may limit diffusion of pathogen-derived elicitors, thus reducing the level of activation of the SA-dependent defense pathway.

The mechanisms of regulation and targeting for PMR4/GSL5 callose deposition are largely unknown. Recently, the barley ADP-ribosylation factor (ARF) GTPase ARFA1b/1c was found to be important for callose deposition at powdery mildew penetration sites (Böhlenius et al., 2010). RNAi knockdown or expression of a dominant negative ARFA1b/1c variant abolished callose accumulation at penetration sites and resulted in increased fungal penetration success. ARFA1b/1c was found to localize to an endosomal multi-vesicular body compartment that accumulated at fungal penetration sites prior to the accumulation of callose. The authors propose a model whereby cell surface callose synthase enzymes are activated throughout the cell and the resulting extracellular callose is internalized into multi-vesicular bodies and delivered to penetration sites in an ARFA1b/1c dependent process. Interestingly, the *Arabidopsis* ARF-GEF (guanine nucleotide exchange factor) MIN7 is required for normal levels of callose deposition in response to the *Pseudomonas syringae* pv. *tomato* (*Pst*) ΔCEL mutant, suggesting that an ARF-dependent process may also play a role in callose deposition at sites of pathogen detection in *Arabidopsis* (Nomura et al., 2006).

In addition to callose, phenolic polymers are incorporated into papillae. Phenolics are thought to contribute to the physical barrier through cross linking to form a hardened wall that cannot be easily degraded by enzymes employed by pathogens (Zeyen et al., 2002). Accumulation of a specific phenolic–polyamine conjugate correlated with resistance in *mlo* barley and was found to have direct antifungal activity, suggesting that phenolic polymers may have multiple defense functions in papillae (von Röpenack et al., 1998). Recently, *Arabidopsis* mutants impaired in specific isoforms of cinnamyl alcohol dehydrogenase (CAD), enzymes that catalyze the final step of mono-lignol biosynthesis, were found to be more susceptible to *Pst* (Tronchet et al., 2010). Increased levels of multiplication and more severe disease symptoms were observed on *cad-b1* single mutants and *cad-C/cad-D* double mutants inoculated with either virulent *Pst* DC3000 or avirulent DC3000 (AvrPphB). The mutants exhibited altered expression profiles for SA biosynthesis and response genes after bacterial inoculation, complicating interpretation of the results. It is not yet clear if phenolic polymers or lignin have a direct effect on antibacterial defenses as a structural barrier and/or through antimicrobial activity or if the effects are indirectly exerted through altered SA biosynthesis or response.

Plant cells respond to invasion by haustorium-forming pathogens through the deposition of several related cell wall-associated structures including papillae, haustorial encasements, and haustorial collars or neckbands (Zeyen et al., 2002; Micali et al., 2011; **Figure 1**). Ultrastructural observations have suggested that haustorial encasements and collars are extensions of or derived from papillae (Zeyen et al., 2002). More recent immunocytochemical characterization of haustorial encasements supports the notion that these structures are extensions of papillae. Immunofluorescence and immunogold labeling detected callose, arabinogalactan proteins, rhamnogalacturonan I, a β-linked galactose-containing protein, and xyloglucan in *Arabidopsis* encasements completely or partially surrounding *G. orontii* haustoria, but failed to detect high- or low-ester homogalacturonans or extensins (Micali et al., 2011). The same epitopes were detected in papillae, reinforcing the notion that these structures are related. In addition to cell wall materials, papillae, and encasements also contain membrane lipid materials and multi-vesicular bodies that may be involved in delivery of materials for construction of the papilla (Meyer et al., 2009). Haustorial encasements appear to be defensive structures rather than playing a role in haustorium accommodation as they are not observed around haustoria in compatible interactions, suggesting that adapted fungi have the ability to suppress formation of encasements. Encasements were observed around ∼20% of *G. orontii* haustoria in *Arabidopsis*, but were not observed around *G. cichoracearum* haustoria, suggesting that *G. cichoracearum* can suppress this defense response more effectively than *G. orontii* (Koh et al., 2005; Meyer et al., 2009). Understanding how haustorium-forming pathogens suppress the formation of encasements and defeat papilla-associated cell wall defenses will be a significant challenge for future research.

PLASMA MEMBRANE-CELL WALL ADHESION

Plant cells, like those of other multicellular organisms, maintain a connection to and communication with their extracellular environment. Mammalian cells connect to and communicate with their extracellular matrix in part through plasma membrane (PM)-localized integrin proteins that recognize components of the extracellular matrix via an Arg-Gly-Asp (RGD) protein motif (Gee et al., 2008). In plants, a similar connection between the PM and cell wall dependent on RGD or similar motifs appears to exist (Canut et al., 1998). RGD-mediated PM-cell wall adhesion appears to play a role in resistance to pathogens. PM-wall adhesion can be disrupted by treatment with peptides containing the RGD motif (Canut et al., 1998). Such treatment causes the disappearance of PM attachment sites, referred to as Hechtian strands, that become visible upon plasmolysis and subsequently changes the appearance of the plasmolyzed protoplasts from a concave morphology to convex. Pretreatment with RGD peptides caused a decrease in callose and H_2O_2 accumulation at penetration sites in both the cowpea rust (*Uromyces vignae*)-pea (*Pisum sativum*) and *G. cichoracearum*-cowpea (*Vigna unguiculata*) interactions

and increased the frequency of penetration by both pathogens (Mellersh and Heath, 2001). A localized decrease in PM-wall adhesion was observed at penetration sites in the cowpea rust–cowpea interaction and the appearance of cell wall defense responses was correlated with the maintenance of PM-wall adhesion. These results suggest that some pathogens may disrupt PM-wall adhesion to promote infection and suppress cell wall-associated defense responses. This notion has been further substantiated by the discovery of an RGD-containing effector protein, IPI-O, from the oomycete pathogen *Phytophthora infestans* (Senchou et al., 2004). Treatment of *Arabidopsis* suspension-cultured cells with recombinant IPI-O disrupted PM-wall adhesion and disruption was dependent on an intact RGD motif. IPI-O was found to bind the *Arabidopsis* lectin receptor kinase LecRK-I.9 (Gouget et al., 2005). *Arabidopsis lecRK-I.9* mutants were susceptible to a normally incompatible isolate of *P. brassicae* whereas lines overexpressing LecRK-I.9 became resistant to a normally virulent *P. brassicae* isolate (Bouwmeester et al., 2011). PM-wall adhesion was also partially compromised in *lecRK-I.9* mutant plants. Similarly, transgenic expression of the IPI-O effector *in planta* resulted in susceptibility to incompatible *P. brassicae* and disruption of PM-wall adhesion. Interestingly, *lecRK-I.9* and 35S::IPI-O plants were also impaired in callose deposition induced by the *Pst* DC3000 *hrcC* mutant and by the flagellin-derived flg22 peptide, further supporting the correlation between disruption of PM-wall adhesion and abrogation of cell wall-associated defense responses. It will be interesting to see whether interfering with PM-wall adhesion is a widespread pathogenesis strategy among phytopathogens. Powdery mildews do not appear to utilize this strategy, as PM-wall adhesion was found to increase slightly in both compatible and incompatible plant–powdery mildew interactions (Mellersh and Heath, 2001).

Recently, the *Arabidopsis* NDR1 protein was found to share structural similarity to mammalian integrins and to contain a solvent exposed Asn-Gly-Asp (NGD) motif (Knepper et al., 2011). NDR1 is a PM-localized protein that is required for HR induction mediated by numerous resistance genes of the coiled coil–nucleotide binding site-leucine-rich repeat class (Aarts et al., 1998). Despite the importance of NDR1 in resistance protein signaling, its cellular function has remained enigmatic. Knepper and colleagues demonstrated that *ndr1* mutants are impaired in PM-wall adhesion and that NGD-containing peptides can disrupt adhesion, suggesting that the NGD motif is functionally equivalent to RGD. Responsiveness of *ndr1* mutants to flg22 peptide was reduced. Additionally, electrolyte leakage was increased in *ndr1* plants inoculated with avirulent *Pst* DC3000 (AvrRpt2), suggesting that NDR1 impacts fluid loss from cells. However, treatment with NGD- or RGD-containing peptides did not alter the HR in response to avirulent *Pst*, indicating that the participation of NDR1 in promoting PM-wall adhesion does not account for its function in resistance protein signaling.

IMPACTS OF CELL WALL ALTERATIONS ON PLANT–MICROBE INTERACTIONS

In addition to pathogen resistance conferred by active reinforcement of plant cell walls, it is expected that differences in cell wall composition between plant species may account, at least in part, for limitations of pathogen host range (Vorwerk et al., 2004). Due to the complexity of the cell wall and the fact that many mutants with alterations in cell wall polymer composition display significant pleiotropic effects, it has proven difficult to directly address this hypothesis. However, analysis of a number of mutants has revealed correlations between altered cell wall compositions and altered susceptibility to pathogens. The *Arabidopsis powdery mildew resistant* mutants *pmr5* and *pmr6* both display similar patterns of cell wall alteration when analyzed by Fourier transform infrared (FTIR) spectroscopy (Vogel et al., 2002, 2004). The FTIR spectra of these mutants suggested an increase in pectin content and decrease in pectin methyl esterification or *O*-acetylation. The resistance of *pmr5* and *pmr6* appears specific to powdery mildews as these mutants were susceptible to *Pst* and *H. arabidopsidis*. *PMR6* was found to encode a putative pectate lyase, consistent with the apparent alteration of pectin content and/or structure in the mutant (Vogel et al., 2002). *PMR5* encodes a protein of unknown function that belongs to a large gene family encoding proteins that contain a plant-specific DUF231 domain (Vogel et al., 2004). Interestingly, DUF231 family proteins were found to contain sequence similarity to the N-terminal domain of *Cryptococcus neoformans* Cas1p, a protein that is involved in polysaccharide acetylation (Janbon et al., 2001; Anantharaman and Aravind, 2010). The C-terminus of Cas1p shares sequence similarity with a small family of *Arabidopsis* proteins that include REDUCED WALL ACETYLATION2 (RWA2). *Arabidopsis rwa2* mutants were found to have reduced acetylation of both pectic and non-pectic wall polysaccharides (Manabe et al., 2011). Interestingly, *rwa2* showed increased resistance to the necrotrophic fungal pathogen *Botrytis cinerea*, but no alteration in susceptibility to the powdery mildew *G. cichoracearum*. Localization of RWA2 to the endoplasmic reticulum and the fact that the reduction in wall acetylation was not specific to a particular wall polysaccharide prompted Manabe and colleagues to hypothesize that RWA2 acts upstream of polysaccharide acetyl transfer. These authors further speculate that DUF231 proteins may be involved in specific polysaccharide acetylation, potentially as transferase enzymes. This notion is supported by the identification of the *Arabidopsis axy4* mutant which specifically lacks xyloglucan *O*-acetylation and is impaired in a DUF231 protein (Gille et al., 2011). It remains to be determined if PMR5 alters pectin acetylation either directly as a transferase enzyme or indirectly in some other manner and, if this is the case, how an alteration in pectin acetylation results in powdery mildew resistance. Vogel et al. (2004) hypothesized that alteration in pectin modification may result in the release of defense elicitor-active fragments upon degradation by powdery mildew hydrolytic enzymes. Ongoing research on pectin modification by RWA2 and DUF231 proteins may soon shed light on this hypothesis.

PERSPECTIVE

The cell wall represents a first line of defense for plant cells against infection by microbial pathogens. Despite over a century of study, many questions about the role of the cell wall in pathogen defense remain to be answered: what are the major factors underlying success or failure of wall-associated defenses in a given plant–microbe interaction? What are the rate-limiting steps that affect

timing of papilla deposition and can these be rationally altered to enhance resistance? How significant a factor are differences in wall composition in determining pathogen host range? How does PM-wall adhesion contribute to defense activation? Answering these questions may provide tools to promote rational engineering of disease resistance through enhancement of wall-associated defense responses and should further our understanding of the dynamic nature of the plant cell wall.

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REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J., and Parker, J. E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10306–10311.

Aist, J. R. (1976). Papillae and related wound plugs of plant cells. *Annu. Rev. Phtyopathol.* 14, 145–163.

An, Q., Ehlers, K., Kogel, K-H., van Bel, A. J. E., and Hückelhoven, R. (2006). Multivesicular compartments proliferate in susceptible and resistant *MLA12*-barley leaves in response to infection by the biotrophic powdery mildew fungus. *New Phytol.* 172, 563–576.

Anantharaman, V., and Aravind, L. (2010). Novel eukaryotic enzymes modifying cell surface biopolymers. *Biol. Direct* 5, 1.

Bayles, C. J., Ghemawat, M. S., and Aist, J. R. (1990). Inhibition by 2-deoxy-D-glucose of callose formation, papilla deposition, and resistance to powdery mildew in an *mlo* barley mutant. *Physiol. Mol. Plant Pathol.* 36, 63–72.

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., and Schulze-Lefert, P. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 232, 101–106.

Böhlenius, H., Mørch, S. M., Godfrey, D., Nielsen, M. E., and Thordal-Christensen, H. (2010). The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley. *Plant Cell* 22, 3831–3844.

Bouwmeester, K., de Sain, M., Weide, R., Gouget, A., Klamer, S., Canut, H., and Govers, F. (2011). The lectin receptor kinase LecRK-I.9 is a novel *Phytophthora* resistance component and a potential host target for an RXLR effector. *PLoS Pathog.* 7, e1001327. doi:10.1371/journal.ppat.1001327

Büttner, D., and He, S. Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiol.* 150, 1656–1664.

Canut, H., Carrasco, A., Galaud, J., Cassan, C., Bouyssou, H., Vita, N., Ferrera, P., and Pont-Lezica, R. (1998). High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* link the cell wall. *Plant J.* 16, 63–71.

Clay, N. K., Adio, A. M., Denoux, C., Jander, G., and Ausubel, F. M. (2009). Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323, 95–100.

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J. L., Huckelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S. C., and Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425, 973–977.

Enrique, R., Siciliano, F., Favaro, M. A., Gerhardt, N., Roeschlin, R., Rigano, L., Sendin, L., Castagnaro, A., Vojnov, A., and Marano, M. R. (2011). Novel demonstration of RNAi in citrus reveals importance of citrus callose synthase in defence against *Xanthomonas citri* subsp. *citri*. *Plant Biotechnol. J.* 9, 394–407.

Gee, E. P., Ingber, D. E., and Stultz, C. M. (2008). Fibronectin unfolding revisited: modeling cell traction-mediated unfolding of the tenth type-III repeat. *PLoS ONE* 3, e2373. doi:10.1371/journal.pone.0002373

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011). O-acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Gouget, A., Senchou, V., Govers, F., Sanson, A., Barre, A., Rougé, P., Pont-Lezica, R., and Canut, H. (2005). Lectin receptor kinases participate in protein-protein interactions to mediate plasma membrane-cell wall adhesions in *Arabidopsis*. *Plant Physiol.* 140, 81–90.

Jacobs, A. K., Lipka, V., Burton, R. A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., and Fincher, G. B. (2003). An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. *Plant Cell* 15, 2503–2513.

Janbon, G., Himmelreich, U., Moyrand, F., Improvisi, L., and Dromer, F. (2001). Cas1p is a membrane protein necessary for the *O*-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. *Mol. Microbiol.* 42, 453–467.

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

Knepper, C., Savory, E. A., and Day, B. (2011). *Arabidopsis* NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *Plant Physiol.* 156, 286–300.

Koh, S., Andre, A., Edwards, H., Ehrhardt, D., and Somerville, S. (2005). *Arabidopsis thaliana* subcellular responses to compatible *Erisyphe cichoracearum* infections. *Plant J.* 44, 516–529.

Laluk, K., and Mengiste, T. (2010). Necrotroph attacks on plants: wanton destruction or covert extortion? *Arabidopsis Book* 8, e0136.

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., Pauly, M., Knox, J. P., Sakuragi, Y., and Scheller, H. V. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078.

Mellersh, D. G., and Heath, M. C. (2001). Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. *Plant Cell* 13, 413–424.

Meyer, D., Pajonk, S., Micali, C., O'Connell, R., and Schulze-Lefert, P. (2009). Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. *Plant J.* 57, 986–999.

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.

Nicaise, V., Roux, M., and Zipfel, C. (2009). Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol.* 150, 1638–1647.

Nishimura, M. T., Stein, M., Hou, B-H., Vogel, J. P., Edwards, H., and Somerville, S. C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science* 301, 969–972.

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

Senchou, V., Weide, R., Carrasco, A., Bouyssou, H., Pont-Lezica, R., Govers, F., and Canut, H. (2004). High affinity recognition of a *Phytophthora* protein by *Arabidopsis* via an RGD motif. *Cell. Mol. Life Sci.* 61, 502–509.

Smith, G. (1900). The haustoria of the Erysipheae. *Bot. Gaz.* 29, 153–184.

Szabo, L. J., and Bushnell, W. R. (2001). Hidden robbers: the role of fungal haustoria in parasitism of plants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7654–7765.

Tronchet, M., Balagué, C., Kroj, T., Jouanin, L., and Roby, D. (2010). Cinnamyl alcohol dehydrogenases-C and D, key enzymes in lignin biosynthesis, play an essential role in disease resistance in *Arabidopsis*. *Mol. Plant Pathol.* 11, 83–92.

Vogel, J., and Somerville, S. (2000). Isolation and characterization of

powdery mildew-resistant *Arabidopsis* mutants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1897–1902.

Vogel, J. P., Raab, T. K., Schiff, C., and Somerville, S. C. (2002). *PMR6*, a pectate-lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. *Plant Cell* 14, 2095–2106.

Vogel, J. P., Raab, T. K., Somerville, C. R., and Somerville, S. C. (2004). Mutations in *PMR5* result in powdery mildew resistance and altered cell wall composition. *Plant J.* 40, 968–978.

von Röpenack, E., Parr, A., and Schulze-Lefert, P. (1998). Structural analysis and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *J. Biol. Chem.* 273, 9013–9022.

Vorwerk, S., Somerville, S., and Somerville, C. (2004). The role of plant cell wall polysaccharide composition in disease resistance. *Trends Plant Sci.* 9, 203–209.

Yun, M. H., Torres, P. S., Oirdi, M. E., Rigano, L. A., Gonzalez-Lamothe, R., Marano, M. R., Castagnaro, A. P., Dankert, M. A., Bouarab, K., and Vojnov, A. A. (2006). Xanthan induces plant susceptibility by suppressing callose deposition. *Plant Physiol.* 141, 178–187.

Zeyen, R. J., Carver, T. L. W., and Lyngkjaer, M. F. (2002). "Epidermal cell papillae," in *The Powdery Mildews: A Comprehensive Treatise*, eds R. R. Belanger, W. R. Buschnell, A. J. Dik, and T. L. W. Carver (St. Paul, MN: APS Press), 107–125.

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The cell walls of green algae: a journey through evolution and diversity

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The green algae represent a large group of morphologically diverse photosynthetic eukaryotes that occupy virtually every photic habitat on the planet. The extracellular coverings of green algae including cell walls are also diverse. A recent surge of research in green algal cell walls fueled by new emerging technologies has revealed new and critical insight concerning these coverings. For example, the late divergent taxa of the Charophycean green algae possess cell walls containing assemblages of polymers with notable similarity to the cellulose, pectins, hemicelluloses, arabinogalactan proteins (AGPs), extensin, and lignin present in embryophyte walls. Ulvophycean seaweeds have cell wall components whose most abundant fibrillar constituents may change from cellulose to β-mannans to β-xylans and during different life cycle phases. Likewise, these algae produce complex sulfated polysaccharides, AGPs, and extensin. Chlorophycean green algae produce a wide array of walls ranging from cellulose–pectin complexes to ones made of hydroxyproline-rich glycoproteins. Larger and more detailed surveys of the green algal taxa including incorporation of emerging genomic and transcriptomic data are required in order to more fully resolve evolutionary trends within the green algae and in relationship with higher plants as well as potential applications of wall components in the food and pharmaceutical industries.

Keywords: cell walls, scales, green algae, sulfated polysaccharides, glycoproteins

INTRODUCTION

The emergence of green algae (Chlorophyta and Streptophyta, Viridiplantae; *sensu* Leliaert et al., 2012) onto land roughly 470 million years ago represents one of the most important events in the history of life on the planet. Their successful colonization of land and subsequent evolution into modern land plants significantly altered the atmosphere, changed terrestrial substrates and paved the way for the evolution of other biota. Today, humans ultimately depend on the evolutionary "offshoots" of green algae (i.e., embryophytes or "land plants") for food, textiles, building material, pharmaceuticals, and fuels. Yet these events and applications represent only parts of a much larger story of green algae. Contemporary green algae are ubiquitous. They are important members of the ocean's phytoplankton, common and sometimes nuisance seaweeds of coastal marine habitats, peculiar symbionts of lichens and flatworms, and inhabitants of just about any freshwater ecosystem ranging from ponds, rivers, lakes, wetlands, and snow banks. In the 1.5 billion years since they first appeared (Lewis and McCourt, 2004; Becker and Marin, 2009; Finet et al., 2010; Leliaert et al., 2011; Wodnick et al., 2011), green algae have successfully adapted to virtually all photic zones of the planet.

Similar to their land plant offspring, the vast majority of extant green algae today are covered by a very large assortment of types of extracellular matrix (ECM). These external coverings are products of complex biosynthetic machineries that often make use of the bulk of the alga's photosynthetically fixed carbon. The ECM is integral to growth and development, affords the alga physical protection and defense against microbial attack, is involved in cell–cell and cell–substrate adhesion and in some cases, is involved in sexual reproduction. Some green algae are covered by multiple layers of intricately sculpted scales while others have crystalline glycoprotein coverings or thick multilaminate fibrillar cell walls. A few taxa though have cell walls with remarkable structural and biochemical similarity to cell walls found in land plants (Sørensen et al., 2010, 2011). How did these diverse extracellular coverings arise and what are the evolutionary links between them? Many analytical approaches and technologies are now being used to study green algal ECMs and are providing new and critical insight into structure, chemistry, and evolution of these coverings (**Table 1**). Nevertheless, we are only in an infancy stage in our understanding of the green algal extracellular coverings. In this review, we describe some of these recent discoveries and comment on future directions for study of the cell walls of green algae.

THE CURRENT STATE OF STUDY OF GREEN ALGAL CELL WALLS AND EXTRACELLULAR COVERINGS

While the green algae display a large and diverse array of ECM-coverings, only a few taxa have been studied in detail. It is widely accepted that taxa of the Ulvophyceae and the Charophycean green algae (CGA) possess fibrillar cell walls (Popper et al., 2011) consisting of various polysaccharide and proteoglycan constituents while other taxa, especially those of the Prasinophyceae,

Table 1 | Major methodologies used today in the study of green algal coverings.

Methodology	Technical aspects	Data obtained/status	Reference
Biochemical	Chemical and enzymatic fractionation; methylation analysis-GC/MS; NMR; electrophoresis	Monosaccharide composition, glycosidic linkage composition, conformational studies, molecular weights of various cell wall polysaccharides	Popper et al. (2011), Popper and Fry (2003), Estevez et al. (2009)
Carbohydrate microarrays	Sequential extraction of polysaccharides; immobilization onto nitrocellulose, mAb probing	Early divergent CGA walls differ from late divergent taxa walls; late divergent taxa possess HGA, RG-I, MLG, various hemicelluloses, AGPs, extensins	Sørensen et al. (2010, 2011), Moller et al. (2007)
Immunocytochemistry	Immunofluorescence and immunogold labeling of live cells and sections of fixed cells	Wall polymer mAbs may be used in live cell studies; *Coleochaete* walls possess lignin-like epitopes	Domozych et al. (2009, 2011), Eder and Lutz-Meindl (2010), Sørensen et al. (2011)
FTIR microspectroscopy	IR spectral arrays obtained from microscopically imaged covering	Analysis of presence and distribution of polymers in the cell wall of Ulvophyceae	Estevez et al. (2009), Fernández et al. (2011a), Carpita et al. (2001)
Molecular	Transcriptome and genome acquisition; annotation of genes	Genomes sequenced in *Volvox carteri*, *Chlamydomonas reinhardtii*, *Micromonas sp. RCC299*, *Ostreococcus tauri*, and *Ostreococcus lucimarinus* (see http://bioinformatics.psb.ugent.be/plaza/) and *Chlorella variabilis* NC64; several transcriptomes analyzed	Blanc et al. (2010), Timme and Delwiche (2010), Vannerum et al. (2011), Timme et al. (2012)

mAb, monoclonal antibody; RG-I, rhamnogalacturonan-I; MLG, mixed linkage glucans; AGP, arabinogalactan protein; FTIR, Fourier transform infrared; CGA, Charophycean green algae.

Chlorodendrophyceae, and some taxa of the Chlorophyceae, produce coverings that are structurally and biochemically unique. Presently, extant green algae are classified into six distinct clades (Delwiche and Timme, 2011; Leliaert et al., 2011, 2012). The following represent brief synopses of the current state of knowledge concerning the ECM of these groups (see also **Table 2**).

THE PRASINOPHYCEAEN MATRIX: SUBTLE TO THE SPECTACULAR!

The Prasinophyceae or prasinophytes represent a group of motile and non-motile unicells that are presently classified in four clades (Leliaert et al., 2012) and are most commonly found in marine habitats. In photic zones of oceans, picoplanktonic prasinophytes (perhaps the smallest extant eukaryotes; 0.8 μm cell size), like *Ostreococcus* and *Micromonas*, exist in very large numbers. Previous microscopy-based research has shown that taxa like these are either covered with scales or do not have any discernable matrix at all (i.e., they are naked; Piganeau et al., 2011). However, recent analysis of the *Ostreococcus* genome plus immunocytochemical investigations in our laboratories together raise questions as to the nakedness of this picoalga. Other prasinophytes are significantly larger and covered with layers of thousands of distinctly shaped scales coating both the cell and flagellar membrane surfaces (Moestrup and Walne, 1979). Biochemical analyses have shown that these scales are comprised primarily of neutral and acidic sugars including 2-keto sugars such as 3-deoxy-lyxo-2-heptulosaric acid (DHA; Becker et al., 1991, 1994). All scales of prasinophytes are believed to be processed in the Golgi apparatus, packaged in secretory vesicles and secreted to the cell surface near the flagellar apparatus or to vacuole-like scale reservoirs before release to the cell surface.

CHLORODENDROPHYCEAE: WALLS FROM A FUSION

Taxa of the small Chlorodendrophyceae group of green algae consist of motile or non-motile and sometimes stalked unicells (*Tetraselmis* and *Scherffelia)* that are covered by a single cell wall or layers of cell walls. Furthermore, the wall or theca consists of regular repeating subunits and unlike the cell walls of other green algae, this wall is believed to be a product of fused scales. The subunits of the theca are scale-like and are processed in the Golgi apparatus like those of scaly prasinophytes. The acid sugars, 2-keto-3-deoxy-D-manno-octulosonic acid, 5-*O*-methyl 2-keto-3-deoxy-D-manno-octulosonic acid, and DHA comprise 60% of the sugars present in the theca (Becker et al., 1991).

TREBOUXIOPHYCEAE: WALLS OF UNUSUAL POLYMERS

The Trebouxiophyceae consists of an assemblage of primarily freshwater and terrestrial forms that exhibit diverse phenotypes ranging from unicells to colonies to filaments as well as representing most of the photobiont green algae of lichens (e.g., *Trebouxia*). Some are considered highly attractive candidate genera for use in algal biofuel production (e.g., *Chlorella*; Rodrigues and da Silva Bon, 2011). Most members of this group possess cell walls but surprisingly little is known about their biosynthesis, composition, or architecture. In *Chlorella*, the wall contains cellulose and in some species, the wall is coated by a highly resistant outer stratum consisting of "algaenan," an aliphatic polymer containing long polymethylenic chains that are decorated with amide and *N*-alkyl substituted pyrroles (Rodrigues and da Silva Bon, 2011). In *Trebouxia* isolated from lichens and grown separately from its fungal partner, β-galactofuranan has been demonstrated, a polysaccharide previously found in fungi but not known from green algae (Cordeiro et al., 2006).

Table 2 | Summary of the composition of extracellular coverings in green algae.

Taxon	Covering type	Biochemical composition	Reference
Prasinophyceae	"Scales," coatings	2-Keto sugars (e.g., DHA), mannans, glycoproteins	Becker et al. (1991, 1994), Moestrup and Walne (1979)
Chlorodendrophyceae	Wall of fused scales	2-Keto sugars (e.g., DHA), proteins	Becker et al. (1991)
Trebouxiophyceae	Cell walls	Cellulose, algaenan, β-galactofuranan	Rodrigues and da Silva Bon (2011), Cordeiro et al. (2006)
Chlorophyceae	Crystalline glycoprotein walls; fibrillar cell walls	Hyp-rich glycoproteins, cellulose pectins, AGP, extensin	Voigt et al. (2001, 2007), Kirk (1998), Estevez et al. (2008)
Ulvophyceae	Cell walls	Cellulose, β-mannans, β-xylans, sulfated (sometimes pyruvylated) polysaccharides or sulfated rhamnogalacturonans, AGP, extensin	Ciancia et al. (2012), Estevez et al. (2009), Percival (1979), Lahaye and Robic (2007)
Charophyceae-early divergent clades	Scales, cell walls	2-Keto sugars, cellulose, homogalacturonans, 1,3 β-glucans, AGP	Sørensen et al. (2011), Domozych et al. (1991)
Charophyceae-late divergent clades	Cell walls	Cellulose, homogalacturonans, RG-I xyloglucans, mannans, xylans, mixed linkage glucans, 1,3 β-glucans, AGP, extensin, lignin	Sørensen et al. (2011, 2012), Popper and Tuohy (2010)

For further detailed information, key references are provided. AGP, arabinogalactan proteins; Hyp, hydroxyproline.

CHLOROPHYCEAE: GLYCOPROTEINS AND CELLULOSE

The Chlorophyceae are the largest group of green algae and exhibit great morphological diversity ranging from motile unicells to large filaments to blade-like thalli. The extracellular coverings of the Chlorophycean algae are also very diverse and consist of a distinct assortment of "cell walls." In *Oedogonium*, the cell wall resembles those of higher plants in containing microfibrillar cellulose, homogalacturonans and rhamnogalacturonan-I, extensin, and arabinogalactan constituents (Estevez et al., 2008). Many of the polysaccharides that are common to embryophyte walls are thought to have evolved within the CGA, so these results were unexpected. Further biochemical study and a much wider screening of this and other Chlorophycean taxa will be required to ascertain the similarity of these polymers with those of the CGA and embryophytes. However, in the *Chlamydomonas–Volvox* assemblage (i.e., volvocalean flagellates), the cell wall does not contain cellulose but is made of crystalline glycoproteins, specifically one based upon aggregates of hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich glycoproteins (Imam et al., 1985; Adair et al., 1987; Kirk, 1998; Voigt et al., 2001, 2007). Extensins of plants are a group of cell wall glycoproteins that probably share at least some glycosylation motives and a common ancestry with the HRGPs of *Chlamydomonas–Volvox* assemblage. The glycosylation motives that govern extensin-type glycosylation comprise the SPPPP sequence (i.e., serine-proline-proline-proline-proline) usually occurring several times. The prolines are hydroxylated by prolyl hydroxylases prior to glycosylation. Showalter et al. (2010) used SPPPSPPP to define the class of extensins in their bioinformatic classification of HRGPs. The genetic encoding of the repetitive structures allows for substantial genetic drift without loss of function (Kieliszewski and Lamport, 1994), this being the reason why clear orthologies between individual vascular plant and chlorophyte extensins cannot be traced. The core arabinosylation machinery is clearly equivalent, however. Egelund et al. (2007) used the phylogenetic distances between family GT77 glycosyltransferases to deduce the function of the *Arabidopsis* GT77 clade A genes. This proposition that the genes encode extensin arabinosyltransferases, was later corroborated by extensin phenotypes of mutants knocked out in these genes (Velasquez et al., 2011). The prolyl hydroxylases described in that paper also have orthologs in the chlorophyte genomes and one from *Chlamydomonas* has been shown to be involved in cell wall biogenesis (Keskiaho et al., 2007) and the CAZy-database classifies *Chlamydomonas* gene BAF46284 to family GT75. The encoded protein is 68.6% identical to *Arabidopsis* At3g02230, one of the mutases that catalyzes the interconversion of UDP-Ara*p* and UDP-Ara*f* (Rautengarten et al., 2011), which we believe to be the donor substrate also of these arabinosyl transferases.

ULVOPHYCEAE: SULFATED POLYSACCHARIDES, FIBRILLAR POLYMERS, AND GLYCOPROTEINS

The Ulvophyceae area diverse assemblage of organisms including the best known marine seaweeds with siphonocladous, siphonous, and filamentous forms (Leliaert et al., 2012). Some of them have cellulose as a major fibrillar component of their cell walls. However, others possess (1→4)-β-mannans or (1→3)-β-xylans (Percival and McDowell, 1981; Painter, 1983; Yamagaki et al., 1997; Dunn et al., 2007; Estevez et al., 2009; Ciancia et al., 2012) and in some, the type of fibrillar polysaccharide is life cycle stage-dependent (Huizing and Rietema, 1975; Wutz and Zetsche, 1976; Huizing et al., 1979; Dunn et al., 2007).

These algae synthesize sulfated polysaccharides, an adaptation to the marine habitats that is also observed in many other marine organisms, including angiosperms and invertebrates (Aquino et al., 2011). These sulfated wall constituents may be classified into one of two groups as originally designated by Percival (1979): (1) uronic acid-rich polysaccharides also containing rhamnose, xylose, and sometimes galactose, and (2) uronic acid-limited polysaccharides consisting of major quantities of galactose, arabinose and, in some cases, xylose. The first group is represented by *Ulva*,

Monostroma, *Gayralia*, and *Acetabularia* (Ray, 2006; Chattopadhyay et al., 2007a; Dunn et al., 2007; Lahaye and Robic, 2007; Cassolato et al., 2008; Mao et al., 2008, 2009) and the second one includes *Caulerpa*, *Codium*, and *Bryopsis* (Bilan et al., 2007; Chattopadhyay et al., 2007b; Estevez et al., 2009; Ciancia et al., 2012).

Cell walls from the first group as represented by several *Ulva* species comprise two major polysaccharide components, soluble ulvans and cellulose, and two minor ones, an alkali-soluble linear xyloglucan and a glucuronan. Ulvan is the family of sulfated polysaccharides that consist of large quantities of glucuronic acid and rhamnose with the main repeating disaccharide being →4)-β-D-GlcA*p*-(1→4)-α-L-Rha*p*-(1→. α-L-Iduronic acid or β-xylose can replace glucuronic acid to a certain extent with the latter sugar also found as a side chain linked to C-2 of some rhamnose residues. Sulfation appears mostly on C-3 of the rhamnose units and on C-2 of the glucuronic acid side chains. Distribution of these polymers in the cell walls of the bi-seriated thallus of *Ulva* was determined by cytochemical and physicochemical data (Lahaye and Robic, 2007). These polymers are structurally related to glycosaminoglycans from animal tissues, like chondroitin sulfate or heparin, but they have glucosamine units instead of rhamnose in their backbone, and the sulfation pattern is different.

The second group is represented by coenocytic *Codium* species, which produce a 4-linked β-D-mannan as fibrillar component. Three different sulfated polysaccharide structures were isolated from *C. vermilara* including: (1) highly ramified sulfated and pyruvylated β-(1→3)-D-galactans, also present in other *Codium* species (Bilan et al., 2007; Ciancia et al., 2007; Farias et al., 2008; Ohta et al., 2009; Fernández et al., 2011a); (2) linear highly sulfated β-(1→3)-L-arabinans, with the arabinose units in the pyranose form (P. V. Fernandez, J. M. Estevez, A. S. Cerezo, and M. Ciancia, unpublished results); and (3) linear β-(1→4)-D-mannans partially sulfated mainly on C-2 of some of the mannose units (Fernández et al., 2011b). Also, HRGPs with characteristics similar to those of arabinogalactan proteins (AGPs) and extensins from vascular plants were detected in these cell walls (Estevez et al., 2009; Fernández et al., 2011a). Distribution of β-mannans and HRGP-like epitopes was shown to be found in two distinct cell wall layers, whereas sulfated polysaccharides were distributed in the middle area of the wall.

CHAROPHYCEAE: CELL WALLS AND THE ORIGIN OF LAND PLANTS

The Charophyceae or CGA are the extant group of green algae most closely related and ancestral to land plants. Over the past decade, a surge of research activity focusing on the walls of CGA taxa has revealed important insight into cell wall structure and evolution (Popper and Fry, 2003; Domozych et al., 2007, 2009; Eder et al., 2008; Eder and Lutz-Meindl, 2010; Popper and Tuohy, 2010; Sørensen et al., 2010, 2011; Popper et al., 2011). First, the cell walls of taxa of the early divergent CGA such as the Chlorokybales and Klebsormidiales, do not possess most of the polymers commonly found in late divergent CGA and land plants. The basal clade of CGA, the Mesostigmales, does not even have a cell wall but rather produces layers of scales (Domozych et al., 1991). Second, the late divergent clades, the Charales, Coleochaetales, and Zygnematales, possess cell walls with notable similarity to those of land plants. Polysaccharides like cellulose, pectins (including homogalacturonans and rhamnogalacturonan-I), β-(1-3) glucans, and hemicellulosic polymers like xyloglucans, mannans, and xylans are found in the cell wall of these advanced CGA. Mixed linkage glucans or MLGs that were once thought to be found only in members of the grass family of angiosperms, were also found in Zygnematalean taxa, demonstrating a more widespread occurrence in green plants. Also surprising is the presence of lignin-like compounds in the cell walls of several species of the genus *Coleochaete*.

Arabinogalactan proteins and extensin have also been described in many CGA taxa. Extensins surprisingly are not detected in *Nitella* and *Chara* (Charales; Gotteli and Cleland, 1968). Arabinosyltransferases involved in extensin arabinosylation belong to the GT77 A- and C-clades sensu Petersen et al. (2011) and it is remarkable that the *Chara* transcriptome (courtesy of Gernot Glöckner, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin) as well as that of *Nitella hyalina* (Timme et al., 2012) each have a putative member in family GT77 clade D, but none in clade A or C. The transcriptome of *Nitella*, but not that of *Chara* comprises putative prolyl hydroxylases allowing for the existence of AGPs even though extensins are missing. It thus appears that members of Charales have evolved and separated themselves significantly from other members of the CGA, notably the Zygnematales and Coleochaetales which feature cell walls that more closely resemble that of vascular plants and which are known to express enzymes involved in cell wall metabolism akin to that observed in angiosperms (e.g., Vannerum et al., 2011). These latter similarities suggest that late divergent taxa of the CGA (i.e., their ancestors 470 million years ago) may have possessed cell wall characteristics that pre-adapted them for successful emergence onto and life on land.

THE NEXT GOALS?

More detailed characterization of the various taxa will be required before we can fully understand the evolution of extracellular coverings of green plants as well as adaptations to ECM chemistry in response to life in marine, freshwater, and terrestrial habitats. Some specific questions and areas of focus for future study include:

(1) Insights into the polysaccharide biosynthetic machinery of CGA are and will at the same time offer insights into the evolutionary events that accompanied adaptation to life on land. This will require full genomic sequencing as transcriptomic analysis is useful for demonstrating the functional expression of genes, but not for the absence. This endeavor offers many challenges as many relevant CGA genomes are estimated to be as big as if not bigger than that of *Arabidopsis* (Kapraun, 2007).
(2) Detailed analysis of the roles of cell wall polymers in the CGA is critical. Although the CGA share many cell wall constituents with their embryophyte descendents, it is not clear if they are used in equivalent roles.
(3) Sulfated polysaccharides from ulvophyte seaweeds have only recently been reexamined and have yet to be widely used in the hydrocolloid industry. Ulvan has been investigated as

potential dietary fiber for human diet (Lahaye and Robic, 2007) due to its medical properties and potentially profitable extraction yields from harvested specimens. In addition, ulvans have some interesting biological activities, including acting as antioxidants, modifying certain macrophage activities, and serving as potential anti-hyperlipidemic agent (Wijesekara et al., 2011). Sulfated polysaccharides from the Bryopsidales are not obtained in large yields (Ciancia et al., 2007, 2012), but have piqued interest as bioactive compounds with several potential pharmacological applications (Ohta et al., 2009; Ciancia et al., 2010; Costa et al., 2010; Lee et al., 2010). More detailed characterization of these molecules is needed in order to fully recognize their potential.

(4) Comprehensive chemical and functional screening of the large but virtually unknown polysaccharide complexes secreted outside the cell walls of many Zygnematalean taxa (Domozych et al., 2005) will be important for understanding their physiology and importance to ecosystem dynamics.

The study of green algal cell walls and other coverings has now entered a truly exciting phase whereby new methodologies especially from the biochemical and molecular fronts are allowing for detailed resolution of wall polymers. While evolutionary and cell biology-based studies have been and will continue to be driving forces for this, the study of green algal coverings represents a critical step for emerging applied technologies as green algae are being, or will be, used as food sources for humans and domesticated animals, hydrocolloids for the food and pharmaceutical industry, bioactive compounds for medicinal use and starting material for biofuels.

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REFERENCES

Adair, W. S., Steinmetz, S. A., Mattson, D. M., Goodenough, U. W., and Heuser, J. E. (1987). Nucleated assembly of *Chlamydomonas* and *Volvox* cell walls. *J. Cell Biol.* 105, 2373–2382.

Aquino, R. S., Grativol, C., and Mourão, P. A. S. (2011). Rising from the sea: correlations between sulfated polysaccharides and salinity in plants. *PLoS ONE* 6, e18862. doi:10.1371/journal.pone.0018862.

Becker, B., Becker, D., Kamerling, J. P., and Melkonian, M. (1991). 2-Keto-sugar acids in green flagellates: a chemical marker for prasinophycean scales. *J. Phycol.* 27, 498–504.

Becker, B., and Marin, B. (2009). Streptophyte algae and the origin of embryophytes. *Ann. Bot.* 103, 999–1004.

Becker, B., Marin, B., and Melkonian, M. (1994). Structure, composition, and biogenesis of prasinophyte cell coverings. *Protoplasma* 181, 233–244.

Bilan, M. I., Vinogradova, E. V., Shashkov, A. S., and Usov, A. I. (2007). Structure of a highly pyruvylated galactan sulfate from the pacific green alga *Codium yezoense* (Bryopsidales, Chlorophyta). *Carbohydr. Res.* 342, 586–596.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., Lindquist, E., Lucas, S., Pangilinan, J., Polle, J., Salamov, A., Terry, A., Yamada, T., Dunigan, D. D., Grigoriev, I. V., Claverie, J. M., and Van Etten, J. L. (2010). The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. *Plant Cell* 22, 2943–2955.

Carpita, N. C., Defernez, M., Findlay, K., Wells, B., Shoue, D. A., Catchpole, G., Wilson, R. H., and McCann, M. C. (2001). Cell wall architecture of the elongating maize coleoptile. *Plant Physiol.* 127, 551–565.

Cassolato, J. E. F., Noseda, M. D., Pujol, C. A., Pellizzari, F. M., Damonte, E. B., and Duarte, M. A. R. (2008). Chemical structure and antiviral activity of the sulfated heterorhamnan isolated from the green seaweed *Gayralia oxysperma*. *Carbohydr. Res.* 343, 3085–3095.

Chattopadhyay, K., Mandal, P., Lerouge, P., Driouich, A., Ghosal, P., and Ray, B. (2007a). Sulphated polysaccharides from Indian samples of *Enteromorpha compressa* (Ulvales, Chlorophyta): isolation and structural features. *Food Chem.* 104, 928–935.

Chattopadhyay, K., Adhikari, U., Lerouge, P., and Ray, B. (2007b). Polysaccharides from *Caulerpa racemosa*: purification and structural features. *Carbohydr. Polym.* 68, 407–415.

Ciancia, M., Alberghina, J., Arata, P. X., Benavides, H., Leliaert, F., Verbruggen, H., and Estevez, J. M. (2012). Characterization of cell wall polysaccharides of the coenocytic green seaweed *Bryopsis plumosa* (Bryopsidaceae, Chlorophyta) from the Argentine coast. *J. Phycol.* 48, 326–335.

Ciancia, M., Quintana, I., and Cerezo, A. S. (2010). Overview of anticoagulant activity of sulfated polysaccharides from seaweeds in relation to their structures, focusing on those of green seaweeds. *Cur. Med. Chem.* 17, 2503–2529.

Ciancia, M., Quintana, I., Vizcargüénaga, M. I., Kasulin, L., de Dios, A., Estevez, J. M., and Cerezo, A. S. (2007). Polysaccharides from the green seaweeds *Codium fragile* and C. *vermilara* with controversial effects on hemostasis. *Int. J. Biol. Macromol.* 41, 641–649.

Cordeiro, L. M., Carbonero, E. R., Sassaki, G. L., Reis, R. A., Stocker-Worgotter, E., Gorin, P. A. J., and Iacomini, M. (2006). A fungus-like β-galactofuranan in the cultivated *Trebouxia* photobiont of the lichen *Ramalina gracilis*. *FEMS Microbiol. Lett.* 244, 193–198.

Costa, L. S., Fidelis, G. P., Cordeiro, S. L., Oliveira, R. M., Sabry, D. A., Câmara, R. B. G., Nobre, L. T. D. B,. Costa, M. S. S. P., Almeida-Lima, J., Farias, E. H. C., Leite, E. L., and Rocha, H. A. O. (2010). Biological activities of sulfated polysaccharides from tropical seaweeds. *Biomed. Pharmacother.* 64, 21–28.

Delwiche, C. F., and Timme, R. E. (2011). Plants. *Cur. Biol.* 21, R417–R423.

Domozych, D. S., Brechka, H., Britton, A., and Toso, M. (2011). Cell wall growth and modulation dynamics in a model unicellular green alga – *Penium margaritaceum*: live cell labeling with monoclonal antibodies. *J. Bot.* doi:10.1155/2011/632165

Domozych, D. S., Kort, S., Benton, S., and Yu, T. (2005). The extracellular polymeric substance of the green alga *Penium margaritaceum* and its role in biofilm formation. *Biofilms* 2, 1–16.

Domozych, D. S., Lambiasse, L., Kiemle, S. N., and Gretz, M. R. (2009). Cell-wall development and bipolar growth in the desmid *Penium margaritaceum* (Zygnematophyceae, Streptophyta). Asymmetry in a symmetric world. *J. Phycol.* 45, 879–893.

Domozych, D. S., Serfis, A., Kiemle, S. N., and Gretz, M. R. (2007). The structure and biochemistry of charophycean cell walls. I. Pectins of *Penium margaritaceum*. *Protoplasma* 230, 99–115.

Domozych, D. S., Wells, B., and Shaw, P. J. (1991). The basket scales of the green alga *Mesostigma viride*: chemistry, immunology and ultrastructure. *J. Cell Sci.* 100, 397–407.

Dunn, E. K., Shoue, D. A., Huang, X., Kline, R. E. L., MacKay, A. L., Carpita, N. C., Taylor, I. E. P., and Mandoli, D. F. (2007). Spectroscopic and biochemical analysis of regions of the cell wall of the unicellular 'mannan weed', *Acetabularia acetabulum*. *Plant Cell Physiol.* 48, 122–133.

Eder, M., and Lutz-Meindl, U. (2010). Analyses and localization of pectin-like carbohydrates in cell wall and mucilage of the green alga *Netrium digitus*. *Protoplasma* 243, 25–38.

Eder, M., Tenhaken, R., Driouich, A., and Lutz-Meindl, U. (2008). Occurrence and characterization of arabinogalactan-like proteins and hemicelluloses in *Micrasterias* (Streptophyta). *J. Phycol.* 44, 1221–1234.

Egelund, J., Obel, N., Ulvskov, P., Geshi, N., Pauly, M., Bacic, A., and Petersen, B. L. (2007). Molecular characterization of two *Arabidopsis thaliana* glycosyltransferase mutants, rra1 and rra2, which have a reduced residual arabinose content in a polymer tightly associated with the cellulosic wall residue. *Plant Mol. Biol.* 64, 439–451.

Estevez, J. M., Leonardi, P. I., and Alberghina, J. S. (2008). Cell wall carbohydrate epitopes in the green alga Oedogonium bharuchae *F. minor* (Oedogoniales, Chlorophyta). *J. Phycol.* 44, 1257–1268.

Estevez, J. M, Kasuli, L., Fernandez, P. V., Dupree, P., and Ciancia, M. (2009). Chemical in situ characterization of macromolecular components of the complex cell walls from the coenocytic green alga *Codium fragile*. *Glycobiology* 18, 250–259.

Farias, E. H. C., Pomin, V. H., Valente, A. P., Nader, H. B., Rocha, H. A. O., and Mourão, P. A. S. (2008). A preponderantly 4-sulfated, 3-linked galactan from the green alga *Codium isthmocladum*. *Glycobiology* 18, 250–259.

Fernández, P. V., Ciancia, M., and Estevez, J. M. (2011a). Cell wall variability in the green seaweed *Codium vermilara* (Bryopsidales Chlorophyta) from the Argentine coast. *J. Phycol.* 47, 802–810.

Fernández, P. V., Ciancia, M., Miravalles, A. B., and Estevez, J. M. (2011b). Cell-wall polymer mapping in the coenocytic macroalga *Codium vermilaria* (Bryopsidales, Chlorophyta). *J. Phycol.* 46, 456–465.

Finet, C., Timme, R. E., Delwiche, C. F., and Marlétaz, F. (2010). Multigene phylogeny of the green lineage reveals the origin and diversification of land plants. *Cur. Biol.* 20, 2217–2222.

Gotteli, L. B., and Cleland, R. (1968). Differences in the occurrence and distribution of hydroxyproline-proteins among the algae. *Am. J. Bot.* 55, 907–914.

Huizing, H. J., and Rietema, H. (1975). Xylan and mannan as cell wall constituents of different stages in the life-histories of some siphoneous green algae. *Br. Phycol. J.* 10, 13–16.

Huizing, H. J., Rietema, H., and Sietsma, J. H. (1979). Cell wall constituents of several siphoneous green algae in relation to morphology and taxonomy. *Br. Phycol. J.* 14, 25–32.

Imam S. H., Buchanan, M. J., Shin, H.-C., and Snell, W. J. (1985). The *Chlamydomonas* cell wall: characterization of the wall framework. *J. Cell Biol.* 101, 1599–1607.

Kapraun, D. F. (2007). Nuclear DNA content estimates in green algal lineages: Chlorophyta and Streptophyta. *Ann. Bot.* 99, 677–701.

Keskiaho, K., Hieta, R., Sormunen, R., and Myllyharju, J. (2007). *Chlamydomonas reinhardtii* has multiple prolyl 4-hydroxylases, one of which is essential for proper cell wall assembly. *Plant Cell* 19, 256–269.

Kieliszewski, M. J., and Lamport, D. T. A. (1994). Extensin: repetitive motifs, functional sites, posttranslational codes and phylogeny. *Plant J.* 5, 157–172.

Kirk, D. L. (1998). *Volvox*. New York: Cambridge University Press.

Lahaye, M., and Robic, A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules* 8, 1766–1774.

Lee, J.-B., Ohta, Y., Hayashi, K., and Hayashi, T. (2010). Immunostimulating effects of a sulfated galactan from *Codium fragile*. *Carbohydr. Res.* 345, 1452–1454.

Leliaert, F., Smith, D. R., Moreau, H., Herron, M., Verbruggen, H., Delwiche, C. F., and De Clerck, O. (2012). Phylogeny and molecular evolution of the green algae. *Crit. Rev. Plant Sci.* 31, 1–46.

Leliaert, F., Verbruggen, H., and Zechman, F. W. (2011). Into the deep: new discoveries at the base of the green plant phylogeny. *Bioessays* 33, 683–692.

Lewis, L. A., and McCourt, R. M. (2004). Green algae and the origin of land plants. *Am. J. Bot.* 91, 1535–1556.

Mao, W., Li, H., Li, Y., Zhang, H., Qi, X., Sun, H., Chen, Y., and Guo, S. (2009). Chemical characteristic and anticoagulant activity of the sulfated polysaccharide isolated from *Monostroma latissimum* (Chlorophyta). *Int. J. Biol. Macromol.* 44, 70–74.

Mao, W.-J., Fang, F., Li, H.-Y., Qi, X.-H., Sun, H.-H., Chen, Y., and Guo, S.-D. (2008). Heparinoid-active two sulfated polysaccharides isolated from marine green algae *Monostroma nitidum*. *Carbohydr. Polym.* 74, 834–839.

Moestrup, O., and Walne, P. L. (1979). Studies on scale morphogenesis in the Golgi apparatus of *Pyramimonas tetrarhynchus* (Prasinophyceae). *J. Cell Sci.* 36, 437–459.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J., Knox, J. P., Bacic, T., and Willats W. G. T. (2007). High-throughput mapping of cell wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Ohta, Y., Lee, J.-B., Hayashi, K., and Hayashi, T. (2009). Isolation of sulfated galactan from *Codium fragile* and its antiviral effect. *Biol. Pharm. Bull.* 32, 892–898.

Painter, T. J. (1983). "Algal polysaccharides," in *The Polysaccharides*, Vol. 2, ed. G. O. Aspinall (New York, NY: Academic Press), 827–837.

Percival, E. (1979). The polysaccharides of green, red, and brown seaweeds: their basic structure, biosynthesis and function. *Br. Phycol. J.* 14, 103–117.

Percival, E., and McDowell, R. H. (1981). "Algal walls. Composition and biosynthesis," in *Encyclopedia of Plant Physiology*, Vol. 13B, eds W. Tanner and F. A. Loewus (Berlin: Springer), 277–316.

Petersen, B. L., Faber, K., and Ulvskov, P. (2011). "Glycosyltransferases of the GT77 family," in *Annual Plant Reviews: Plant Polysaccharides, Biosynthesis, and Bioengineering*, ed. P. Ulvskov (New York: Blackwell), 305–320.

Piganeau, G., Grimsley, N., and Moreau, H. (2011). Genome diversity in the smallest marine photosynthetic eukaryotes. *Res. Microbiol.* 62, 570-577

Popper, Z. A., and Fry, S. C. (2003). Primary cell wall composition of bryophytes and charophytes. *Ann. Bot.* 91, 1–12.

Popper, Z. A., Michel, G., Herve, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant Biol.* 62, 567–590.

Popper, Z. A., and Tuohy, M. G. (2010). Beyond the green: understanding the evolutionary puzzle of plant and algal cell walls. *Plant Physiol.* 153, 373–383.

Rautengarten, C., Ebert, B., Herter, T., Petzoldc, C. J., Ishii, T., Mukhopadhyay, A., Usadel, B., and Schellera, H. V. (2011). The interconversion of UDP-Arabinopyranose and UDP-Arabinofuranose is indispensable for plant development in *Arabidopsis*. *Plant Cell* 23, 1373–1390.

Ray, B. (2006). Polysaccharides from *Enteromorpha compressa*: isolation, purification and structural features. *Carbohydr. Polym.* 66, 408–416.

Rodrigues, M. A., and da Silva Bon, E. P. (2011). Evaluation of *Chlorella* (Chlorophyta) as source of fermentable sugars via cell wall enzymatic hydrolysis. *Enzyme Res.* doi:4061/2011/405603

Showalter, A. M., Keppler, B., Lichtenberg, J., Gu, D., and Welch, L. R. (2010). A bioinformatics approach to the identification, classification, and analysis of hydroxyproline-rich glycoproteins. *Plant Physiol.* 153, 485–513.

Sørensen, I., Domozych, D., and Willats, W. G. T. (2010). How have plant cells walls evolved? *Plant Physiol.* 153, 366–372.

Sørensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F., O'Neill, M. A., Fei, Z., Rose, J. K. C., Domozych, D. S., and Willats, W. G. T. (2011). The Charophycean green algae provide insights into early origin of plant cell walls. *Plant J.* 153, 366–372.

Sørensen, I., Rose, J. K. C., Doyle, J. J., Domozych, D. S., and Willats, W. G. T. (2012). The Charophycean green algae as model systems to study plant cell walls and other evolutionary adaptations that gave rise to land plant. *Plant Signal. Behav.* 7, 1–3.

Timme, R. E., Bachvaroff, T. R., and Delwiche, C. F. (2012). Broad phylogenomic sampling and the sister lineage of land plants. *PLoS ONE* 7, e29696. doi: 10.1371/journal.pone.0029696

Timme, R. E., and Delwiche, C. F. (2010). Uncovering the evolutionary origin of plant molecular processes: comparison of *Coleochaete* (Coleochaetales) and *Spirogyra* (Zygnematales) transcriptomes. *BMC Plant Biol.* 10, 96. doi:10.1186/1471-2229-10-96

Vannerum, K., Huysman, M. J. J., De Rycke, R., Vuylsteke, M., Leilaert, F., Pollier, J., Lutz-Meindl, U., Gillard, J., DeVeylder, L., Goossens, A., Inze, D., and Vyverman, W. (2011). Transcriptional analysis of cell growth and morphogenesis in the unicellular green alga *Micrasterias* (Streptophyta), with emphasis on the role of expansin. *BMC Plant Biol.* 11, 128. doi:10.1186/1471-2229-11-128

Velasquez, S. M., Ricardi, M. M., Dorosz, J. G., Fernandez, P. V., Nadra, A. D., Pol-Fachin, L., Egelund, J., Gille, S., Harholt, J., Ciancia, M., Verli, H., Pauly, M., Bacic, A., Olsen, C. E., Ulvskov, P., Petersen, B. L., Somerville, C., Iusem, N. D., and Estevez, J. M. (2011). O-glycosylated cell wall proteins are essential in root hair growth. *Science* 332, 1401–1403.

Voigt, J., Liebich, I., Kieß, M., and Frank, R. (2001). Subcellular distribution of 14-3-3 proteins in the unicellular green alga *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 268, 6449–6457.

Voigt, J., Woestemeyer, J., and Frank, R. (2007). The chaotrope-soluble glycoprotein GP2 is a precursor of the insoluble glycoprotein framework of the *Chlamydomonas* cell wall. *J. Biol. Chem.* 282, 30381–30392.

Wijesekara, I., Pangestuti, R., and Kim, S.-K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydr. Polym.* 84, 14–21.

Wodnick, S., Brinkmann, H., Glockner, G., Heidel, A. J., Philippe, H., Melkonian, M., and Becker, B. (2011). Origin of land plants: do conjugating green algae hold the key? *BMC Evol. Biol.* 11, 104. doi:10.1186/1471-2148-11-104

Wutz, M., and Zetsche, K. (1976). Zur Biochimie und Regulation des Heteromorphen Generationswechsels der Grünalge. *Planta* 129, 211–216.

Yamagaki, T., Maeda, M., Kanazawa, K., Ishizuka, Y., and Nakanishi, H. (1997). Structural clarification of Caulerpa cell wall β-(1→3)-xylan by NMR spectroscopy. *Biosci. Biotechnol. Biochem.* 61, 1077–1080.

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The cell wall-associated kinases, WAKs, as pectin receptors

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The wall-associated kinases, WAKs, are encoded by five highly similar genes clustered in a 30-kb locus in *Arabidopsis*. These receptor-like proteins contain a cytoplasmic serine threonine kinase, a transmembrane domain, and a less conserved region that is bound to the cell wall and contains a series of epidermal growth factor repeats. Evidence is emerging that WAKs serve as pectin receptors, for both short oligogalacturonic acid fragments generated during pathogen exposure or wounding, and for longer pectins resident in native cell walls. This ability to bind and respond to several types of pectins correlates with a demonstrated role for WAKs in both the pathogen response and cell expansion during plant development.

Keywords: WAKs, pectin, cell wall, receptor kinase, pathogen response, cell expansion

INTRODUCTION

The wall-associated kinases, or WAKs, are receptor-like kinases that are linked to the pectin fraction of the cell wall, and have a cytoplasmic protein kinase domain. WAKs are required for cell expansion, are involved in the pathogen response, and their expression is activated by numerous environmental stimuli. Recent work supports the idea that WAKs are receptors for both pectin in the cell wall, and for pectin fragments, oligogalacturonic acids (OGs), generated during some pathogen attacks. The history of WAK isolation, and their characterization that led to the idea that WAKs are OG and pectin receptors, follows.

IDENTIFICATION OF WAKs

The WAKs were identified though the chance isolation of their cDNAs that subsequently led to the recognition of their significance to the cell wall (Kohorn et al., 1992; He et al., 1996; Kohorn, 2000, 2001). The WAKs are defined as cell wall-associated receptors by several key observations. WAKs could only be extracted from cells by boiling in detergent and reductant and appeared cross-linked to insoluble material. In addition, electron micrographs with specific WAK-kinase antiserum showed WAK protein in the cell wall and on the plasma membrane. Yet protease treatment of protoplasts and Western blots revealed that the kinase domain was cytoplasmic, and the receptor crossed the membrane such that the epidermal growth factor (EGF) domain is placed in the extracellular space (He et al., 1996). The five *WAK* genes are tightly clustered in tandem in a 30-kb locus on chromosome 1 (He et al., 1999), their encoded kinase domains are 85% identical, and the extracellular regions shows up to 65% identity (He et al., 1999). However, all WAKs contain the same conserved spacing of cysteine residues in the extracellular domain, the hallmark of the EGF repeat of metazoans (Sampoli Benitez and Komives, 2000).

WAK-LIKE GENES

There are at least 21 other genes for receptor-like kinases that contain EGF repeats in *Arabidopsis*, and these have been coined WAK-like or *WAKL* genes (Verica and He, 2002; Verica et al., 2003). In crop plants, the *WAKLs* have greatly expanded in number and are often present in several large clusters. The expansion and size of this family indicates their importance, and some have suggested they play a role in pathogen resistance (Verica and He, 2002; Zhang et al., 2005). Stronger evidence for this idea is provided by the observation that dominant mutant alleles of *WAKLs* in *Arabidopsis* provide resistance to *Fusarium*, and a rice *WAKL* allele is less sensitive to rice blast disease (Diener and Ausubel, 2005; Li et al., 2009). Despite their importance and interesting biology, there is insufficient evidence to conclude that the WAKL proteins are wall-associated. The *WAKLs* instead may describe a class of plant receptor that is defined by the presence of EGF repeats, and whose additional domains and perhaps function has diverged from the *WAKs*. One might expect, therefore, their functions to be accordingly diverse.

WAKs ARE REQUIRED FOR CELL EXPANSION

Several lines of evidence point to a role of WAKs in cell expansion. Antisense *WAK* RNA to the conserved WAK-kinase domain can be induced using the Dex system, and this leads to a 50% reduction in WAK protein levels and a smaller cell size, rather then fewer cells. Thus, WAKs are required for cell elongation (Anderson et al., 2001; Lally et al., 2001; Wagner and Kohorn, 2001). A *WAK2* null allele, *wak2-1*, causes a loss of cell expansion in roots, but only under limiting sugar and salt conditions (Kohorn et al., 2006b). Individual loss of function alleles in any of the four other *WAKs* do not result in an obvious phenotype, and unfortunately genetic analysis of the *Arabidopsis WAKs* has been greatly hampered by the fact that all five genes lie in a 30-kb cluster, making it difficult to create double or triple mutants of the isoforms (He et al., 1999).

A survey of enzyme activity showed that in roots of the *wak2-1* null mutants there was a reduction in vacuolar invertase activity. Since invertase RNA levels are also affected, it suggests that WAK2 can regulate invertase at the transcriptional level. These results support a model where WAKs regulate cell

expansion through a control of sugar concentration and thus perhaps turgor control. Consistent with this model is the observation that the *wak2-1* phenotype could also be rescued by the expression of sucrose phosphate synthase which alters sugar sinks (Kohorn et al., 2006b). What remained was to establish how WAKs might be sensing the cell wall to effect the turgor regulated cell expansion.

WAKs BIND TO PECTIN

Wall-associated kinases are plasma membrane receptors, appear to be cross-linked to cell wall material, and can be seen by electron microscopy to lie in the cell wall. Pectinase, but not cellulase or other cell wall degrading enzymes release WAKs from the cell wall material. This led to the initial suggestion that WAKs are bound to pectins (Anderson et al., 2001; Wagner and Kohorn, 2001). Indeed, extracted WAK protein is still bound to a pectin epitope on denaturing gels, suggesting a covalent binding to a pectic fragment (Anderson et al., 2001; Wagner and Kohorn, 2001). The purified extracellular domains of WAK1 or 2 bind to pectin *in vitro*, and have a higher affinity for de-esterified pectin than to esterified molecules that did not have as negative a charge. Moreover, short pectin fragments of degree of polymerization (dp) 9–15 effectively competed with longer pectins for WAK binding (Decreux and Messiaen, 2005; Kohorn et al., 2006b, 2009). Mutation of the positively charged residues in WAK1 to neutral amino acids lead to a loss of binding to de-esterified pectin suggesting that the interaction was in part charge based (Decreux et al., 2006). These *in vitro* results provided confirmation of a WAK–pectin association, but also a puzzle as to why the WAK isolated from plants after pectinase treatment, had a covalent attachment to pectin. Moreover, it raised the question as to what type of pectin WAK was binding to and was perhaps responsive to in plants. *In vitro*, both WAK1 and 2 bind to a variety of pectins including polymers of homogalacturonan (HA), OGs, and to rhamnogalacturonans (RG) I and II (Kohorn et al., 2009). The common feature of these molecules is presence of a galacturonic acid backbone, and this is therefore the predicted target of WAKs. However, the backbone of RGII is not HA, suggesting that the binding requirements are not to a simple polymer of HA, but perhaps the presence galacturonic acid.

An analysis of WAK-GFP expression noted that WAK is cross-linked into a detergent-insoluble complex within the Golgi which contains pectin, and this is independent of fucose modification or cellulose synthesis. Thus, the assembly and crosslinking of WAKs may begin at an early stage within a cytoplasmic compartment rather than in the cell wall itself, and is coordinated with synthesis of surface cellulose (Kohorn et al., 2006a).

The biological activity of pectin fragments, or OGs, has been recorded for many years, with suggestions of a role in both defense and stress responses, and in developmental processes (Yamazaki et al., 1983; Willats et al., 2001; Mohnen, 2008; Harholt et al., 2010). Pathogen invasion, physical wounding, and herbivory have the ability to generate OGs in the cell wall and it has been suggested by many that there was a specific receptor for these OGs. WAKs may indeed be this receptor, but how it distinguishes OGs from nascent pectins is not clear. The *in vitro* binding data raise several other interesting questions. Pectin can be de-esterified once secreted into the cell wall by a family of methyl esterases, revealing a negative charge that permits a calcium-induced crosslinking with other pectins, and perhaps other carbohydrates and proteins in the wall (Bosch and Hepler, 2005; Krichevsky et al., 2007). Numerous reports have also documented an unequal distribution of esterified and de-esterified pectins in a variety of cell types, and correlations between the degree of esterification and cell enlargement have been observed (Willats et al., 2001; Mohnen, 2008; Caffall and Mohnen, 2009; Wolf et al., 2009; Harholt et al., 2010). How the differential binding of WAKs to esterified and de-esterified pectin relates to the unequal distribution has yet to be explored. WAKs in most cells appear to be uniformly distributed (He et al., 1996) as viewd by low resolution microscopy, but further imaging is needed. In the emerging pollen tube WAK epitopes are only detected near the tip. WAKs are present in the growing pollen tube, and its relationship to pectin deposition and directional growth also needs more exploration.

WAKs ARE RESPONSIVE TO PECTINS

Several recent lines of evidence indicate that the binding of pectin to WAKs activates several signaling pathways. The first is that pectin treatment of protoplasts causes the induction and repression of hundreds of genes involved in cell wall biogenesis and stress responses, and this response is blocked in cells lacking WAK2 (Kohorn et al., 2006b, 2009). An invertase promoter-RFP reporter in protoplasts was used to show that pectin can activate invertase expression, but not in a *wak2-1* null. While there may be additional proteins in the membrane that mediate the pectin response, the data are consistent with WAK1 and 2 serving as pectin receptors. This suggestion received more support from experiments in which the WAK1 extracellular domain was fused to a different cytoplasmic kinase, EFR, creating a novel chimeric receptor. The EFR kinase domain is known to activate ROS accumulation, a rise in ethylene levels, and a change in gene expression and these served as a recordable down stream pathway for the WAK extracellular domain (Brutus et al., 2010). Indeed, OGs activated a pathway downstream of the chimeric WAK-EFR kinase, when the chimera was transiently expressed in tobacco leaves. While these experiments entail a chimeric and hence novel receptor in a heterologous tissue, and the downstream readouts are also in part activated by both the native EFR kinase and the WAK kinase, they do support the idea that WAKs are an OG receptor. Detailed structural analysis and kinase activation studies are needed to understand how OG, or longer pectins can bind to WAKs to actually activate the protein kinase domain. These reports do not address the question of how WAKs might distinguish longer pectin polymers present in the cell wall from newly generated and bioactive OGs. Indeed, the ability to bind different types of pectins mirrors the bi-functional role of WAKs in pathogenesis and normal development, and is discussed further below.

PROTEIN LIGANDS?

In addition to OGs and pectins, WAK may also bind to glycine-rich proteins (GRPs) of the cell wall. GRP3 interacts in a yeast two-hybrid assay with an extracellular domain of WAK1 that does not contain the EGF repeats (Park et al., 2001). The WAK–GRP3

interaction is specific to WAK1 but not WAK2 or 4 in the yeast two-hybrid assay (Anderson et al., 2001). GRPs are a large family of secreted proteins in *Arabidopsis* and it is possible that different family members interact with different WAKs (Anderson et al., 2001; Mousavi and Hotta, 2005; Mangeon et al., 2010). It is believed that GRPs are structural cell wall proteins (Ringli et al., 2001), and since they might bind WAKs they may also have a signaling capacity. Both GRP3 and WAK1 can be isolated in the same 450 kDa complex in *Arabidopsis* extracts, yet this result is surprising given that WAK1 is normally not detected as a water soluble protein, and further analysis is needed to confirm this result (Park et al., 2001). Blue native gel analysis of protein complexes fails to identify this WAK–GRP complex, and indeed the relationship between GRP, pectin, and WAKs warrants more exploration (Anderson et al., 2001).

WAKs AND THE STRESS RESPONSE

Wall-associated kinases are clearly required for cell expansion but they are also involved in the response to pathogen and stress. WAK expression is induced by wounding, pathogen infection, and by many stresses such as ozone and heavy metals (He et al., 1998; Anderson et al., 2001; Kohorn, 2001; Wagner and Kohorn, 2001; Sivaguru et al., 2003). This induced expression, however, is late in the signaling response and does not necessarily indicate a role for the WAK receptor itself. Dominant alleles of *WAK1* that lack the extracellular domain did cause a resistance to high levels of salicylate, and antisense *WAK1* plants had increased sensitivity to salicylate (He et al., 1998). While it was not clear how WAKs were involved, the work did establish a connection with pathogenesis, and reinforced the need to explore the role that pathogen-induced OGs might have in regulating WAKs. More recently a direct role for WAKs in the pathogen response was established. A dominant allele of *WAK2*, *WAK2cTAP*, was observed to cause ectopic lesions, ROS accumulation, curling of leaves, and stunted growth, all hallmarks of a pathogen effect, yet in the absence of pathogen (Kohorn et al., 2009, 2011). *WAK2cTAP* alleles mutant in either the pectin binding domain, the EGF region, or in the catalytic kinase site do not induce these phenotypes indicating that active receptor function is required. Moreover, the WAK2cTAP receptor is still responsive to pectin stimulation, and appears to be a hyper-activated allele (Kohorn et al., 2011).

MPKs

MPK3 activity is elevated in cells treated with long polymers of pectin (Kohorn et al., 2009), and OGs have been shown to activate MPK3 (Moscatiello et al., 2006; Andreasson and Ellis, 2010). The idea that WAKs serve as pectin receptors is supported by the observation that plants homozygous for the null allele *wak2-1* showed a reduction in the activation of MPK3 relative to wild-type (Kohorn et al., 2009). Moreover, plants expressing the *WAK2cTAP* dominant allele have a constitutively active MPK3 and 6. A *WAK2cTAP* allele in a *mapk3*$^{-/-}$ background had more severe growth defects than *WAK2cTAP* alone, supporting the concept that MPK3 is required for downstream WAK2 signaling (Kohorn et al., 2009). In contrast, a *mpk6* null allele is able to suppress the effects of this dominant *WAK2* mutation, thus distinguishing MPK3 and MPK6 whose activity previously was thought to be redundant (Andreasson and Ellis, 2010; Kohorn et al., 2011). These results suggest that WAKs can activate different downstream pathways depending upon the state of the wall, and a model is presented below. However, an analysis of other MPKs is still needed to fully understand WAK-MPK signaling.

WAKs DISTINGUISH THE STATE OF PECTIN: A MODEL

Wall-associated kinases are bound to pectin in native cell walls and their activity is required for normal cell expansion, yet OGs also bind to WAKs and mediate a response to pathogens and wounding (Kohorn et al., 2006b, 2009, 2011; Denoux et al., 2008; Brutus et al., 2010). Taken together, the results are consistent with a model presented in **Figure 1**. Here the type and concentration of pectin present in the wall could lead to a WAK-dependent activation of different signaling pathways. Unchallenged but expanding walls would preferentially activate via WAKs a cell expansion path that includes MPK3 (that other receptors are signaling via MPK3 and 6 is indicated by additional arrows). When OGs are generated by a wall disturbance, the WAKs may alter their signaling path to help effect the stress response by now also activating MPK6 and a new downstream response (**Figure 1**, right side). That the *in vitro* binding assays reveal a higher binding affinity of OGs than longer polymers for WAK (Decreux and Messiaen, 2005; Decreux et al., 2006) suggests a mechanism by which WAKs can switch from binding the native cell wall pectin to OGs, thus distinguishing types of pectin. Differential activation by various pectins might be achieved by the specific pectin affinity of an individual receptor, or perhaps combinations of WAKs with as yet unidentified partners.

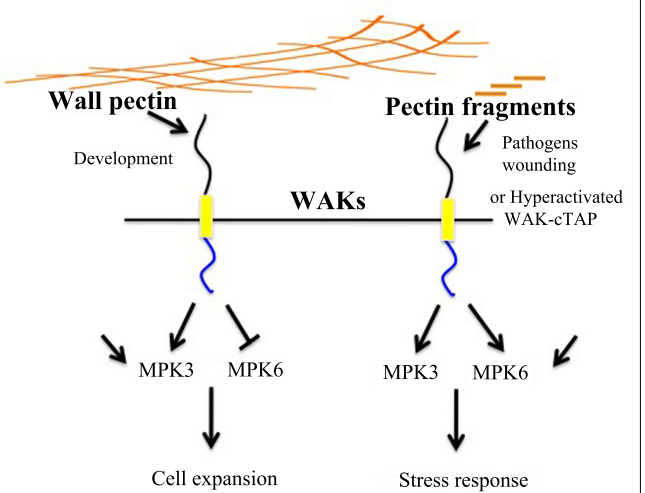


FIGURE 1 | A model for pectin–WAK regulation of growth and the stress response. Cartoon of pectin in the cell wall (long brown lines) activating WAK in the plasma membrane, and subsequent activation of MPK3. MPK6 is either repressed or not activated, and downstream gene activation leads to cell growth. In the presence of OGs, fragmented pectins, generated by pathogens or wounding, WAKs now activate MPK6 as well as MPK3, and the subsequent change in gene expression leads to a stress response. MPK6 and MPK3 are activated by many other pathways, as indicated by black arrows not originating from WAK.

CELL WALL "SENSORS"

Plant cell walls arise through a complex, developmentally regulated coordination of synthesis, turnover, and interactions between protein and carbohydrates. Screens for mutants in developmental processes have not surprisingly then revealed numerous alleles of cell wall biosynthesis genes, and conversely mutations in cell wall function have identified alleles in genes normally associated with a variety of metabolic and developmental pathways (Seifert and Blaukopf, 2010). These genes include receptor kinases such as *THESEUS* (*THE1*; Hematy et al., 2007; Hematy and Hofte, 2008), *FERONIA* (*FER*), *HERKULES* (*HERK*), and *ANXUR* (Hematy and Hofte, 2008; Guo et al., 2009a,b; Miyazaki et al., 2009). This class of receptor has been reviewed extensively elsewhere (Seifert and Blaukopf, 2010; Cheung and Wu, 2011) and they have been termed cell wall sensors. However, it may be more accurate at this point to describe them as regulators of pathways that are linked physiologically, biochemically, or physically to aspects of cell wall biology.

The WAKs are like many other plant receptors in that they regulate some aspect of cell division or growth, but also affect aspects of the cell wall; perhaps WAKs are also cell wall sensors. But WAKs remain unique in that they are known to bind directly to a major structural component of the cell wall, pectin, which appears to influence a WAK-dependent signaling pathway regulating cell expansion. WAKs are also involved in the pathogen and stress responses, but are likely activated in this case by fragmented pectins. An understanding of how WAKs are regulated by the various forms of pectin in cell walls will be greatly aided by a detailed knowledge of their structural requirements for binding pectin, and by an analysis of the distribution of WAKs and pectins in the cell wall. With the realization that the cell wall is so intimately involved in plant development and the pathogen response, and in turn knowing that there are numerous signaling pathways that regulate these events, it would not be surprising to discover that WAKs form networks with numerous other receptor pathways.

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REFERENCES

Anderson, C. M., Wagner, T. A., Perret, M., He, Z. H., He, D., and Kohorn, B. D. (2001). WAKs: cell wall-associated kinases linking the cytoplasm to the extracellular matrix. *Plant Mol. Biol.* 47, 197–206.

Andreasson, E., and Ellis, B. (2010). Convergence and specificity in the *Arabidopsis* MAPK nexus. *Trends Plant Sci.* 15, 106–113.

Bosch, M., and Hepler, P. K. (2005). Pectin methylesterases and pectin dynamics in pollen tubes. *Plant Cell* 17, 3219–3226.

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.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Cheung, A. Y., and Wu, H. M. (2011). THESEUS 1, FERONIA and relatives: a family of cell wall-sensing receptor kinases? *Curr. Opin. Plant Biol.* 14, 632–641.

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.

Decreux, A., Thomas, A., Spies, B., Brasseur, R., Van Cutsem, P., and Messiaen, J. (2006). *In vitro* characterization of the homogalacturonan-binding domain of the wall-associated kinase WAK1 using site-directed mutagenesis. *Phytochemistry* 67, 1068–1079.

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

Diener, A. C., and Ausubel, F. M. (2005). RESISTANCE TO FUSARIUM OXYSPORUM 1, a dominant *Arabidopsis* disease-resistance gene, is not race specific. *Genetics* 171, 305–321.

Guo, H., Li, L., Ye, H., Yu, X., Algreen, A., and Yin, Y. (2009a). Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7648–7653.

Guo, H., Ye, H., Li, L., and Yin, Y. (2009b). A family of receptor-like kinases are regulated by BES1 and involved in plant growth in *Arabidopsis thaliana*. *Plant Signal. Behav.* 4, 784–786.

Harholt, J., Suttangkakul, A., and Vibe Scheller, H. (2010). Biosynthesis of pectin. *Plant Physiol.* 153, 384–395.

He, Z. H., Cheeseman, I., He, D., and Kohorn, B. D. (1999). A cluster of five cell wall-associated receptor kinase genes, Wak1-5, are expressed in specific organs of *Arabidopsis*. *Plant Mol. Biol.* 39, 1189–1196.

He, Z. H., Fujiki, M., and Kohorn, B. D. (1996). A cell wall-associated, receptor-like protein kinase. *J. Biol. Chem.* 271, 19789–19793.

He, Z. H., He, D., and Kohorn, B. D. (1998). Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. *Plant J.* 14, 55–63.

Hematy, K., and Hofte, H. (2008). Novel receptor kinases involved in growth regulation. *Curr. Opin. Plant Biol.* 11, 321–328.

Hematy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J. P., and Hofte, H. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931.

Kohorn, B. D. (2000). Plasma membrane-cell wall contacts. *Plant Physiol.* 124, 31–38.

Kohorn, B. D. (2001). WAKs; cell wall associated kinases. *Curr. Opin. Cell Biol.* 13, 529–533.

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., and Obregon, P. (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60, 974–982.

Kohorn, B. D., Kobayashi, M., Johansen, S., Friedman, H. P., Fischer, A., and Byers, N. (2006a). Wall-associated kinase 1 (WAK1) is crosslinked in endomembranes, and transport to the cell surface requires correct cell-wall synthesis. *J. Cell Sci.* 119, 2282–2290.

Kohorn, B. D., Kobayashi, M., Johansen, S., Riese, J., Huang, L. F., Koch, K., Fu, S., Dotson, A., and Byers, N. (2006b). An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* 46, 307–316.

Kohorn, B. D., Kohorn, S. L., Todorova, T., Baptiste, G., Stansky, K., and McCullough, M. (2011). A dominant allele of *Arabidopsis* pectin-binding wall-associated kinase induces a stress response suppressed by MPK6 but not MPK3 mutations. *Mol. Plant* doi: 10.1093/mp/ssr096 [Epub ahead of print].

Kohorn, B. D., Lane, S., and Smith, T. A. (1992). An *Arabidopsis* serine/threonine kinase homologue with an epidermal growth factor repeat selected in yeast for its specificity for a thylakoid membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10989–10992.

Krichevsky, A., Kozlovsky, S. V., Tian, G. W., Chen, M. H., Zaltsman, A., and Citovsky, V. (2007). How pollen tubes grow. *Dev. Biol.* 303, 405–420.

Lally, D., Ingmire, P., Tong, H. Y., and He, Z. H. (2001). Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. *Plant Cell* 13, 1317–1331.

Li, H., Zhou, S. Y., Zhao, W. S., Su, S. C., and Peng, Y. L. (2009). A novel wall-associated receptor-like protein kinase gene, OsWAK1, plays important roles in rice blast disease resistance. *Plant Mol. Biol.* 69, 337–346.

Mangeon, A., Junqueira, R. M., and Sachetto-Martins, G. (2010). Functional diversity of the plant glycine-rich proteins superfamily. *Plant Signal. Behav.* 5, 99–104.

Miyazaki, S., Murata, T., Sakurai-Ozato, N., Kubo, M., Demura, T., Fukuda, H., and Hasebe, M. (2009). ANXUR1 and 2, sister genes to FERONIA/SIRENE, are male factors for coordinated fertilization. *Curr. Biol.* 19, 1327–1331.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* 11, 266–277.

Moscatiello, R., Mariani, P., Sanders, D., and Maathuis, F. J. (2006). Transcriptional analysis of calcium-dependent and calcium-independent signalling pathways induced by oligogalacturonides. *J. Exp. Bot.* 57, 2847–2865.

Mousavi, A., and Hotta, Y. (2005). Glycine-rich proteins: a class of novel proteins. *Appl. Biochem. Biotechnol.* 120, 169–174.

Park, A. R., Cho, S. K., Yun, U. J., Jin, M. Y., Lee, S. H., Sachetto-Martins, G., and Park, O. K. (2001). Interaction of the *Arabidopsis* receptor protein kinase Wak1 with a glycine-rich protein, AtGRP-3. *J. Biol. Chem.* 276, 26688–26693.

Ringli, C., Keller, B., and Ryser, U. (2001). Glycine-rich proteins as structural components of plant cell walls. *Cell. Mol. Life Sci.* 58, 1430–1441.

Sampoli Benitez, B. A., and Komives, E. A. (2000). Disulfide bond plasticity in epidermal growth factor. *Proteins* 40, 168–174.

Seifert, G. J., and Blaukopf, C. (2010). Irritable walls: the plant extracellular matrix and signaling. *Plant Physiol.* 153, 467–478.

Sivaguru, M., Ezaki, B., He, Z. H., Tong, H., Osawa, H., Baluska, F., Volkmann, D., and Matsumoto, H. (2003). Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in *Arabidopsis*. *Plant Physiol.* 132, 2256–2266.

Verica, J. A., Chae, L., Tong, H., Ingmire, P., and He, Z. H. (2003). Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in *Arabidopsis*. *Plant Physiol.* 133, 1732–1746.

Verica, J. A., and He, Z. H. (2002). The cell wall-associated kinase (WAK) and WAK-like kinase gene family. *Plant Physiol.* 129, 455–459.

Wagner, T. A., and Kohorn, B. D. (2001). Wall-associated kinases are expressed throughout plant development and are required for cell expansion. *Plant Cell* 13, 303–318.

Willats, W. G., McCartney, L., Mackie, W., and Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant Mol. Biol.* 47, 9–27.

Wolf, S., Mouille, G., and Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. *Mol. Plant* 2, 851–860.

Yamazaki, N., Fry, S. C., Darvill, A. G., and Albersheim, P. (1983). Host-pathogen interactions: XXIV. Fragments isolated from suspension-cultured sycamore cell walls inhibit the ability of the cells to incorporate [C]leucine into proteins. *Plant Physiol.* 72, 864–869.

Zhang, S., Chen, C., Li, L., Meng, L., Singh, J., Jiang, N., Deng, X. W., He, Z. H., and Lemaux, P. G. (2005). Evolutionary expansion, gene structure, and expression of the rice wall-associated kinase gene family. *Plant Physiol.* 139, 1107–1124.

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The synthesis and origin of the pectic polysaccharide rhamnogalacturonan II – insights from nucleotide sugar formation and diversity

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There is compelling evidence showing that the structurally complex pectic polysaccharide rhamnogalacturonan II (RG-II) exists in the primary cell wall as a borate cross-linked dimer and that this dimer is required for the assembly of a functional wall and for normal plant growth and development. The results of several studies have also established that RG-II structure and cross-linking is conserved in vascular plants and that RG-II likely appeared early in the evolution of land plants. Two features that distinguish RG-II from other plant polysaccharides are that RG-II is composed of 13 different glycoses linked to each other by up to 22 different glycosidic linkages and that RG-II is the only polysaccharide known to contain both apiose and aceric acid. Thus, one key event in land plant evolution was the emergence of genes encoding nucleotide sugar biosynthetic enzymes that generate the activated forms of apiose and aceric acid required for RG-II synthesis. Many of the genes involved in the generation of the nucleotide sugars used for RG-II synthesis have been functionally characterized. By contrast, only one glycosyltransferase involved in the assembly of RG-II has been identified. Here we provide an overview of the formation of the activated sugars required for RG-II synthesis and point to the possible cellular and metabolic processes that could be involved in assembling and controlling the formation of a borate cross-linked RG-II molecule. We discuss how nucleotide sugar synthesis is compartmentalized and how this may control the flux of precursors to facilitate and regulate the formation of RG-II.

Keywords: RG-II biosynthesis, UDP-apiose, CMP-kdo, aceric acid, Golgi, wall evolution, borate, dimer

INTRODUCTION

The pectic matrix of the plant primary cell wall is comprised of at least three polysaccharide domains, homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (RG-II), that are believed to be covalently linked to one another (Caffall and Mohnen, 2009). Rhamnogalacturonan II is a low-molecular-weight (see **Figure 1A**), structurally complex pectic polysaccharide that is released when plant cell walls are treated with endo-α-(1,4)-polygalacturonase (Darvill et al., 1978). RG-II contains 13 different monosaccharides (Darvill et al., 1978; Spellman et al., 1983a; York et al., 1985; Stevenson et al., 1988a) and has a backbone composed of 1,4-linked α-D-Gal*p*A residues (Puvanesarajah et al., 1991; see **Figure 1A**). A series of structural studies (Spellman et al., 1983b; York et al., 1985; Melton et al., 1986; Stevenson et al., 1988b; Thomas et al., 1989; Puvanesarajah et al., 1991; Whitcombe et al., 1995; Pellerin et al., 1996; Vidal et al., 2000; Glushka et al., 2003) established that four different oligosaccharide side chains (A–D, see **Figure 1A**) are linked to this backbone, although their order along the backbone is not known with certainty (du Penhoat et al., 1999; Glushka et al., 2003; Pérez et al., 2003). RG-II has also been isolated from the cells walls of gymnosperms (Edashige and Ishii, 1998; Shimokawa et al., 1999), lycopodiophytes, and pteridophytes (Matsunaga et al., 2004). Thus, RG-II is likely present in the walls of all vascular plants. Nevertheless, RG-II remained an enigmatic component of the cell wall with no known function for almost 20 years after its discovery.

The results of several studies had led to the suggestion that an interaction between borate and pectin is important for wall structure and for plant growth and development (Matoh et al., 1993; Hu and Brown, 1994; Hu et al., 1996). The first clues that this interaction involved RG-II began to emerge when it was shown that the amounts of RG-II present in the cell wall are correlated with the boron requirements of flowering plants (Matoh et al., 1996) and that two RG-II molecules are cross-linked to one another by a borate diester (Kobayashi et al., 1996; see **Figures 1B,C**). This diester bond is located between the apiosyl residue of side chain A in two separate RG-II molecules (Ishii et al., 1999; see **Figures 1B,C**). Together the studies of borate cross-linking of RG-II *in vitro* (O'Neill et al., 1996, 2001; Fleischer et al., 1999; Ishii et al., 1999; Kobayashi et al., 1999) led to the hypothesis that borate diester formation and stability *in vivo* are likely influenced by the primary structure of RG-II, wall pH, and calcium ions.

At least 90% of the RG-II in the primary cell wall is cross-linked when plants are grown with an adequate supply of borate (Kobayashi et al., 1996; O'Neill et al., 1996; Matoh et al., 2000). By contrast, virtually none of the RG-II is cross-linked when

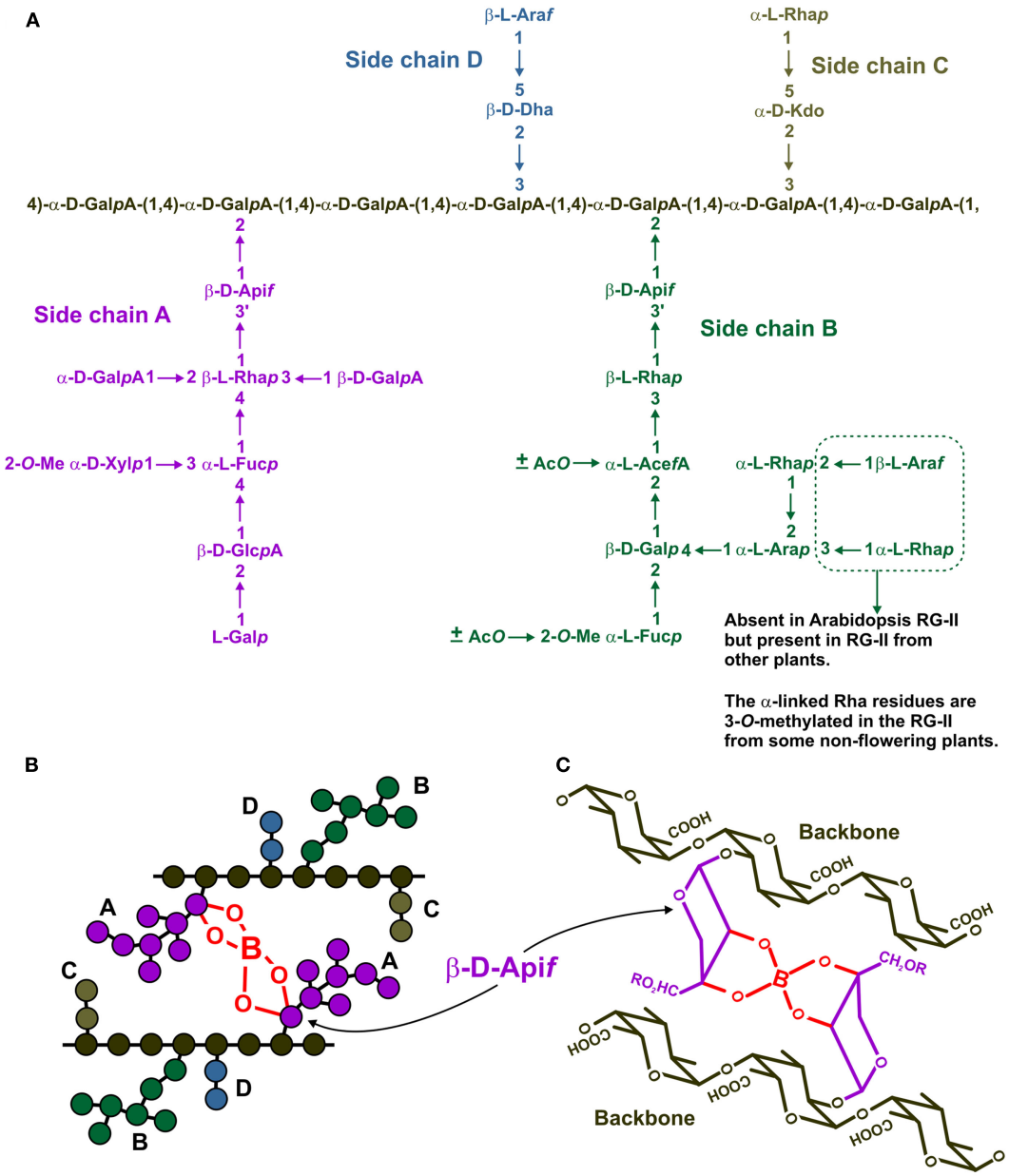


FIGURE 1 | Rhamnogalacturonan II. (A) The glycosyl sequence of RG-II. The nominal mass for the RG-II monomer is 4,612, assuming a backbone of 7 GalA residues and fully decorated side chains. **(B)** Cartoon showing the cross-linking of two RG-II molecules. **(C)** The borate diester cross-link. When two apiosyl residues are linked by a borate diester, the boron atom is chiral, and two diastereoisomers (bis[3-*C*-(hydroxymethyl)-β-L *threo*-furanoside]-(R)-2,3:2′,3′ borate) and (bis[3-*C*-(hydroxymethyl)-β-L-*threo*-furanoside]-(S)-2,3:2′,3′ borate may form (O'Neill et al., 2004). It is not known if one or both diastereoisomers are formed when RG-II is cross-linked in the cell wall.

suspension-cultured *Chenopodium album* cells are adapted to grow in the absence of added borate (Fleischer et al., 1999). Moreover, the walls of these cells have pore sizes that are ∼60% larger than their borate-grown counterparts. Pore size and RG-II cross-linking return to near-normal values when the cells are supplied with borate suggesting that RG-II cross-linking affects the size of molecules that can diffuse through the wall (Fleischer et al., 1999). The lack of RG-II cross-linking also reduces the strength of the wall, which ruptures when the borate-deficient cells enter the stationary growth phase (Fleischer et al., 1999). Reduced borate cross-linking of RG-II is associated with reduced growth, brittle tissues, and swollen cell walls when pumpkin plants are grown in the absence of added borate (Ishii et al., 2001, 2002). Similarly, tobacco cells adapted to grow in low-borate media have much thicker walls than is normal and most of the RG-II is not cross-linked (Matoh et al., 2000). Mutations that alter RG-II structure and thereby decrease its ability to cross-link with borate have also been shown to be deleterious to plant growth and development

(O'Neill et al., 2001; Noguchi et al., 2003; Reuhs et al., 2004; Ahn et al., 2006; Delmas et al., 2008; Voxeur et al., 2011). Such results when taken together with evidence that RG-II, homogalacturonan, and rhamnogalacturonan I are covalently linked to one another (Ishii and Matsunaga, 2001), are consistent with the hypothesis that cross-linking of RG-II results in the formation of a pectic network within the primary cell wall (Brown and Hu, 1997; Matoh and Kobayashi, 1998; O'Neill et al., 2004). The inability to form this network likely alters the mechanical and physical properties of the cell wall and may account for many of the growth defects observed when vascular plants do not have an adequate supply of boron (Loomis and Durst, 1992).

The conserved structure of RG-II likely ensures that two RG-II molecules can interact with one another and with borate to self-assemble into a dimer. This dimer apparently is essential for the formation of a functional wall. Nevertheless, there is evidence that the glycosyl sequences of RG-II isolated from different plants are not identical. Such natural differences typically involve variations in the external monosaccharides of a side chain (see **Figure 1A**) and have no discernible effect on borate cross-linking or plant growth. For example, side chain B exists predominantly as a heptasaccharide in *Arabidopsis* (Glushka et al., 2003) and rice (Thomas et al., 1989) RG-II and predominantly as a nonasaccharide in sycamore (Whitcombe et al., 1995), wine (Glushka et al., 2003), and citrus (Séveno et al., 2009). The B side chain of the RG-II isolated from the walls of several non-flowering plants contains terminal non-reducing 3-*O*-methyl rhamnose rather than terminal non-reducing rhamnose (Matsunaga et al., 2004). There are no published data showing structural variations in side chains A, C, and D. Nevertheless, other glycoforms may exist as RG-II has only been isolated from a limited number of plants and structurally characterized. Furthermore, the possibility cannot be discounted that RG-II structure varies in different plant tissues and cell types. If other RG-II glycoforms do exist they may provide insight into the minimum glycosyl sequence that is required for borate cross-linking and for a functional wall.

RG-II is the only glycan known in nature to consist of such a large number of different sugars linked to one another by so many different glycosidic linkages. However, it is not known if this structural complexity is required solely to ensure that RG-II can interact with borate to form a functional wall or if RG-II has other as yet unidentified functions. To fully define the role(s) of RG-II in the cell wall requires in-depth knowledge of the molecular processes leading to the initiation of RG-II synthesis, the elongation of the backbone, and the formation and modification of the four side chains. Here we briefly review recent genetic and biochemical studies that have contributed to a better understanding of some of the factors involved in RG-II formation.

NUCLEOTIDE SUGARS REQUIRED FOR RG-II BIOSYNTHESIS

RG-II synthesis is believed to occur in the Golgi apparatus (Mohnen, 2008) and, due to its structural complexity, requires a large number of different activated sugars, specific glycosyltransferases and additional enzymes required for methylation and acetylation of side chain residues. RG-II formation requires the following nucleotide sugars: UDP-D-Xyl*p*, UDP-D-Gal*p*, UDP-D-Gal*p*A, UDP-D-Glc*p*A, CMP-D-Kdo (2-keto-3-deoxy-D-*manno*-octulosonic acid), UDP-L-Rha*p*, UDP-L-Ara*p*, UDP-L-Ara*f*, GDP-L-Fuc*p*, and GDP-L-Gal*p* (**Figure 2**). In addition, UDP-D-Api*f* [3-*C*-(hydroxymethyl)-D-*glycero*-tetrose], a plant-specific activated branched-chain sugar (Mølhøj et al., 2003; Guyett et al., 2009), and the as yet unidentified activated forms of Dha (2-keto-3-deoxy-D-*lyxo*-heptulosaric acid) and aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) are also necessary. Several studies have shown that mutations that affect the formation of nucleotide sugars involved in RG-II synthesis may severely impact plant growth and development. For example, the lack of UDP-Api (Ahn et al., 2006) or CMP-Kdo (Delmas et al., 2008; Kobayashi et al., 2011) is lethal. By contrast, reducing the amounts of GDP-L-Fuc (O'Neill et al., 2001) and GDP-L-Gal (Voxeur et al., 2011) that are formed results in decreased plant growth. This is likely due to reduced RG-II cross-linking as plant growth is rescued by feeding the plants with exogenous supply of borate (O'Neill et al., 2001; Voxeur et al., 2011).

Glycosyltransferases (GTs) are enzymes that transfer a sugar from the nucleotide sugar in a linkage-specific manner to extend an acceptor. We estimate that at least 22 specific GTs are needed to synthesize RG-II, based on the assumption that one GT is required for the formation of each different glycosidic linkage. Additionally, two *O*-methyl transferases, two *O*-acetyl transferases and the enzymes that generate their respective donors are required for the modification of side chain monosaccharides.

Many of the activated sugars used for the synthesis of RG-II are also used for the synthesis of other plant glycans including polysaccharides, glycoproteins, proteoglycans, glycolipids, and low-molecular-weight glycosides. By contrast, RG-II and apiogalacturonan, which has a backbone composed of 1,4-linked α-D-Gal*p*A residues that are substituted at *O*-2 with apiosyl and di-apiosyl side chains (Hart and Kindel, 1970; Ovodov et al., 1971; Longland et al., 1989; Gloaguen et al., 2010), are the only polysaccharides known to require UDP-Api for their formation. To date, apiogalacturonans have only detected in the cell walls of selected monocots including *Lemna* and plants referred to as "seagrasses" (Hart and Kindel, 1970; Ovodov et al., 1971; Gloaguen et al., 2010).

CMP-Kdo is required for the formation of RG-II and may also be used for the synthesis of glycolipids (Li et al., 2011), whereas the activated forms of AceA and Dha appear to be used only for the synthesis of RG-II. Thus, for RG-II to be produced by early land plants, either existing NDP-sugar biosynthetic genes/pathways evolved new functions to allow for the formation of different sugars or new genes were recruited to expand the repertoire of activated sugars. With the exception of UDP-Api and the activated form of AceA, sequence data provides evidence that genes involved in the formation of the remaining nucleotide sugars were present in green plants prior to the existence of RG-II (see **Table 1**). For example, CMP-Kdo synthetase has been proposed to have appeared in plants through an ancient horizontal gene transfer event from endoparasitic eubacteria similar to the genus *Chlamydia* (Royo et al., 2000). The development of hypotheses describing the sequence of molecular events that allowed plants to synthesize RG-II requires further phylogenetic analysis of NDP-sugar

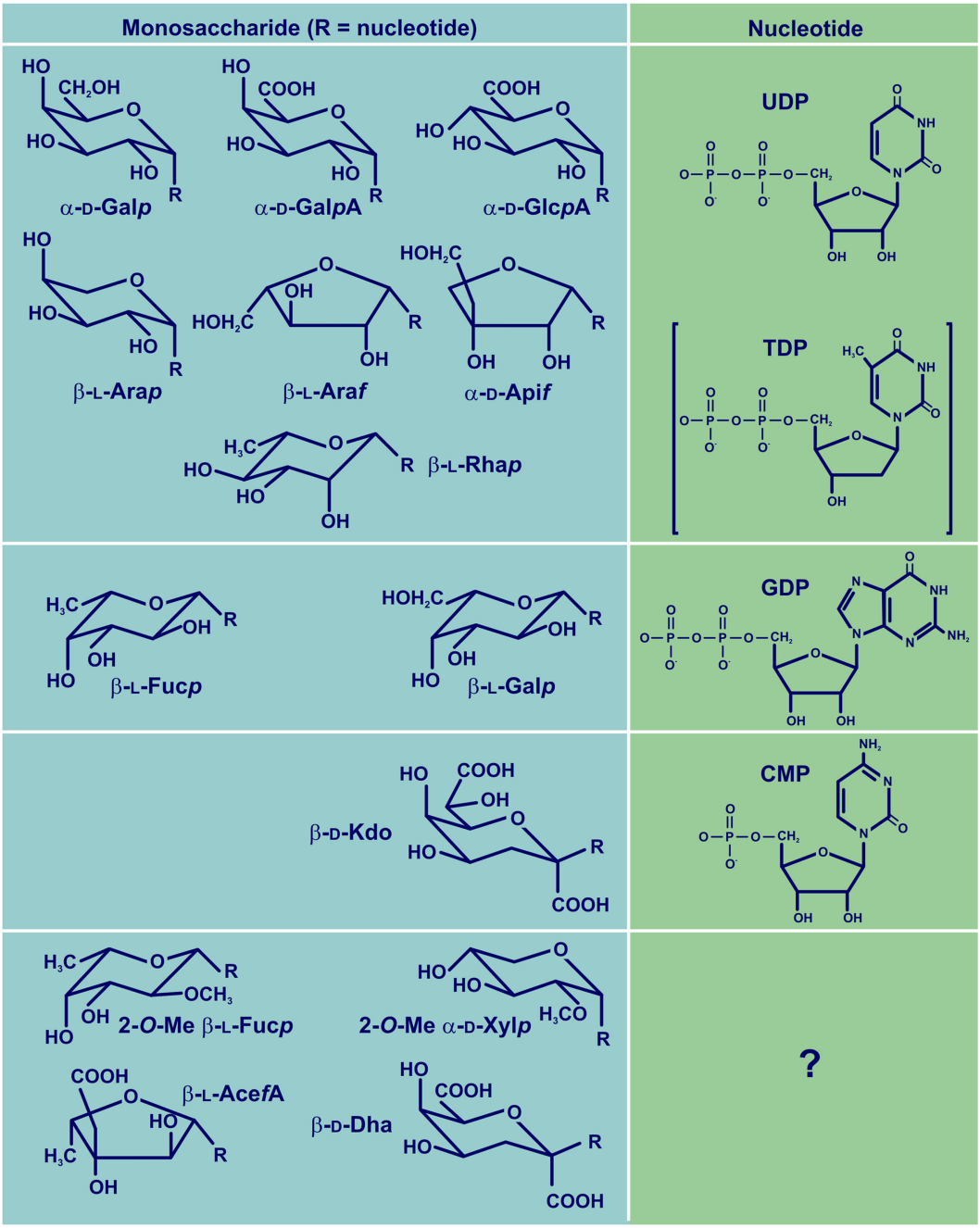


FIGURE 2 | The monosaccharides of RG-II and their corresponding nucleotides. TDP-Rha is the activated form of rhamnose in bacteria. It is not known if fucose and xylose are *O*-methylated when they exist as nucleotide sugars or after they have been transferred to the side chains of RG-II. The activated forms of aceric acid and Dha have not been determined.

biosynthetic pathway enzymes from taxonomically diverse plants and prokaryotes.

THE SUBCELLULAR DISTRIBUTION OF NUCLEOTIDE SUGARS

Nucleotide sugars are used in diverse cellular compartments to form glycans and low-molecular-weight glycosides. For example, UDP-Rha is likely used for the synthesis of RG-II and rhamnogalacturonan I (RG-I) in the Golgi (Mohnen, 2008) but is also used in the cytosol for the formation of rhamnose-containing secondary metabolites including flavonoids, and terpenoids (Bar-Peled et al., 1991). The enzymes that form UDP-Rha are present in the cytosol, therefore mechanisms must exist to transport this activated sugar to the appropriate organelle. By contrast, other nucleotide sugars including UDP-Xyl, UDP-Ara*p*, and UDP-GalA are made in both the cytosol and in the lumen of the Golgi (Bar-Peled and O'Neill, 2011). For example, UDP-GalA is formed from

Table 1 | The occurrence of selected nucleotide sugar biosynthetic genes and pectic polysaccharides in green plants.

Plant	UDP-Api	UDP-Rha	UDP-GalA	UDP-Xyl	UDP-Ara*f*	CMP-Kdo	GDP-Gal	Pectic polysaccharide		
	Nucleotide sugar biosynthetic genes[1]							RG-I	RG-II	HG
VASCULAR										
Arabidopsis thaliana	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	+	+	+
Oryza sativa	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	+	+	+
Selaginella moellendorffii	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+	G+/E+	+	+	+
AVASCULAR										
Physcomitrella patens	G+/E−	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	G+/E+	+	?	+
ALGAE STREPTOPHYTE										
Mesostigma viride	Nd	Nd	Nd	E−	E−	E +	E−	?	?	?
CHLOROPHYTES										
Chlamydomonas reinhardtii	Nd	G+/E+	G+/E+	G+/E+	G+/E+	Nd	G+/E+	?	?	?
Volvox carteri	Nd	G+/E+	G+/E +	G+/E−	G+/E+	G−/E−	G+/E +	?	?	?
Scherffelia dubia	Nd	Nd	Nd	Nd	Nd	E+[2]	Nd	?	?	?
Chlorella sp.	Nd	G+/E−	Nd	G+/E−	G+/E−	G+/E−	Nd	?	?	?
Micromonas sp.	Nd	G+/E+	G+/E−	G+E−	Nd	G+/E−	G+/E−	?	?	?
Ostreococcus tauri/lucima	Nd	G+/E+	G+/E+	G+/E+	Nd	G+/E−	G+/E+	?	?	?

[1]Genes used for database queries were the functionally characterized Arabidopsis genes: UDP-apiose synthase (UAXS, AXS, At2g27860); UDP-Rha synthase (URS, Rhm, At1g78570); UDP-GalA 4-epimerase (UGlcAE, GAE, At2g45310, for UDP-GalA); UDP-xylose synthase (UXS, AT3g62830); UDP-arabinose mutase (UAM, RGP, At3g02230); CMP-Kdo synthase (CKS, AT1G53000); GDP-Man 3,5-epimerase (GME, AT5g28840, for GDP-L-Gal). G, presence (+) or absence (−) of a sequence homolog in publically available genomic data base (NCBI, January 2012). E, presence (+) or absence (−) of sequence homolog in expressed sequence tag (EST) data (NCBI, January 2012). ?, indicates not detected or no data available. Nd, gene, or EST not detected in database. [2]The scaly walls of Scherffelia dubia contain a sulfated KDO polymer. No KDO synthase sequences were identified, although an EST was reported by Becker et al. (2001). No GalA-kinase detected in Physcomitrella and the green algae. The possibility cannot be excluded that a transcript of the gene is expressed during a specific stage in the life of the plant.

UDP-GlcA by six 4-epimerase isoforms (UGlcAe) that reside in the Golgi lumen (Gu and Bar-Peled, 2004). However, UDP-GalA is also generated from GalA by a cytosolic GalA-kinase and a UDP-sugar pyrophosphorylase referred to as "Sloppy" (Yang et al., 2009). This raises the question of whether the pools of UDP-GalA formed in the cytosol and in the Golgi are used for the synthesis of specific polysaccharides. Some Golgi-localized glycosyltransferases have been shown to have their catalytic domain facing the cytosol and thus likely use cytosolic nucleotide sugars for glycan synthesis (Davis et al., 2010), whereas others including a GalA transferase have their catalytic domain in the Golgi lumen (Sterling et al., 2001). Additional studies are required to determine the biological significance of segregating the formation of some but not all UDP-sugars in plants. Such compartmentalization may provide an additional level of regulation to control the channel of sugars for the synthesis of specific polysaccharides.

Recent reports suggest that the catalytic domain of CMP-Kdo synthetase is localized to the lumen of mitochondria (Kobayashi et al., 2011). Thus, mitochondria are a site of CMP-Kdo formation, where it is presumably used for the synthesis of Kdo-containing glycolipids (Séveno et al., 2010; Li et al., 2011). If mitochondria are the only site of CMP-Kdo formation then mechanisms must exist to export CMP-Kdo to the Golgi for RG-II synthesis. Interestingly, there are reports that there is vesicular trafficking between the mitochondria, the endoplasmic reticulum and the Golgi (Jouhet et al., 2009). Yet, the possibility cannot be discounted that CMP-Kdo is transported to the Golgi via the cytosol or that an altogether different CMP-Kdo synthase exists in the Golgi.

DO NUCLEOTIDE SUGARS FORMED IN THE CYTOSOL AND GOLGI CONTRIBUTE EQUALLY TO RG-II SYNTHESIS?

There is compelling evidence that plants contain multiple genes encoding enzymes involved in the synthesis of the same NDP-sugar (Bar-Peled and O'Neill, 2011). Several hypotheses can been put forward for the existence of multiple isoforms: (i) Isoforms are redundant with one another, (ii) isoforms are expressed in a cell and tissue specific manner, (iii) each isoform is localized to a specific compartment within a single cell, (iv) individual isoforms can function in different Golgi cisternae or within subdomains of an individual cisternae, (v) individual isoforms are required for the formation of glycan synthase complexes, and (vi) the activity of individual isoforms may be differentially affected by other nucleotide sugars. One cannot exclude the possibility that not all Golgi are "created equal" since they possess different complements of isoforms.

The interconversion of UDP-Ara*p* and UDP-Ara*f* is catalyzed by a mutase. At least two mutase isoforms are found in rice (Konishi et al., 2007) and at least three mutases exist in *Arabidopsis* (Rautengarten et al., 2011). These mutases have been shown to exist predominantly in the cytosol although they may also be closely associated with the cytosolic face of the Golgi (Konishi et al., 2007; Rautengarten et al., 2011). Multiple isoforms located exclusively in the cytosol generate pools of UDP-Rha, UDP-Api, UDP-Gal, and GDP-Fuc. Thus, these activated sugars must be imported into the lumen of the Golgi by nucleotide sugar transporters (Reyes and Orellana, 2008) if they are to be used as substrates by those glycosyltransferases that have their catalytic domains facing the Golgi

lumen. UDP-Xyl, UDP-GalA, and UDP-Ara*p* are also formed by multiple isoforms. However these isoform exist in both the cytosol and in the Golgi lumen (Bar-Peled and O'Neill, 2011). Such subcellular compartmentalization may provide a mechanism to regulate the channeling of precursor sugars for the synthesis of specific polysaccharides.

Enzymes involved in plant *N*-glycan biosynthesis and processing (Schoberer and Strasser, 2011) and cell wall polysaccharide formation (Chevalier et al., 2010) have been reported to be targeted to and localized in different Golgi sub-compartments. For example, the glycosyltransferases involved in forming the side chains of xyloglucan are differentially distributed across the compartments of Golgi stacks. The XylT that adds Xyl to the glucan backbone resides in the *cis* and medial Golgi cisternae, the GalT the adds Gal to this Xyl resides in the medial cisternae and the FucT that fucosylates the Gal resides in the *trans* cisternae (Chevalier et al., 2010). Thus, it is probable that specifically localized transporters import UDP-Gal and GDP-Fuc into the cisternae where the distinct glycosyltransferase activities exist. However, the source of the UDP-Xyl used by the XylT is not known as UDP-Xyl is produced in both the cytosol and in the Golgi lumen.

Based on analyses of transcriptomic data for individual root cells (Birnbaum et al., 2003; Brady et al., 2007), the genes encoding the multiple isoforms of the NDP-sugar-forming enzymes are in large part expressed in all cells, albeit at different levels (unpublished results of the authors). This suggests that multiple isoforms of the same enzyme activity are essential for the normal functioning of a single cell. The challenge will be to decipher the role of each isoform.

The flux of precursors between and within different subcellular compartments is likely to have an important role in regulating the synthesis of RG-II as well as other cell wall glycans. Sugars may be channeled to different glycan metabolic routes through different pools of nucleotide sugars. Alternatively, partitioning may provide a mechanism to control, at the metabolite level (e.g., UDP-GalA), the amounts of RG-II, or other polysaccharides produced. Another possibility is that the amount of an activated sugar available is a bottleneck for wall polysaccharide synthesis. For example, the amounts of UDP-apiose present in a cell may be key to controlling the amounts of RG-II formed. Altering the balance of nucleotide sugars may also have multiple effects on wall compositions. For example, analyses of the developing endosperm in the ADP-Glc transporter mutant (*lys*) of barley showed that the amounts of mixed-linkage β-glucan increased, presumably due to a shift of the ADP-Glc pool via Glc-1-P to UDP-Glc (Christensen and Scheller, 2012). Interestingly, rice plants carrying a null mutation in a gene encoding a Golgi-localized UDP-Glc transporter (*OsNST1*) have walls that are thinner than normal and contain reduced amounts of cellulose and xyloglucan and increased amounts of arabinoxylan and pectin (Zhang et al., 2011).

UDP-GlcNAc is used as a donor substrate for the formation of glycoproteins (Kornfeld and Kornfeld, 1985). In animals, UDP-GlcNAc is also used by *O*-GlcNAc transferases for the post-translational modification of regulatory proteins (Comer and Hart, 2000). Such *O*-GlcNAc modification has been shown to affect gene expression and contributes to changes in metabolism and growth (Slawson et al., 2010). Plants contain two *O*-GlcNAc transferases referred to as SECRET AGENT (SEC) and SPINDLY (SPY) that are functionally similar to their animal counterparts (Hartweck et al., 2002; Olszewski et al., 2010). Mutant analysis has indicated that SEC and SPY-dependent *O*-GlcNAcylation may have a role in multiple plant metabolic pathways and growth processes, although their target proteins remain largely unknown. SPY has been proposed to modify transcription factors, which in turn affect a plants response to cytokinin (Steiner et al., 2012). Thus, plants may be utilizing a *O*-GlcNAc modification to sense and control diverse plant biochemical pathways. It will be of interest to determine if the activities of wall biosynthetic enzymes are directly or indirectly affected by *O*-GlcNAc modification. It also remains to be determined if other nucleotide sugars have a role in as yet unidentified signaling pathways.

RG-II BIOSYNTHESIS – INITIATION AND ASSEMBLY

Current models of pectin structure depict a macromolecule composed of homogalacturonan, rhamnogalacturonan I and RG-II domains linked to one another by covalent bonds (Caffall and Mohnen, 2009), although the precise arrangement of each component remains a subject of debate (Vincken et al., 2003). Irrespective of how the individual components are arranged, the pectin macromolecules are believed to be cross-linked to one another in the walls of growing cells by the formation of borate diesters between RG-II molecules. Other types of molecular interactions including Ca^{2+} cross-links between GalA residues and hydrophobic interactions between methyl-esterified GalA residues likely stabilize the pectin network. Such a model of pectin structure, if correct, raises numerous questions regarding pectin synthesis in general and RG-II synthesis in particular.

In aqueous environments, pectic polysaccharides typically exist as large extended molecules. Light scattering and atomic force microscopy studies suggest that a single pectin molecule may have sizes in the range of 20–170 nm (Round et al., 2001, 2010) whereas a RG-II molecule may have a radius of up to 18 nm (Pérez et al., 2003). Pectic polysaccharides are believed to be synthesized in Golgi cisternae, which are typically flattened disk-like organelles with a diameter of up to a 1000-nm long and a height of 40 nm (Seguí-Simarro and Staehelin, 2006; Staehelin and Kang, 2008). How many pectin molecules can such a cisternae accommodate? Is this number increased by packing or folding of pectin molecules in a manner comparable to the compacting of DNA? Are pectins assembled on molecules that anchor them to the Golgi membrane or are they free to diffuse throughout the cisternae? Are the individual pectin domains synthesized together or in separate zones of the same cisternae or in different cisternae? If the individual domains are synthesized separately, when are they linked to one another and when does borate cross-linking of RG-II occur? Clearly, the volume of the Golgi, the dimensions of the polysaccharides being synthesized, the copy numbers of nucleotide sugar-forming enzymes, glycosyltransferases, and modifying enzymes along with all the other molecules that transit through this organelle must be taken into account to understand how pectin is assembled.

Here, we will for convenience, consider the synthesis of RG-II as occurring in three distinct steps: first, initiation and extension of the homogalacturonan backbone, followed by side chain formation and addition to the backbone, and finally, side chain

modification. The borate cross-link may form during RG-II synthesis or during the incorporation of RG-II into the growing wall. Under normal growth conditions the apoplast likely contains adequate amounts of borate for cross-linking RG-II. If the borate cross-link is formed during RG-II synthesis, then mechanisms must exist to transport borate into the Golgi. Although several borate transporters have been identified in plants, they are present in the plasma membrane and are believed to be involved in boron uptake from the soil (Miwa and Fujiwara, 2010). Irrespective of where cross-linking of RG-II occurs, the currently available evidence suggests that borate diester formation is a spontaneous self-assembly process that does not require enzymes (Ishii et al., 1999).

Numerous genes that encode putative GalA transferases have been identified (Sterling et al., 2006; Yin et al., 2010). Only one of these (GAUT1) has been shown to be involved in the formation of the homogalacturonan backbone (Sterling et al., 2006). To date no GalAT has been identified and shown to be involved in the initiation and elongation of the RG-II backbone. Moreover, it is not known if RG-II side chains are attached to a pre-existing homogalacturonan, or if backbone extension and side chain addition are coupled to one another.

The formation of RG-II side chains and their attachment to the backbone may occur through at least two different mechanisms. The side chains may be assembled directly on the backbone by addition of one sugar at a time or the side chains could be assembled on a lipid intermediate and then transferred *en bloc* to the backbone. Lipid-linked oligosaccharides are known to be used for the formation of *N*- and *O*-linked oligosaccharides of glycoproteins (Sharma et al., 1974; Weerapana and Imperiali, 2006). Arabinose and xylose-containing lipids have been reported to be formed by pea membranes (Hayashi and Maclachlan, 1984), but their role in the formation of cell wall polysaccharides remains to be demonstrated.

Rhamnogalacturonan II xylosyltransferase (RGXT) is the only glycosyltransferase identified to date that has been predicted to be involved in the synthesis of an RG-II side chain (Egelund et al., 2006; Liu et al., 2011). The C-terminal catalytic domain of RGXT2 has been shown to be localized to the lumen of the Golgi apparatus (Søgaard et al., 2012). Recombinant versions of this transferase have been reported to catalyze the formation of the α-Xyl-1,3-Fuc linkage of side chain A (see **Figure 1**) using RG-II as an acceptor. Thus, at least *in vitro*, xylose can be added to a pre-existing side chain attached to RG-II and suggests that *O*-methylation of xylose occurs after it has been added to side chain A. Identifying other GTs involved in RG-II synthesis is hampered by the lack of viable RG-II-defective plants. Reverse-genetic approaches have also been of limited success. Thus, it is likely that identifying the genes and the corresponding enzymes involved in RG-II biosynthesis will require the use of classical biochemistry.

Studies of the structure of RG-II synthesized by the fucose-deficient *mur1* mutant suggest that *in vivo*, the transferase that forms the α-Xyl-1,3-Fuc linkage is less effective when the L-Fuc is replaced by L-Gal (Reuhs et al., 2004; Séveno et al., 2009). By contrast, the transferase that adds β-GlcA to the same Fuc residue does not appear to be affected by the *mur1* mutation. This GlcA serves as the acceptor for the addition of a L-Gal residue (see **Figure 1**). Interestingly, silencing genes involved in GDP-L-Gal formation results in reduced amounts of this L-Gal, decreased borate cross-linking of RG-II and reduced seedling growth (Voxeur et al., 2011). However, when these same mutants are grown in the presence of borate, seedling growth, and RG-II cross-linking is restored to wild-type. This result when taken together with studies of the *mur1* mutant (O'Neill et al., 2001) indicate that altering RG-II structure results in reduced borate cross-linking of apiose, which is detrimental to plant growth. Knowledge of the three-dimensional structure of RG-II together with insight into how the side chains interact with one another and with borate and how these interactions are affected by changes in side chain structure are required to understand the structure-function relationships of RG-II and borate (Pérez et al., 2003).

Some of the sugars present in the side chains of RG-II are *O*-methylated and/or *O*-acetylated (see **Figure 1**). However, it is not known if these modifications affect the structure-function relationship of RG-II, since little is known about the genes and enzymes involved in such modifications. Early studies of the synthesis of grass 4-*O*-methyl glucuronoxylans indicated that the GlcA is *O*-methylated after it has been transferred to the xylan backbone (Kauss and Hassid, 1967). Acetyl-CoA has been shown to be a donor for *O*-acetylation of plant cell wall polysaccharides (Pauly and Scheller, 2000), although no protein has been isolated and shown to acetylate a specific plant polysaccharide. However, recent genetic studies have begun to shed light on some of the genes that may be involved in *O*-acetylation of plant polysaccharides (Gille et al., 2011; Manabe et al., 2011).

RG-II DISASSEMBLY

During plant cell growth and development a portion of the wall is believed to be recycled. Some tissues including, germinating seeds, developing fruits, and senescing leaves as well as pollen tubes have higher polysaccharide hydrolytic activity than other tissues and cells (Hadfield and Bennett, 1998; Buckeridge et al., 2000; Vicente et al., 2007). The released monosaccharides are believed to be made available for other metabolic processes or for conversion to nucleotide sugars and subsequent reincorporation into the growing wall. Nevertheless, the amounts of cell wall polysaccharides that are salvaged and the significance of these processes for plant growth remains to be determined. *Arabidopsis* mutants that are unable to salvage fucose by converting it to GDP-Fuc, accumulate large amounts of free fucose but appear to grow normally (Kotake et al., 2008). By contrast, *Arabidopsis* mutants that lack arabinose kinase and thus are unable to convert Ara to Ara-1-P germinate in the presence of arabinose but fail to grow (Dolezal and Cobbett, 1991). Numerous enzymes and genes involved in the salvage of GalA, Gal, Fuc, Ara, and GlcA have been described suggesting that numerous sugar salvage pathways exist in plants (Bar-Peled and O'Neill, 2011). However, it is not known if any of the RG-II sugars are recycled. It will be of interest to determine if plants produce enzymes that fragment the RG-II backbone and the side chains. Such enzymes would also be of value for structure-function studies of RG-II, and for the generation of acceptor molecules for biosynthesis studies, since very few microbial enzymes have been indentified that fragment RG-II (Vidal et al., 1999).

WHEN DID RG-II FIRST APPEAR IN GREEN PLANTS – INSIGHT FROM NUCLEOTIDE SUGAR BIOSYNTHETIC GENES

There is persuasive evidence that RG-II is present in the cell walls of all major groups of extant vascular plants including lycopodiophytes, pteridophytes, gymnosperms, and angiosperms (Matsunaga et al., 2004). By contrast, it is far from clear whether RG-II is present in the cell walls of avascular land plants (mosses, liverworts, and hornworts), and green algae (Matsunaga et al., 2004; Sørensen et al., 2011). Bryophytes have been reported to contain over 100-fold less RG-II than dicots on the basis of the boron contents of their cell walls (Matsunaga et al., 2004). Interestingly, the genome of the moss *Physcomitrella patens* contains a gene encoding a protein with high sequence identity (73%) to *Arabidopsis* UDP-Api synthase (see **Table 1**). We identified no transcripts for this gene in publically available EST databases, nor have we been able to detect apiose in the cell walls isolated from *Physcomitrella* leafy gametophores or protonema (unpublished results of the authors). Thus, the function of the product of this gene in *Physcomitrella* and the biosynthetic pathways that it is involved in remains to be determined. No monosaccharides characteristic of RG-II have been detected in the cell walls of charophycean green algae (Sørensen et al., 2011). By contrast, there is evidence that the cell walls of these algae and the cell walls bryophytes do contain homogalacturonan (Moller et al., 2007; Sørensen et al., 2011), whereas rhamnogalacturonan I may be restricted to land plant cell walls (Sørensen et al., 2011).

The walls of several monocots (Alismatales) including *Lemna* and the "seagrasses" contain apiogalacturonans (Hart and Kindel, 1970; Golovchenko et al., 2002; Gloaguen et al., 2010), but the relationship between RG-II and these polysaccharides is not known. Apiogalacturonans appear to have a limited distribution in vascular plants and may be a relatively recent addition to the cell wall as there are no studies describing their presence in the walls of non-flowering vascular plants. Nevertheless, "absence of evidence" is not "evidence of absence". Indeed, mixed-linkage β-glucans were once believed to be present only in grass cell walls, but are now known to be a component of the walls of horsetails, a non-flowering vascular plant (Fry et al., 2008; Sørensen et al., 2008).

Rhamnose is a component of RG-II as well as rhamnogalacturonan I and plant glycosides. It is likely that in plants UDP-Rha is the predominate activated form of rhamnose (Bar-Peled and O'Neill, 2011). Genes encoding enzymes for UDP-Rha exist in diverse algae and avascular plants (**Table 1**). Somewhat unexpectedly, genes with high sequence similarity to plant UDP-Rha biosynthetic genes have been identified in viruses that inhabit both Chlorella and ameba and in selected fungi (Parakkottil Chothi et al., 2010; Martinez et al., 2012). Moreover, many of these organisms have been shown to produce UDP-Rha. By contrast prokaryotes utilize dTDP-Rha in the synthesis of Rha-containing glycans (Trezer et al., 1999). Interestingly the plant enzymes that form UDP-Rha can also form TDP-Rha *in vitro*, albeit at lower efficiency (Watt et al., 2004). Such studies suggest that using different forms of activated rhamnose for the synthesis of rhamnose-containing glycans provides some, as yet unidentified, evolutionary advantages for prokaryotes and eukaryotes.

Currently available data is consistent with the notion that RG-II appeared early in the evolution of land plants (Matsunaga et al., 2004; Sørensen et al., 2011). However, this data is insufficient to determine if this occurred before or after the divergence of avascular and vascular plants. Irrespective of when RG-II appeared in plants, the question remains as to whether the activated sugars required for RG-II synthesis appeared at the same time as RG-II or were already present in plants but used for the formation of other carbohydrate-containing molecules. An examination of publically available sequence data (genomic and expression sequence tags) revealed that genes with homology to UDP-apiose synthase (UAXS/AXS) exist in land plants but not in green algae (**Table 1**). By contrast, genes for the formation of CMP-Kdo are widely distributed in green plants (**Table 1**). However, this alone is not evidence for the presence of RG-II. For example, the walls of some members of the Prasinophyceaen algae (Chlorophyta) contain sulfated polysaccharides composed of Kdo, 5-*O*-methyl Kdo, Dha, and GalA (Becker et al., 1989, 1991, 1998, 2001). Dha has also been reported to be a present in the scales of the Streptophyte alga *Mesostigma viride* (Domozych et al., 1991). It is believed that these ancient green algae or their ancestors acquired the genes for the formation of the activated forms of Kdo and Dha from prokaryotes (Royo et al., 2000). Interestingly, the Prasinophyte polysaccharide, which has been reported to have a backbone composed of 2,8-linked Kdo residues, has structural features similar to the polysialic acids produced by animals and bacteria (Muhlenhoff et al., 1998). The presence in land plants of genes encoding Kdo transferases can also no longer be considered a marker for RG-II, as the studies by Raetz and colleagues have shown that *Arabidopsis* has orthologs of several enzymes involved in the biosynthesis bacterial Kdo-containing glycolipids (Li et al., 2011).

Clearly, our knowledge of the changes in the types and amounts of polysaccharides in the cell wall during the evolution of land plants is rudimentary (Niklas, 2004; Popper and Tuohy, 2010; Sørensen et al., 2010; Popper et al., 2011). How such changes affected wall architecture and function also need to be elucidated. If substantial advances are to be made in plant cell wall biology we will require a combination of chemical, biochemical, and genetic data from a much wider taxonomic range of Chlorophyta and Streptophyta (Viridiplantae) than is currently available. Such metadata can then be evaluated using computational biology to reveal candidate genes and proteins involved in wall biology in general as well as in RG-II formation.

FUTURE CHALLENGES

Although there has been some progress in understanding RG-II structure and function, numerous challenges remain for the current and future generations of cell wall researchers. Clearly, a complete description of RG-II formation will require the identification of the activated forms of aceric acid and Dha as well as the functional characterization of the myriad genes encoding the glycosyl, acetyl, and methyl transferases involved in RG-II assembly. Progress in this area will be dependent on the ability to generate the appropriate acceptor substrates and to develop facile assays for the individual transferases activities. Success in these endeavors will provide an opportunity to determine if RG-II is either formed "one sugar at a time" or built in manner analogous to the

N-linked oligosaccharides of glycoproteins. With this information in hand it will be possible to identify the factors that initiate RG-II synthesis and to determine the order of side chain assembly. This in turn will lead to insight into whether the amounts of RG-II formed and inserted into the wall is controlled by the activities of individual glycosyltransferases, the availability of specific nucleotide sugars or other yet to be discovered mechanisms. Since RG-II cross-linking is sensitive to changes in RG-II structure, it is important to determine if there are "quality control processes" in place to ensure that only the correct type of RG-II is deposited in the cell wall. Additional studies are required to address if ester formation *in vivo* is an entirely spontaneous chemical reaction or a reaction catalyzed by other molecules. Irrespective of where borate cross-linking occurs, there is a need to determine how two RG-II molecules "find one another" and then align so that the borate ester can form. Molecular modeling studies are likely to provide clues as to the ability of RG-II molecules to form complementary structures that interact with each other and thereby facilitate and stabilize cross-linking. In addition, studies are required to determine how plant cells detect changes in cross-linking of wall pectins and how such changes are translated into altered growth. Another major challenge will be to identify the mechanisms that coordinate the synthesis of RG-II and the other pectic polysaccharides present in the cell wall. There is increasing awareness that plant cell wall pectic polysaccharides may be formed by enzymes complexes (Atmodjo et al., 2011). If such complexes do exist, plant scientists must determine if all three pectin domains are synthesized by a "mega-complex" in the Golgi and then secreted into the apoplast. Alternatively, there may be complexes within the Golgi that synthesize the individual pectin domains. These domains would then need to be linked to one another by enzymes located either in the Golgi or the apoplast. A combination of genetic, biochemical, and physicochemical studies will be required to understand the biology of RG-II and will allow researchers to determine if the structural complexity of RG-II evolved solely to ensure the "correct" borate cross-linking of pectin in the primary wall or if this unusual polysaccharide has other as yet unidentified functions in plant growth and development.

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REFERENCES

Ahn, J. W., Verma, R., Kim, M., Lee, J. Y., Kim, Y. K., Bang, J. W., Reiter, W. D., and Pai, H. S. (2006). Depletion of UDP-D-apiose/UDP-D-xylose synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. *J. Biol. Chem.* 281, 13708–13718.

Atmodjo, M. A., Sakuragi, Y., Zhu, X., Burrell, J. A., Mohanty, S. S., Atwood, J. A. III, Orlando, R., Scheller, H. V., and Mohnen, D. (2011). Galacturonosyltransferase (GAUT)1 and GAUT7 are the core of a plant cell wall pectin biosynthetic homogalacturonan:galacturonosyltransferase complex. *Proc. Nat. Acad. Sci. U.S.A.* 108, 20225–20230.

Bar-Peled, M., Lewinsohn, E., Fluhr, R., and Gressel, J. (1991). UDP-rhamnose: flavanone-7-O-glucoside -2-O-rhamnosyltransferase. Purification and characterization of an enzyme catalyzing the production of bitter compounds in citrus. *J. Biol. Chem.* 266, 20953–20960.

Bar-Peled, M., and O'Neill, M. A. (2011). Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. *Annu. Rev. Plant Biol.* 62, 127–155.

Becker, B., Becker, D., Kamerling, J. P., and Melkonian, M. (1991). 2-keto-sugar acids in green flagellates: a chemical marker for Prasinophycean scales. *J. Phycol.* 27, 498–504.

Becker, B., Feja, N., and Melkonian, M. (2001). Analysis of expressed sequence tags (ESTs) from the scaly green flagellate *Scherffelia dubia* Pascher emend. Melkonian et Preisig. *Protist* 152, 139–147.

Becker, B., Hard, K., Melkonian, M., Kamerling, J. P., and Vligenthart, F. G. V. (1989). Identification of 3-deoxy-manno-2-octulosonic acid, 3-deoxy-5-O-methyl-manno-2-octulosonic acid and 3-deoxy-lyxo-2-heptulosaric acid in the cell wall (theca) of the green alga *Tetraselmis striata* Buthcher (Prasinophyceae). *Eur. J. Biochem.* 182, 153–160.

Becker, B., Melkonian, M., and Kamerling, J. P. (1998). The cell wall (theca) of *Tetraselmis striata* (Chlorophyta): macromolecular composition and structural elements of the complex polysaccharides. *J. Phycol.* 34, 779–787.

Birnbaum, K., Shasha, D. E., Wang, J. Y., Jung, J. W., Lambert, G. M., Galbraith, D. W., and Benfey, P. N. (2003). A gene expression map of the *Arabidopsis* root. *Science* 302, 1956–1960.

Brady, S. M., Orlando, D. A., Lee, J. Y., Wang, J. Y., Koch, J., Dinneny, J. R., Mace, D., Ohler, U., and Benfey, P. N. (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. *Science* 318, 801–806.

Brown, P. H., and Hu, H. (1997). Does boron play only a structural role in the growing tissues of higher plants? *Plant Soil* 196, 211–215.

Buckeridge, M. S., Pessoa dos Santos, H., and Tiné, M. A. S. (2000). Mobilisation of storage cell wall polysaccharides in seeds. *Plant Physiol. Biochem.* 38, 141–156.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Chevalier, L., Bernard, S., Ramdani, Y., Lamour, R., Bardor, M., Lerouge, P., Follet-Gueye, M. L., and Driouich, A. (2010). Subcompartment localization of the side chain xyloglucan-synthesizing enzymes within Golgi stacks of tobacco suspension-cultured cells. *Plant J.* 64, 977–989.

Christensen, U., and Scheller, H. V. (2012). Regulation of (1, 3;1, 4) β-d-glucan synthesis in developing endosperm of barley lys mutants. *J. Cereal Sci.* 55, 69–76.

Comer, F. I., and Hart, G. W. (2000). O-Glycosylation of nuclear and cytosolic proteins. *J. Biol. Chem.* 275, 29179–29182.

Darvill, A. G., McNeil, M., and Albersheim, P. (1978). Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiol.* 62, 418–427.

Davis, J., Brandizzi, F., Liepman, A. H., and Keegstra, K. (2010). Arabidopsis mannan synthase CSLA9 and glucan synthase CSLC4 have opposite orientations in the Golgi membrane. *Plant J.* 64, 1028–1037.

Delmas, F., Séveno, M., Northey, J. G. B., Hernould, M., Lerouge, P., McCourt, P., and Chevalier, C. (2008). The synthesis of the rhamnogalacturonan II component 3-deoxy-D-manno-2-octulosonic acid (Kdo) is required for pollen tube growth and elongation. *J. Exp. Bot.* 59, 2639–2647.

Dolezal, O., and Cobbett, C. S. (1991). Arabinose kinase-deficient mutant of *Arabidopsis thaliana*. *Plant Physiol.* 96, 1255–1260.

Domozych, D. S., Wells, B., and Shaw, P. J. (1991). Basket scales of the green alga, *Mesostigma viride*: chemistry and ultrastructure. *J. Cell Sci.* 100, 397–407.

du Penhoat, C. H., Gey, C., Pellerin, P., and Perez, S. (1999). An NMR solution study of the mega-oligosaccharide, rhamnogalacturonan II. *J. Biomol. NMR* 14, 253–271.

Edashige, Y., and Ishii, T. (1998). Rhamnogalacturonan II from cell walls of *Cryptomeria japonica*. *Phytochemistry* 49, 681–690.

Egelund, J., Petersen, B. L., Motawia, M. S., Damager, I., Faik, A., Olsen, C. E., Ishii, T., Clausen, H., Ulvskov, P., and Geshi, N. (2006). *Arabidopsis thaliana* RGXT1 and RGXT2 encode Golgi-localized (1,3)-α-D-xylosyltransferases involved in the synthesis of pectic rhamnogalacturonan-II. *Plant Cell* 18, 2593–2607.

Fleischer, A., O'Neill, M. A., and Ehwald, R. (1999). The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan II. *Plant Physiol.* 121, 829–839.

Fry, S. C., Nesselrode, B. H. W. A., Miller, J. G., and Mewburn, B. R. (2008). Mixed-linkage (1→3, 1→4)-β-D-glucan is a major hemicellulose of Equisetum (horsetail) cell walls. *New Phytol.* 179, 104–115.

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011). O-acetylation of *Arabidopsis* hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Gloaguen, V., Brudieux, V., Closs, B., Barbat, A., Krausz, P., Sainte-Catherine, O., Kraemer, M., Maes, E., and Guerardel, Y. (2010). Structural characterization and cytotoxic properties of an apiose-rich pectic polysaccharide obtained from the cell wall of the marine phanerogam *Zostera marina*. *J. Nat. Prod.* 73, 1087–1092.

Glushka, J. N., Terrell, M., York, W. S., O'Neill, M. A., Gucwa, A., Darvill, A. G., Albersheim, P., and Prestegard, J. H. (2003). Primary structure of the 2-O-methyl-α-fucose-containing side chain of the pectic polysaccharide, rhamnogalacturonan II. *Carbohydr. Res.* 338, 341–352.

Golovchenko, V. V., Ovodova, R. G., Shashkov, A. S., and Ovodov, Y. S. (2002). Structural studies of the pectic polysaccharide from duckweed *Lemna minor* L. *Phytochemistry* 60, 89–97.

Gu, X., and Bar-Peled, M. (2004). The biosynthesis of UDP-galacturonic acid in plants. Functional cloning and characterization of *Arabidopsis* UDP-D-glucuronic acid 4-epimerase. *Plant Physiol.* 136, 4256–4262.

Guyett, P., Glushka, J., Gu, X., and Bar-Peled, M. (2009). Real-time NMR monitoring of intermediates and labile products of the bifunctional enzyme UDP-apiose/UDP-xylose synthase. *Carbohydr. Res.* 344, 1072–1078.

Hadfield, K. A., and Bennett, A. B. (1998). Polygalacturonases: many genes in search of a function. *Plant Physiol.* 117, 337–343.

Hart, D. A., and Kindel, P. K. (1970). Isolation and partial characterization of apiogalacturonans from the cell wall of *Lemna minor*. *Biochem. J.* 116, 569–579.

Hartweck, L. M., Scott, C. L., and Olszewski, N. E. (2002). Two O-linked N-acetylglucosamine transferase genes of *Arabidopsis thaliana* L. *Heynh.* Have overlapping functions necessary for gamete and seed development. *Genetics* 161, 1279–1291.

Hayashi, T., and Maclachlan, G. (1984). Biosynthesis of pentosyl lipids by pea membranes. *Biochem. J.* 217, 791–803.

Hu, H., and Brown, P. H. (1994). Localization of boron in cell walls of squash and tobacco and its association with pectin (evidence for a structural role of boron in the cell wall). *Plant Physiol.* 105, 681–689.

Hu, H., Brown, P. H., and Labavitch, J. M. (1996). Species variability in boron requirement is correlated with cell wall pectin. *J. Exp. Bot.* 47, 227–232.

Ishii, T., and Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry* 57, 969–974.

Ishii, T., Matsunaga, T., and Hayashi, N. (2001). Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiol.* 126, 1698–1705.

Ishii, T., Matsunaga, T., Iwai, H., Satoh, S., and Taoshita, J. (2002). Germanium does not substitute for boron in cross-linking of rhamnogalacturonan II in pumpkin cell walls. *Plant Physiol.* 130, 1967–1973.

Ishii, T., Matsunaga, T., Pellerin, P., O'Neill, M. A., Darvill, A., and Albersheim, P. (1999). The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. *J. Biol. Chem.* 274, 13098–13104.

Jouhet, J., Dubots, E., Marechal, E., and Block, M. A. (2009). "Lipid trafficking in plant photosynthetic cells," in *Lipids in Photosynthesis: Essential and Regulatory Functions*, eds W. Hajime and M. Norio (Berlin: Springer Science), 349–372.

Kauss, H., and Hassid, W. (1967). Biosynthesis of the 4-O-methyl-D-glucuronic acid unit of hemicellulose B by transmethylation from S-adenosyl-L-methionine. *J. Biol. Chem.* 242, 1680–1685.

Kobayashi, M., Kouzu, N., Inami, A., Toyooka, K., Konishi, Y., Matsuoka, K., and Matoh, T. (2011). Characterization of *Arabidopsis* CTP: 3-deoxy-D-manno-2-octulosonate cytidylyltransferase (CMP-KDO synthetase), the enzyme that activates KDO during rhamnogalacturonan II biosynthesis. *Plant Cell Physiol.* 52, 1832–1843.

Kobayashi, M., Matoh, T., and Azuma, J. (1996). Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol.* 110, 1017–1020.

Kobayashi, M., Nakagawa, H., Asaka, T., and Matoh, T. (1999). Borate-rhamnogalacturonan II bonding reinforced by Ca^{2+} retains pectic polysaccharides in higher-plant cell walls. *Plant Physiol.* 119, 199–204.

Konishi, T., Takeda, T., Miyazaki, Y., Ohnishi-Kameyama, M., Hayashi, T., O'Neill, M. A., and Ishii, T. (2007). A plant mutase that interconverts UDP-arabinofuranose and UDP-arabinopyranose. *Glycobiology* 17, 345–352.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54, 631–664.

Kotake, T., Hojo, S., Tajima, N., Matsuoka, K., Koyama, T., and Tsumuraya, Y. (2008). A bifunctional enzyme with L-fucokinase and GDP-L-fucose pyrophosphorylase activities salvages free L-fucose in *Arabidopsis*. *J. Biol. Chem.* 283, 8125–8135.

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. Nat. Acad. Sci. U.S.A.* 108, 11387–11392.

Liu, X. L., Liu, L., Niu, Q. K., Xia, C., Yang, K. Z., Li, R., Chen, L. Q., Zhang, X. Q., Zhou, Y., and Ye, D. (2011). MALE GAMETOPHYTE DEFECTIVE 4 encodes a rhamnogalacturonan II xylosyltransferase and is important for growth of pollen tubes and roots in *Arabidopsis*. *Plant J.* 65, 647–660.

Longland, J. M., Fry, S. C., and Trewavas, A. J. (1989). Developmental control of apiogalacturonan biosynthesis and UDP-apiose production in a duckweed. *Plant Physiol.* 90, 972–978.

Loomis, W., and Durst, R. (1992). Chemistry and biology of boron. *Biofactors* 3, 229–239.

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., and Pauly, M. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078.

Martinez, V., Ingwers, M., Smith, J., Glushka, J., Yang, T., and Bar-Peled, M. (2012). Biosynthesis of UDP-4-keto-6-deoxyglucose and UDP-rhamnose in the pathogenic fungi *Magnaporthe grisea* and *Botryotinia fuckeliana*. *J. Biol. Chem.* 287, 879–892.

Matoh, T., Ishigaki, K., Ohno, K., and Azuma, J. (1993). Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol.* 34, 639–642.

Matoh, T., Kawaguchi, S., and Kobayashi, M. (1996). Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls of higher plants. *Plant Cell Physiol.* 37, 636–644.

Matoh, T., and Kobayashi, M. (1998). Boron and calcium, essential inorganic constituents of pectic polysaccharides in higher plant cell walls. *J. Plant Res.* 111, 179–190.

Matoh, T., Takasaki, M., Kobayashi, M., and Takabe, K. (2000). Boron nutrition of cultured tobacco BY-2 cells. III. Characterization of the boron-rhamnogalacturonan II complex in cells acclimated to low levels of boron. *Plant Cell Physiol.* 41, 363–366.

Matsunaga, T., Ishii, T., Matsumoto, S., Higuchi, M., Darvill, A., Albersheim, P., and O'Neill, M. A. (2004). Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. Implications for the evolution of vascular plants. *Plant Physiol.* 134, 339–351.

Melton, L. D., McNeil, M., Darvill, A. G., Albersheim, P., and Dell, A. (1986). Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydr. Res.* 146, 279–305.

Miwa, K., and Fujiwara, T. (2010). Boron transport in plants: co-ordinated regulation of transporters. *Ann. Bot.* 105, 1103–1108.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* 11, 266–277.

Mølhøj, M., Verma, R., and Reiter, W. D. (2003). The biosynthesis of the branched-chain sugar D-apiose in plants: functional cloning and characterization of a UDPα-D-apiose/UDP-D-xylose synthase from *Arabidopsis. Plant J.* 35, 693–703.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., and Knox, J. P. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Muhlenhoff, M., Eckhardt, M., and Gerardy-Schahn, R. (1998). Polysialic acid: three-dimensional structure, biosynthesis and function. *Curr. Opin. Struct. Biol.* 8, 558–564.

Niklas, K. J. (2004). The cell walls that bind the tree of life. *Bioscience* 54, 831–841.

Noguchi, K., Ishii, T., Matsunaga, T., Kakegawa, K., Hayashi, H., and Fujiwara, T. (2003). Biochemical properties of the cell wall in the *Arabidopsis* mutant bor1-1 in relation to boron nutrition. *J. Plant Nutr. Soil Sci.* 166, 175–178.

Olszewski, N. E., West, C. M., Sassi, S. O., and Hartweck, L. M. (2010). O-GlcNAc protein modification in plants: evolution and function. *Biochim. Biophys. Acta* 1800, 49–56.

O'Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 294, 846.

O'Neill, M. A., Ishii, T., Albersheim, P., and Darvill, A. G. (2004). Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu. Rev. Plant Biol.* 55, 109–139.

O'Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G., and Albersheim, P. (1996). Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cells, forms a dimer that is covalently cross-linked by a borate ester. *J. Biol. Chem.* 271, 22923–22931.

Ovodov, Y. S., Ovodova, R., Bondarenko, O., and Krasikova, I. (1971). The pectic substances of zosteraceae: part IV. Pectinase digestion of zosterine. *Carbohydr. Res.* 18, 311–318.

Parakkottil Chothi, M., Duncan, G. A., Armirotti, A., Abergel, C., Gurnon, J. R., Van Etten, J. L., Bernardi, C., Damonte, G., and Tonetti, M. (2010). Identification of an L-rhamnose synthetic pathway in two nucleocytoplasmic large DNA viruses. *J. Virol.* 84, 8829–8838.

Pauly, M., and Scheller, H. V. (2000). O-Acetylation of plant cell wall polysaccharides: identification and partial characterization of a rhamnogalacturonan O-acetyl-transferase from potato suspension-cultured cells. *Planta* 210, 659–667.

Pellerin, P., Doco, T., Vida, S., Williams, P., Brillouet, J. M., and O'Neill, M. A. (1996). Structural characterization of red wine rhamnogalacturonan II. *Carbohydr. Res.* 290, 183–197.

Pérez, S., Rodriguez-Carvajal, M., and Doco, T. (2003). A complex plant cell wall polysaccharide: rhamnogalacturonan II. A structure in quest of a function. *Biochimie* 85, 109–121.

Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant Biol.* 62, 567–590.

Popper, Z. A., and Tuohy, M. G. (2010). Beyond the green: understanding the evolutionary puzzle of plant and algal cell walls. *Plant Physiol.* 153, 373–383.

Puvanesarajah, V., Darvill, A. G., and Albersheim, P. (1991). Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. *Carbohydr. Res.* 218, 211–222.

Rautengarten, C., Ebert, B., Herter, T., Petzold, C. J., Ishii, T., Mukhopadhyay, A., Usadel, B., and Scheller, H. V. (2011). The interconversion of UDP-arabinopyranose and UDP-arabinofuranose is indispensable for plant development in *Arabidopsis. Plant Cell* 23, 1373–1390.

Reuhs, B. L., Glenn, J., Stephens, S. B., Kim, J. S., Christie, D. B., Glushka, J. G., Zablackis, E., Albersheim, P., Darvill, A. G., and O'Neill, M. A. (2004). L-Galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient mur1 *Arabidopsis* mutant. *Planta* 219, 147–157.

Reyes, F., and Orellana, A. (2008). Golgi transporters: opening the gate to cell wall polysaccharide biosynthesis. *Curr. Opin. Plant Biol.* 11, 244–251.

Round, A. N., Rigby, N. M., MacDougall, A. J., and Morris, V. J. (2010). A new view of pectin structure revealed by acid hydrolysis and atomic force microscopy. *Carbohydr. Res.* 345, 487–497.

Round, A. N., Rigby, N. M., MacDougall, A. J., Ring, S. G., and Morris, V. J. (2001). Investigating the nature of branching in pectin by atomic force microscopy and carbohydrate analysis. *Carbohydr. Res.* 331, 337–342.

Royo, J., Gimez, E., and Hueros, G. (2000). CMP-KDO synthetase: a plant gene borrowed from gram-negative eubacteria. *Trends Genet.* 16, 432–433.

Schoberer, J., and Strasser, R. (2011). Sub-compartmental organization of Golgi-resident N-glycan processing enzymes in plants. *Mol. Plant* 4, 220–228.

Seguí-Simarro, J. M., and Staehelin, L. A. (2006). Cell cycle-dependent changes in Golgi stacks, vacuoles, clathrin-coated vesicles and multivesicular bodies in meristematic cells of *Arabidopsis thaliana*: a quantitative and spatial analysis. *Planta* 223, 223–236.

Séveno, M., Séveno-Carpentier, E., Voxeur, A., Menu-Bouaouiche, L., Rihouey, C., Delmas, F., Chevalier, C., Driouich, A., and Lerouge, P. (2010). Characterization of a putative 3-deoxy-D-manno-2-octulosonic acid (Kdo) transferase gene from *Arabidopsis thaliana. Glycobiology* 20, 617–628.

Séveno, M., Voxeur, A., Rihouey, C., Wu, A. M., Ishii, T., Chevalier, C., Ralet, M. C., Driouich, A., Marchant, A., and Lerouge, P. (2009). Structural characterisation of the pectic polysaccharide rhamnogalacturonan II using an acidic fingerprinting methodology. *Planta* 230, 947–957.

Sharma, C. B., Babczinksi, P., Lehle, L., and Tanner, W. (1974). The role of dolichol monophosphate in glycoprotein biosynthesis in *Saccharomyces cerevisiae. Eur. J. Biochem.* 46, 35–41.

Shimokawa, T., Ishii, T., and Matsunaga, T. (1999). Isolation and structural characterization of rhamnogalacturonan II-borate complex from *Pinus densiflora. J. Wood Sci.* 45, 435–439.

Slawson, C., Copeland, R., and Hart, G. (2010). O-GlcNAc signaling: a metabolic link between diabetes and cancer? *Trends Biochem. Sci.* 35, 547–555.

Søgaard, C., Stenbæk, A., Bernard, S., Hadi, M., Driouich, A., Scheller, H. V., and Sakuragi, Y. (2012). GO-PROMTO illuminates protein membrane topologies of glycan biosynthetic enzymes in the Golgi apparatus of living tissues. *PLoS ONE* 7, e31324. doi:10.1371/journal.pone.0031324

Sørensen, I., Domozych, D., and Willats, W. G. T. (2010). How have plant cell walls evolved? *Plant Physiol.* 153, 366–372.

Sørensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F., O'Neill, M. A., Fei, Z., Rose, J. K. C., Domozych, D. S., and Willats, W. G. T. (2011). The Charophycean green algae provide insights into the early origins of plant cell walls. *Plant J.* 68, 201–211.

Sørensen, I., Pettolino, F. A., Wilson, S. M., Doblin, M. S., Johansen, B., Bacic, A., and Willats, W. G. T. (2008). Mixed linkage (1→3),(1→4)-β-D-glucan is not unique to the Poales and is an abundant component of Equisetum arvense cell walls. *Plant J.* 54, 510–521.

Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P., and Henrick, K. (1983a). Isolation and characterization of 3-C-carboxy-5-deoxy-L-xylose, a naturally occurring, branched-chain, acidic monosaccharide. *Carbohydr. Res.* 122, 115–129.

Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P., and Dell, A. (1983b). Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydr. Res.* 122, 131–153.

Staehelin, L. A., and Kang, B. H. (2008). Nanoscale architecture of endoplasmic reticulum export sites and of Golgi membranes as determined by electron tomography. *Plant Physiol.* 147, 1454–1468.

Steiner, E., Efroni, I., Gopalraj, M., Saathoff, K., Tseng, T. S., Kieffer, M., Eshed, Y., Olszewski, N., and Weiss, D. (2012). The *Arabidopsis* O-linked N-acetylglucosamine transferase SPINDLY interacts with class I TCPs to facilitate cytokinin responses in leaves and flowers. *Plant Cell* 24, 96–108.

Sterling, J. D., Atmodjo, M. A., Inwood, S. E., Kumar Kolli, V., Quigley, H. F., Hahn, M. G., and Mohnen, D. (2006). Functional identification of an *Arabidopsis* pectin biosynthetic homogalacturonan galacturonosyltransferase. *Proc. Nat. Acad. Sci. U.S.A.* 103, 5236–5241.

Sterling, J. D., Quigley, H. F., Orellana, A., and Mohnen, D. (2001). The catalytic site of the pectin

biosynthetic enzyme (-1,4-galacturonosyltransferase is located in the lumen of the Golgi. *Plant Physiol.* 127, 360–371.

Stevenson, T. T., Darvill, A. G., and Albersheim, P. (1988a). 3-Deoxy-D-lyxo-2-heptulosaric acid, a component of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydr. Res.* 179, 269–288.

Stevenson, T. T., Darvill, A. G., and Albersheim, P. (1988b). Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydr. Res.* 182, 207–226.

Thomas, J. R., Darvill, A. G., and Albersheim, P. (1989). Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Carbohydr. Res.* 185, 261–277.

Trezer, A., Salas, J. A., and Bechtold, A. (1999). Genes and enzymes involved in deoxysugar biosynthesis in bacteria. *Nat. Prod. Rep.* 16, 283–299.

Vicente, A. R., Saladie, M., Rose, J. K. C., and Labavitch, J. M. (2007). The linkage between cell wall metabolism and fruit softening: looking to the future. *J. Sci. Food Agric.* 87, 1435–1448.

Vidal, S., Doco, T., Williams, P., Pellerin, P., York, W. S., O'Neill, M. A., Glushka, J., Darvill, A. G., and Albersheim, P. (2000). Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydr. Res.* 326, 277–294.

Vidal, S., Salmon, J. M., Williams, P., and Pellerin, P. (1999). *Penicillium daleae*, a soil fungus able to degrade rhamnogalacturonan II, a complex pectic polysaccharide. *Enzyme Microb. Technol.* 24, 283–290.

Vincken, J. P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., and Visser, R. G. F. (2003). If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol.* 132, 1781–1789.

Voxeur, A., Gilbert, L., Rihouey, C., Driouich, A., Rothan, C., Baldet, P., and Lerouge, P. (2011). Silencing of the GDP-D-mannose 3,5-epimerase affects the structure and cross-linking of the pectic polysaccharide rhamnogalacturonan II and plant growth in tomato. *J. Biol. Chem.* 286, 8014–8023.

Watt, G., Leoff, C., Harper, A. D., and Bar-Peled, M. (2004). A bifunctional 3, 5-epimerase/4-keto reductase for nucleotide-rhamnose synthesis in *Arabidopsis*. *Plant Physiol.* 134, 1337–1346.

Weerapana, E., and Imperiali, B. (2006). Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems. *Glycobiology* 16, 91R–101R.

Whitcombe, A. J., O'Neill, M. A., Steffan, W., Albersheim, P., and Darvill, A. G. (1995). Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II. *Carbohydr. Res.* 271, 15–29.

Yang, T., Bar-Peled, L., Gebhart, L., Lee, S. G., and Bar-Peled, M. (2009). Identification of galacturonic acid-1-phosphate kinase, a new member of the GHMP kinase superfamily in plants, and comparison with galactose-1-phosphate kinase. *J. Biol. Chem.* 284, 21526–21532.

Yin, Y., Chen, H., Hahn, M. G., Mohnen, D., and Xu, Y. (2010). Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *Plant Physiol.* 153, 1729–1746.

York, W. S., Darvill, A. G., McNeil, M., and Albersheim, P. (1985). 3-Deoxy-D-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. *Carbohydr. Res.* 138, 109–126.

Zhang, B., Liu, X., Qian, Q., Liu, L., Dong, G., Xiong, G., Zeng, D., and Zhou, Y. (2011). Golgi nucleotide sugar transporter modulates cell wall biosynthesis and plant growth in rice. *Proc. Nat. Acad. Sci. U.S.A.* 108, 5110–5115.

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Small molecule probes for plant cell wall polysaccharide imaging

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Plant cell walls are composed of interlinked polymer networks consisting of cellulose, hemicelluloses, pectins, proteins, and lignin. The ordered deposition of these components is a dynamic process that critically affects the development and differentiation of plant cells. However, our understanding of cell wall synthesis and remodeling, as well as the diverse cell wall architectures that result from these processes, has been limited by a lack of suitable chemical probes that are compatible with live-cell imaging. In this review, we summarize the currently available molecular toolbox of probes for cell wall polysaccharide imaging in plants, with particular emphasis on recent advances in small molecule-based fluorescent probes. We also discuss the potential for further development of small molecule probes for the analysis of cell wall architecture and dynamics.

Keywords: plant cell wall, cell wall dynamics, cellulose, pectin, click chemistry

INTRODUCTION

Terrestrial plants annually assimilate 1.3×10^{11} metric tons of CO_2 through the process of photosynthesis (Beer et al., 2010), and the majority of the resulting photosynthate is used to produce the polysaccharide-rich walls that surround plant cells (Pauly and Keegstra, 2008). These complex extracellular matrices are composed of cellulose microfibrils (CMFs), neutral hemicelluloses, acidic pectins, and proteins and must simultaneously be strong enough to resist the substantial cellular osmotic pressure necessary to support turgor-mediated cell growth and dynamic enough to allow cell expansion to occur (Somerville et al., 2004; Cosgrove, 2005). As a result, the ordered deposition of plant cell walls determines the patterning and extent of plant growth and development at the cellular level (Desnos et al., 1996; Arioli et al., 1998; Nicol et al., 1998).

Despite the physiological importance of cell wall deposition and dynamics, relatively little is known about cell wall structure in living cells, primarily due to a paucity of effective labeling strategies for polysaccharides in their native environment. Genetically encoded fluorescent tags for polysaccharides do not exist, necessitating novel approaches for the specific labeling of cell wall glycans. Although chemical (Reiter et al., 1997; Brown et al., 2007) and biochemical (Lerouxel et al., 2002; Barton et al., 2006; Bauer et al., 2006) techniques can accurately determine the primary structures of polysaccharides, these methods provide few spatial details due to the necessity for sample homogenization. Imaging approaches, such as transmission electron microscopy (Moore et al., 1991; Lynch and Staehelin, 1992), Raman microspectroscopy (Schmidt et al., 2010), and fluorescence microscopy with an array of polysaccharide-specific antibodies, lectins, and carbohydrate binding domains (Pattathil et al., 2010) have provided qualitative information regarding the localization and relative abundance of numerous cell wall polysaccharides. However, these techniques are limited by long sample preparation times, potential fixation artifacts, loss of temporal information, lack of epitope specificity (Pattathil et al., 2010), and complications in interpretation due to epitope masking (Marcus et al., 2008). In addition, protein-based probes, particularly intact 160 kDa IgG molecules, are very large compared to the approximately 3 nm pore size of cell walls (Chesson et al., 1997) and may not efficiently label some cell wall components (Marcus et al., 2010). As a result, many developmental changes in cell wall architecture cannot be fully characterized using these approaches.

Polysaccharide-specific fluorescent probes and metabolic labels augment the currently available cell wall imaging toolbox, with the added benefit that these probes are compatible with live-cell imaging and/or pulse-chase experiments. In this review, we summarize the recent development of specific small molecule chemical probes for cell wall imaging, and the insights that these new probes have provided concerning plant cell wall structure.

CELL WALL POLYSACCHARIDE BIOSYNTHESIS

The structural complexity of the plant cell wall arises in part due to the variety of polysaccharides used to assemble this organelle, the spatial and temporal separation of their synthesis, and the various chemical modifications that occur within these polysaccharides. Cell wall polysaccharides are often grouped into three functional categories: cellulose, hemicelluloses, and pectins. Cellulose is composed of 30–40 β-(1 $\rightarrow$ 4)-linked glucan chains that associate to produce semi-crystalline CMFs (Somerville, 2006). These structural polysaccharides are synthesized at the plasma membrane by 30 nm "rosette" complexes (Arioli et al., 1998; Kimura et al., 1999) containing multiple cellulose synthase A (CesA) catalytic subunits (Taylor et al., 2003; Desprez et al., 2007; Persson et al., 2007). Live-cell imaging has indicated that CesA-containing complexes co-align with cortical microtubules underlying the plasma

membrane as they produce nascent CMFs (Paredez et al., 2006; Li et al., 2012), which are deposited in the apoplast.

In contrast to CMFs, hemicelluloses and pectins are synthesized in the Golgi (Mohnen, 2008; Caffall and Mohnen, 2009) and delivered to the apoplast by vesicle-mediated exocytosis via an incompletely described pathway (Moore et al., 1991; Lynch and Staehelin, 1992). Once deposited in the apoplast, hemicelluloses, such as xyloglucan, are thought to associate with and crosslink CMFs in the cell wall (Vincken et al., 1995; Lima et al., 2004), forming a cellulose–hemicellulose network. The interaction between these polysaccharides is proposed to prevent individual CMFs from interacting with each other and to help organize CMFs into a cohesive, dynamic network, although this model has recently been challenged (Park and Cosgrove, 2012b). Hemicelluloses and CMFs are embedded in a gel-like matrix composed of the pectic polysaccharides homogalacturonan, xylogalacturonan (XGA), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) which together control aspects of cell adhesion, wall extensibility, and wall porosity (Krupkova et al., 2007; Mohnen, 2008).

Plant cell wall polysaccharides can also be modified during synthesis or after deposition in the apoplast by the activity of various enzymes. Pectins can be reversibly acetylated at the O-2 or O-3 hydroxyl groups of their galacturonic acid backbone sugars and/or methylesterified at the O-6 carboxyl group (Ishii, 1997; Manabe et al., 2011). Hemicelluloses can also be acetylated (Pauly et al., 1999; Kabel et al., 2003; Gibeaut et al., 2005; Gille et al., 2011) or have their glycan side chains modified during plant development (Günl et al., 2011). Finally, transglycosylase enzymes, such as xyloglucan endotransglycosylase (XET), cut and religate individual xyloglucan chains during wall extension (Xu et al., 1995; Maris et al., 2009). These modifications can significantly affect the physical properties of individual wall polymers (Huang et al., 2002; Ngouemazong et al., 2012) and of the cell wall as a whole (Krupkova et al., 2007; Peaucelle et al., 2008).

POLYSACCHARIDE-BINDING DYES AND METABOLIC LABELS FOR CELL WALL POLYSACCHARIDE IMAGING

Cell wall polysaccharides are structurally diverse, exist in complex three-dimensional arrangements, and are synthesized, deposited, modified, and degraded in highly dynamic processes. Therefore, probes that are highly specific for an individual polysaccharide type and compatible with live-cell imaging can effectively capture this complexity. Various polysaccharide-binding dyes, including Calcofluor white (Fagard et al., 2000; Homann et al., 2007; Harpaz-Saad et al., 2011), Congo red (Kerstens and Verbelen, 2002), ruthenium red (Western et al., 2001; Marks et al., 2008; Harpaz-Saad et al., 2011), and Aniline blue (Adam and Somerville, 1996; Nishikawa et al., 2005; Nguyen et al., 2010; Xie et al., 2011), have been used as imaging tools for plant cell walls, but these dyes cross-react with multiple polysaccharides, and their direct glycan targets are often unknown. To address this issue, Anderson et al. (2010) assayed the previously described fungal cell wall-binding dyes Pontamine Fast Scarlet S4B (S4B) and Solophenyl Flavine 7GFE (7GFE; Hoch et al., 2005) for their ability to bind isolated cell wall polysaccharides. S4B (**Figure 1A**) exhibits several useful imaging properties, including increased fluorescence intensity in the presence of cellulose, increased glycan specificity over Calcofluor white, and spectral properties compatible with 532 and 561 nm lasers. S4B is also non-toxic at concentrations that are compatible with fluorescent imaging and can thus be used as a specific probe to dynamically image cellulose in living plant tissues. 7GFE (**Figure 1B**) exhibits a characteristic shift in its fluorescence emission maximum in the presence of xyloglucan, but not cellulose. Whereas 7GFE is slightly more toxic than S4B, this dye could potentially serve as an orthogonal fluorescent label for xyloglucan because its excitation and emission maxima do not overlap with those of S4B (Anderson et al., 2010).

S4B, 7GFE, and other fluorescent polysaccharide-binding probes are valuable tools that should be explored further, but the biophysical details of many dye–polysaccharide interactions are currently unknown, preventing the rational design of new dyes. However, alternative labeling strategies can be employed to design metabolic probes that can be targeted to individual cell wall polysaccharide networks. For example, fluorescently labeled xyloglucan fragments are metabolically incorporated into subdomains of xyloglucan by XETs, and these oligosaccharides have been used to image the sites of XET activity during cell expansion (Vissenberg et al., 2000). Additionally, molecules containing terminal alkynyl or azido functional groups can participate in a copper-catalyzed [2 + 3] cycloaddition referred to as the Huisgen "click" reaction (**Figure 1C**), which is bio-orthogonal, proceeds rapidly at room temperature with high specificity, and results in the formation of a stable triazole ring (Kolb et al., 2001; Rostovtsev et al., 2002; Tornoe et al., 2002). Exploiting this reaction, small, biologically relevant molecules containing these functional groups have been synthesized and incorporated into proteins, fatty acids, nucleic acids, and sugars which are then covalently labeled with useful probes, such as fluorophores, epitope tags, or other affinity reagents that contain the opposite functional group (Chin et al., 2002; Kiick et al., 2002; Deiters et al., 2003; Kho et al., 2004; Link et al., 2006; Rabuka et al., 2006; Hang et al., 2007; Hsu et al., 2007; Jao and Salic, 2008; Salic and Mitchison, 2008; Chang et al., 2009; Kaschani et al., 2009). These chemical methods have been particularly useful for imaging glycan conjugates in animal, bacterial, and fungal systems (Prescher et al., 2004; Dube et al., 2006; Laughlin et al., 2008), but had not previously been explored in the context of the plant cell wall.

Anderson et al. (2012) investigated the potential of click chemistry to label cell wall polysaccharides using per-acetylated fucose alkyne (FucAl; **Figure 1D**) as a suitable click sugar monosaccharide analog. FucAl was metabolically incorporated into *Arabidopsis* root tissue and labeled with Alexa-488 azide using the copper-catalyzed click reaction (**Figures 1D–F**). Cytological and biochemical analyses indicated that the majority of the FucAl label was incorporated into the pectic fraction of *Arabidopsis* cell walls, most likely in arabinogalactan side chains of rhamnogalacturonan-I (RG-I). Fucose is present in multiple cell wall polysaccharides (Nakamura et al., 2001; Lerouxel et al., 2002; Glushka et al., 2003; Strasser et al., 2004; Wu et al., 2010), but *Arabidopsis* mutants lacking fucosyltransferases for xyloglucan (Vanzin et al., 2002), arabinogalactan protein (Wu et al., 2010), and N-linked glycoproteins

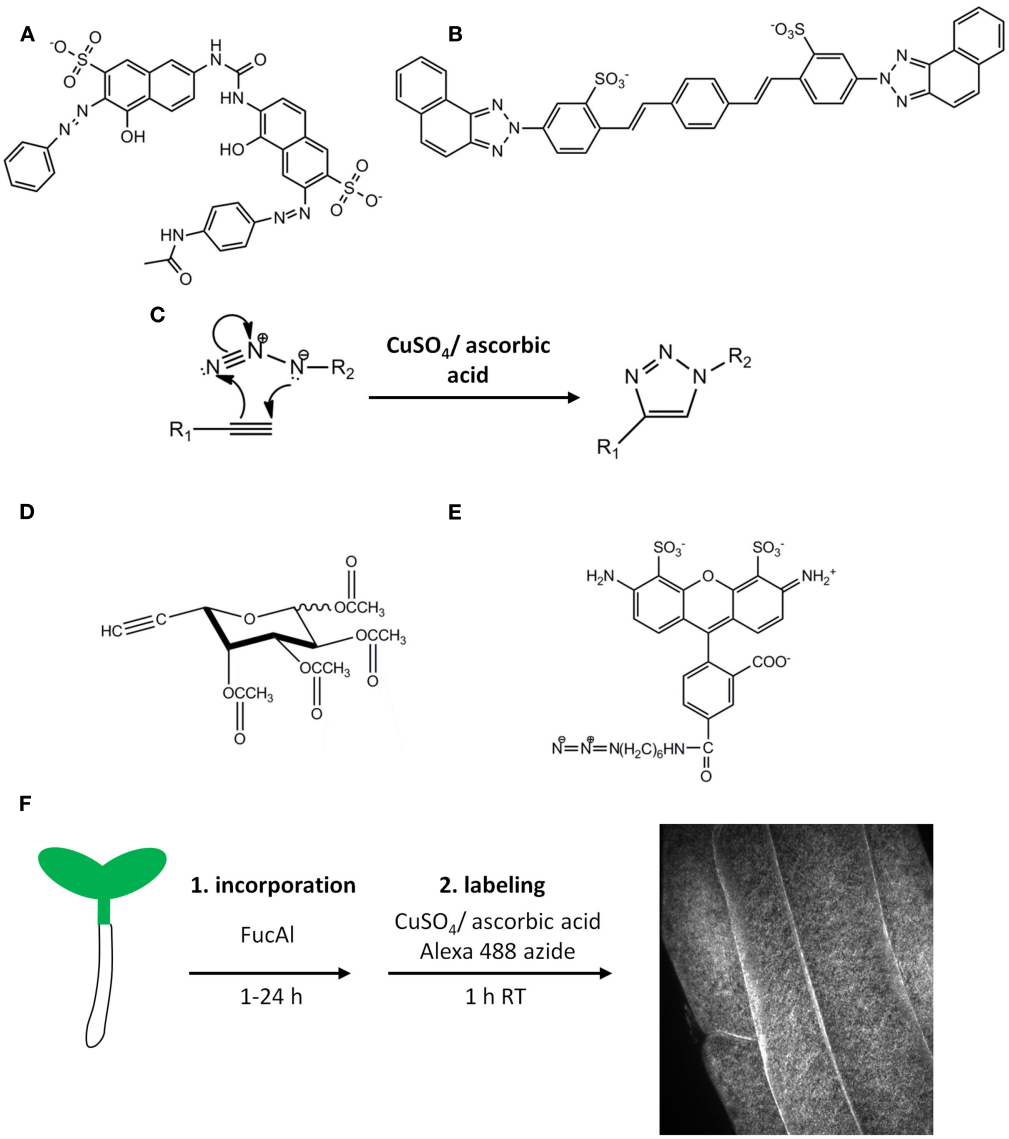


FIGURE 1 | Fluorescent probes for cell wall polysaccharide imaging. The structures of the cellulose-binding dye Pontamine Fast Scarlet 4B (S4B) **(A)** and the xyloglucan-binding dye Solophenyl Flavine 7GFE **(B)**. **(C)** A generalized version of the copper-catalyzed Huisgen [2 + 3] cycloaddition reaction. This reaction is useful for coupling molecules containing terminal alkynyl or azido groups, such as per-acetylated fucose alkyne (FucAl) **(D)** and Alexa-488 azide **(E)**. Sugar analogs such as FucAl can be metabolically incorporated into glycans via the action of sugar salvage pathways and cognate glycosyltransferases, producing modified polysaccharides that can be labeled with azido-containing fluorophores by the click reaction. This strategy was used in Anderson et al. (2012) to metabolically label pectins by incorporating FucAl into the root tissue of *Arabidopsis* followed by copper-catalyzed labeling as shown in **(F)**. A representative image of root epidermal cells in the late elongation zone after labeling is shown in the right panel.

(Strasser et al., 2004) incorporated FucAl at levels similar to wild-type plants, suggesting that FucAl does not evenly label all fucosylated cell wall glycans (Anderson et al., 2012). We hypothesize that this selectivity might arise from conformational differences in the active sites of different fucosyltransferases, suggesting that click chemistry-compatible sugar analogs might be selectively incorporated into specific polysaccharide networks even if the parent monosaccharide is present in several polymers. Structure–activity relationships of click sugar variants containing the alkyne or azide at different positions may elucidate the structural requirements for selective monosaccharide analog incorporation.

POLYSACCHARIDE IMAGING PROBES HIGHLIGHT THE STRUCTURAL DYNAMICS OF CELL WALLS DURING DEVELOPMENT

S4B staining and spinning disk confocal microscopy were used to image CMFs in *Arabidopsis* root tissues (Anderson et al., 2010), which represent a developmental continuum of dividing, expanding, and differentiated cells (Dolan et al., 1993). S4B-stained CMFs were transversely oriented with respect to the major cellular expansion axis in recently divided cells near the root cap and cells in the early elongation zone. However, older cells in the elongation zone exhibited diagonally oriented CMFs, and fully differentiated cells

that were no longer expanding contained longitudinally oriented CMFs (**Figure 2A**). This developmental gradient of CMF orientation agrees with previous electron microscopy studies describing developmental changes in CMF orientation along *Arabidopsis* roots and hypocotyls (Sugimoto et al., 2000; Refregier et al., 2004), suggesting that S4B staining provides a rapid and specific method for CMF imaging.

FucAl incorporation and labeling has been used to image pectic networks along the same root developmental continuum. After 1 h of incorporation, FucAl labeling in the root elongation zone appeared as small puncta in the cell wall (Anderson et al., 2012), potentially representing the locations of vesicle-mediated pectin delivery to the apoplast. After longer incorporation times (e.g., 12 h), the pattern of FucAl labeling in *Arabidopsis* roots exhibited a developmental gradient from the early elongation zone to the differentiation zone. In recently divided cells, FucAl-associated fluorescence was homogeneous, likely as a result of cumulative evenly distributed vesicle-mediated polysaccharide delivery. However, cells in the late elongation zone exhibit more organized patterns of puncta that transition to diagonally oriented fibrillar structures in the differentiation zone (Anderson et al., 2012). These observations suggest that pectic wall material is delivered and organized in distinct patterns during cellular development and that this developmental gradient parallels the reorientation of CMFs during cell elongation (**Figure 2B**).

Polysaccharide-specific probes can also serve as useful tools for the detailed characterization of mutants affecting cell wall polymer networks. For example, S4B staining has revealed new aspects of wall morphology in previously described *Arabidopsis* mutants defective in cellulose and xyloglucan biosynthesis. The *CesA6* mutant *Procuste1-1* (*prc1-1*; Desnos et al., 1996) produces less crystalline cellulose in its cell walls (Fagard et al., 2000), and *prc1-1* seedlings exhibited reduced S4B staining compared to wild-type seedlings (Anderson et al., 2010). Additionally, microfibrils in the root elongation zone were more defined but less prevalent, suggesting that fewer intact microfibrils were synthesized, leading to compromised cell walls and increased isotropic expansion of root cells. S4B staining of the *xxt1*; *xxt2* mutant, which lacks detectable amounts of xyloglucan (Cavalier et al., 2008; Park and Cosgrove, 2012a), labeled CMF fibers that were more prominently defined and thicker than wild-type CMF fibers, supporting the hypothesis that hemicelluloses prevent CMFs from interacting with each other in wild-type primary cell walls. Furthermore, S4B was recently used to characterize cellulose architecture in *Arabidopsis* seed coat mucilage (Harpaz-Saad et al., 2011), leading to the identification of *Cellulose Synthase A5* (*CesA5*), *FEI2*, and *Salt Overly Sensitive 5* (*SOS5*) as genes responsible for mucilage cellulose deposition. This study indicates that fluorescent probes like S4B will be useful for cell wall imaging and mutant phenotype identification in multiple developmental contexts.

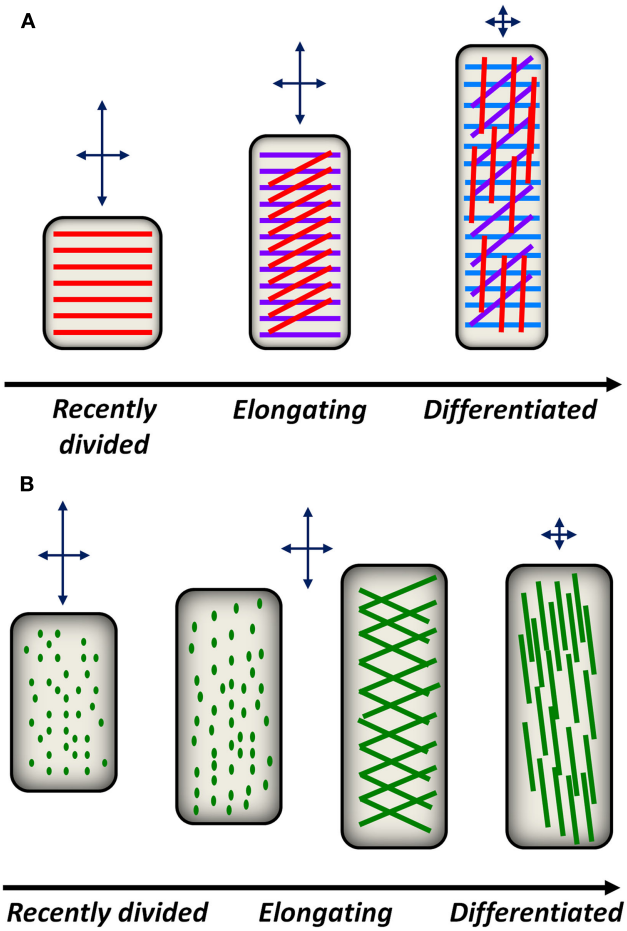


FIGURE 2 | Conceptual models for cell wall polysaccharide deposition and reorientation during plant development. (A) A model for cellulose deposition and reorientation in anisotropically elongating root epidermal cells. Shortly after division in the root meristematic region, cellulose synthase complexes in epidermal cells deposit cellulose (red lines) in a transverse orientation with respect to the elongation axis. This deposition pattern constrains radial expansion of the cell but permits axial expansion as indicated by the arrows above each cell. As epidermal cells elongate, new cellulose layers (magenta lines, blue lines) are deposited transversely, while older layers in the apoplast passively reorient from transverse to diagonal, then longitudinal orientations. The reorientation of older cellulose layers reduces the ability of differentiating cells to expand in the axial direction. **(B)** A model for pectin deposition and remodeling in the apoplast. In recently divided cells, pectic polysaccharides are delivered to the apoplast by secretory vesicles. Putative sites of pectin delivery (green dots) can be imaged with FucAl and are initially distributed evenly across the cell. As epidermal cells stop elongating, diagonal fibrils begin to appear that eventually reorient toward the longitudinal axis, potentially constraining further axial expansion.

DYNAMIC REORIENTATION OF CELL WALL POLYSACCHARIDE NETWORKS

Due to the load-bearing function of CMFs in the cell wall, differences in the orientation of these polymers with respect to the growth axis of anisotropically expanding cells has been proposed to critically affect cell shape (Green, 1960). When CMFs are deposited transversely, they resist radial expansion but allow cells to expand axially. However, as CMFs become longitudinally oriented and parallel to the elongation axis, cells are also expected to encounter expansion resistance in the axial direction (**Figure 2A**). Previous observations suggest that varying CMF angles may result from reorientation of these polysaccharides after deposition in the apoplast (Refregier et al., 2004). Optical sectioning of S4B-stained tissue indicates that CMFs in the outer cell wall layers of differentiated root epidermal cells are oriented longitudinally with respect

to the cellular growth axis, whereas CMFs near the inner face of the wall are oriented transversely (Anderson et al., 2010). Time-lapse imaging of S4B-stained CMFs revealed that the observed changes in orientation are attributable to the rotation of CMFs after they are deposited in the apoplast of root epidermal cells.

FucAl incorporation and labeling was also used to examine the dynamics of pectic networks in *Arabidopsis* (Anderson et al., 2012). Whereas the amounts of FucAl required for incorporation experiments had no effect on normal *Arabidopsis* seedling development, the Cu (I) necessary to catalyze the cycloaddition reaction was toxic, in agreement with previous *in vitro* and *in vivo* observations in other organisms (Agard et al., 2004; Brewer, 2010; Lallana et al., 2011). As an alternative to *in vivo* imaging, plants were pulsed with FucAl for 1 h and labeled at various chase times (Anderson et al., 2012). These experiments revealed that FucAl-associated fluorescence was initially punctate in the apoplast, but appeared as longitudinal fibers after 12 or 24 h chase periods, suggesting that, like CMFs, pectins become longitudinally oriented over time. As a whole, these observations suggest that polysaccharide networks are highly dynamic during cellular development and highlight the importance of specific polysaccharide-directed probes that are capable of capturing these events.

CONCLUSION AND FUTURE DIRECTIONS

The use of multiple labeling strategies for plant cell wall polysaccharides is altering the perception of the cell wall from that of a rigid case to that of a highly dynamic extracellular organelle that changes during development. However, the complexity of the cell wall necessitates the design of specific imaging probes for these dynamic events. Polysaccharide-binding dyes, such as S4B and 7GFE, highlight the utility of fluorescent dyes that are compatible with live-cell imaging and exemplify one avenue for the implementation of new cell wall imaging tools for the future. Indeed, we believe that novel cell wall imaging probes will most likely be isolated via high-throughput fluorescent interaction assays against isolated wall components, structure–activity studies of existing fluorophores, or array-based selectivity analyses that have been described for polysaccharide-binding antibodies (Moller et al., 2008; Pattathil et al., 2010; Sorensen and Willats, 2011). These experiments may lead to the identification of novel fluorophores that label unique polysaccharide populations or networks, so that multiple cell wall polysaccharides can be imaged simultaneously.

Monosaccharide analogs compatible with click chemistry have some advantages over polysaccharide-specific fluorophores that suggest their utility in a variety of experiments. Because click sugars are incorporated metabolically, they label only those polysaccharides synthesized during the incorporation period and are therefore distinct from probes that label all of the existing polysaccharides of a given type. These sugars have the potential to serve as valuable tools for examining the trafficking of polysaccharides from the Golgi to the apoplast, elucidating extracellular events that occur after deposition of polysaccharides in the wall, and the isolation of mutants that affect these processes. These experimental goals will be furthered by the application of click sugars that label other polysaccharide networks, and it may be possible to tune the selective incorporation of a given sugar analog by changing the position or identity of the alkynyl or azido functional group. Membrane permeable click-compatible coumarin and napthalamide fluorescent probes have been described (Sawa et al., 2006; Hsu et al., 2007), and could be used to image intracellular sites of polysaccharide synthesis to complement surface-specific labeling by membrane impermeable fluorophores, such as Alexa Fluor dyes. Additionally, *in vivo* click labeling using either copper-free strain-promoted [2 + 3] cycloaddition reactions (Prescher et al., 2004; Dube et al., 2006; Laughlin et al., 2008) or chelators that sequester toxic Cu (I) (Chan et al., 2004; Lewis et al., 2004) might be applicable to plant systems and could further facilitate the imaging of cell wall glycans in living cells. By adding a temporal dimension to studies of cell wall architecture, the approaches described here provide new ways of examining the synthesis, deposition, and dynamics of plant cell walls that will further our understanding of these remarkable bio-materials, both in the context of plant development and as renewable resources for human use.

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REFERENCES

Adam, L., and Somerville, S. C. (1996). Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana*. *Plant J.* 9, 341–356.

Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004). A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* 126, 15046–15047.

Anderson, C. T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. *Plant Physiol.* 152, 787–796.

Anderson, C. T., Wallace, I. S., and Somerville, C. R. (2012). Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in *Arabidopsis* cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1329–1334.

Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Hofte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998). Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* 279, 717–720.

Barton, C. J., Tailford, L. E., Welchman, H., Zhang, Z., Gilbert, H. J., Dupree, P., and Goubet, F. (2006). Enzymatic fingerprinting of *Arabidopsis* pectic polysaccharides using polysaccharide analysis by carbohydrate electrophoresis (PACE). *Planta* 224, 163–174.

Bauer, S., Vasu, P., Persson, S., Mort, A. J., and Somerville, C. R. (2006). Development and application of a suite of polysaccharide-degrading enzymes for analyzing plant cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11417–11422.

Beer, C., Reichstein, M., Tomelleri, E., Ciais, P., Jung, M., Carvalhais, N., Rodenbeck, C., Arain, M. A., Baldocchi, D., Bonan, G. B., Bondeau, A., Cescatti, A., Lasslop, G., Lindroth, A., Lomas, M., Luyssaert, S., Margolis, H., Oleson, K. W., Roupsard, O., Veenendaal, E., Viovy, N., Williams, C., Woodward, F. I., and Papale, D. (2010). Terrestrial gross carbon dioxide uptake: global distribution and covariation with climate. *Science* 329, 834–838.

Brewer, G. J. (2010). Risks of copper and iron toxicity during aging in humans. *Chem. Res. Toxicol.* 23, 319–326.

Brown, D. M., Goubet, F., Wong, V. W., Goodacre, R., Stephens, E., Dupree, P., and Turner, S. R. (2007). Comparison of five xylan synthesis mutants reveals new insight into the mechanism of xylan synthesis. *Plant J.* 52, 1154–1168.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Chan, T. R., Hilgraf, R., Sharpless, K. B., and Fokin, V. V. (2004). Polytriazoles as copper(I)-stabilizing ligands in catalysis. *Org. Lett.* 6, 2853–2855.

Chang, P. V., Chen, X., Smyrniotis, C., Xenakis, A., Hu, T., Bertozzi, C. R., and Wu, P. (2009). Metabolic labeling of sialic acids in living animals with alkynyl sugars. *Agnew. Chem. Int. Ed. Engl.* 48, 4030–4033.

Chesson, A., Gardener, P. T., and Wood, T. J. (1997). Cell wall porosity and available surface area of wheat straw and wheat grain fractions. *J. Sci. Food Agric.* 75, 289–295.

Chin, J. W., Santoro, S. W., Martin, A. B., King, D. S., Wang, L., and Schultz, P. G. (2002). Addition of p-azido-L-phenylalanine to the genetic code of *Escherichia coli*. *J. Am. Chem. Soc.* 124, 9026–9027.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861.

Deiters, A., Cropp, T. A., Mukherji, M., Chin, J. W., Anderson, J. C., and Schultz, P. G. (2003). Adding amino acids with novel reactivity to the genetic code of *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* 125, 11782–11783.

Desnos, T., Orbovic, V., Bellini, C., Kronenberger, J., Caboche, M., Traas, J., and Hofte, H. (1996). Procuste 1 mutants identify two distinct genetic pathways controlling hypocotyl cell elongation respectively in dark- and light-grown *Arabidopsis* seedlings. *Development* 122, 683–693.

Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15572–15577.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organization of the *Arabidopsis thaliana* root. *Development* 119, 71–84.

Dube, D. H., Prescher, J. A., Quang, C. N., and Bertozzi, C. R. (2006). Probing mucin-type O-linked glycosylation in living animals. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4819–4824.

Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., McCann, M., Rayon, C., Vernhettes, S., and Hofte, H. (2000). PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. *Plant Cell* 12, 2409–2424.

Gibeaut, D. M., Pauly, M., Bacic, A., and Fincher, G. B. (2005). Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. *Planta* 221, 729–738.

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011). O-acetylation of *Arabidopsis* hemicelluloses xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Glushka, J. N., Terrell, M., York, W. S., O'Neill, M. A., Gucwa, A., Darvill, A. G., Albersheim, P., and Prestegard, J. H. (2003). Primary structure of the 2-O-methyl-alpha-L-fucose-containing side chain of the pectic polysaccharide, rhamnogalacturonan-II. *Carbohydr. Res.* 338, 341–352.

Green, P. B. (1960). Multinet growth in the cell wall of *Nitella*. *J. Biophys. Biochem. Cytol.* 7, 289–297.

Günl, M., Neumetzler, L., Kramer, F., de Souza, A., Schultink, A., Pena, M., York, W. S., and Pauly, M. (2011). AXY8 encodes an α-fucosidase, underscoring the importance of apoplastic metabolism on the fine structure of *Arabidopsis* cell wall polysaccharides. *Plant Cell* 23, 4025–4040.

Hang, H. C., Geutjes, E. J., Grotenbreg, G., Pollington, A. M., Bijlmakers, M. J., and Ploegh, H. L. (2007). Chemical probes for the rapid detection of fatty-acylated proteins in mammalian cells. *J. Am. Chem. Soc.* 129, 2744–2745.

Harpaz-Saad, S., McFarlane, H. E., Xu, S., Divi, U. K., Forward, B., Western, T. L., and Kieber, J. J. (2011). Cellulose synthesis via the FEI2 RLK/SOS5 pathway and CELLULOSE SYNTHASE 5 is required for the structure of seed coat mucilage. *Plant J.* 68, 941–953.

Hoch, H. C., Galvani, C. D., Szarowski, D. H., and Turner, J. N. (2005). Two new fluorescent dyes applicable for visualization of fungal cell walls. *Mycologia* 97, 580–588.

Homann, U., Meckel, T., Hewing, J., Hutt, M. T., and Hurst, A. C. (2007). Distinct fluorescent pattern of KAT1::GFP in the plasma membrane of *Vicia faba* guard cells. *Eur. J. Cell Biol.* 86, 489–500.

Hsu, T. L., Hanson, S. R., Kishikawa, K., Wang, S. K., Sawa, M., and Wong, C. H. (2007). Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2614–2619.

Huang, L., Takahasi, R., Kobayashi, S., Kawase, T., and Nishinari, K. (2002). Gelation behavior of native and acetylated konjac glucomannan. *Biomacromolecules* 3, 1296–1303.

Ishii, T. (1997). O-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiol.* 113, 1265–1272.

Jao, C. Y., and Salic, A. (2008). Exploring RNA transcription and turnover in vivo by using click chemistry. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15779–15784.

Kabel, M. A., de Waard, P., Schols, H. A., and Voragen, A. G. J. (2003). Location of O-acetylated oligosaccharides isolated from tomato (*Lycopersicon esculentum*) xyloglucan. *Carbohydr. Res.* 340, 1818–1825.

Kaschani, F., Verhelst, S. H., van Swieten, P. F., Verdoes, M., Wong, C. S., Wang, Z., Kaiser, M., Overkleeft, H. S., Bogyo, M., and van der Hoorn, R. A. (2009). Minitags for small molecules: detecting targets of reactive small molecules in living plant tissues using "click chemistry." *Plant J.* 57, 373–385.

Kerstens, S., and Verbelen, J. P. (2002). Cellulose orientation in the outer epidermal wall of angiosperm roots: implications for biosystematics. *Ann. Bot.* 90, 669–676.

Kho, Y., Kim, S. C., Jiang, C., Barma, D., Kwon, S. W., Cheng, J., Jaunbergs, J., Weinbaum, C., Tamanoi, F., Falck, J., and Zhao, Y. (2004). A tagging-via-substrate technology for detection and proteomics of farnesylated proteins. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12479–12484.

Kiick, K. L., Saxon, E., Tirrell, D. A., and Bertozzi, C. R. (2002). Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 19–24.

Kimura, S., Laosinchai, W., Itoh, T., Cui, X., Linder, C. R., and Brown, R. M. Jr. (1999). Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis*. *Plant Cell* 11, 2075–2086.

Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001). Click chemistry: diverse chemical functions from a few good reactions. *Angew. Chem. Int. Ed. Engl.* 40, 2004–2021.

Krupkova, E., Immerzeel, P., Pauly, M., and Schmulling, T. (2007). The TUMOROUS SHOOT DEVELOPMENT2 gene of *Arabidopsis* encoding a putative methyltransferase is required for cell adhesion and co-ordinated plant development. *Plant J.* 50, 735–750.

Lallana, E., Riguera, R., and Fernandez-Megia, E. (2011). Reliable and efficient procedures for the conjugation of biomolecules through Huisgen azide-alkyne cycloadditions. *Angew. Chem. Int. Ed. Engl.* 50, 8794–8804.

Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008). In vivo imaging of membrane-associated glycans in developing zebrafish. *Science* 320, 664–667.

Lerouxel, O., Choo, T. S., Seveno, M., Usadel, B., Faye, L., Lerouge, P., and Pauly, M. (2002). Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. *Plant Physiol.* 130, 1754–1763.

Lewis, W. G., Magallon, F. G., Fokin, V. V., and Finn, M. G. (2004). Discovery and characterization of catalysts for alkyne-azide cycloaddition by fluorescence quenching. *J. Am. Chem. Soc.* 126, 9152–9153.

Li, S., Lei, L., Somerville, C. R., and Gu, Y. (2012). Cellulose synthase interactive protein (CSI1) links microtubules and cellulose synthase complexes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 185–190.

Lima, D. U., Loh, W., and Buckeridge, M. S. (2004). Xyloglucan-cellulose interaction depends on the sidechains and molecular weight of xyloglucan. *Plant Physiol. Biochem.* 42, 389–394.

Link, A. J., Vink, M. K., Agard, N. J., Prescher, J. A., Bertozzi, C. R., and Tirrell, D. A. (2006). Discovery of aminoacyl-tRNA synthetase activity through cell-surface display of non-canonical amino acids. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10180–10185.

Lynch, M. A., and Staehelin, L. A. (1992). Domain-specific and cell-type specific localization of two types of cell wall matrix polysaccharides in the clover root tip. *J. Cell Biol.* 118, 467–479.

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C., Marcus, S. E., Somerville, S., Pauly, M.,

Knox, J. P., Sakuragi, Y., and Scheller, H. V. (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiol.* 155, 1068–1078.

Marcus, S. E., Blake, A. W., Benians, T. A., Lee, K. J., Poyser, C., Donaldson, L., Leroux, O., Rogowski, A., Petersen, H. L., Boraston, A., Gilbert, H. J., Willats, W. G., and Konx, J. P. (2010). Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *Plant J.* 64, 191–203.

Marcus, S. E., Verhertbruggen, Y., Herve, C., Ordaz-Ortiz, J. J., Farkas, V., Pederson, H. L., Willats, W. G., and Knox, J. P. (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biol.* 8, 60. doi:10.1186/1471-2229-8-60

Maris, A., Suslov, D., Fry, S. C., Verbelen, J. P., and Vissenberg, K. (2009). Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis* and their effect on root growth and cell wall extension. *J. Exp. Bot.* 60, 3959–3972.

Marks, M. D., Betancur, L., Gilding, E., Chen, F., Bauer, S., Wenger, J. P., Dixon, R. A., and Haigler, C. H. (2008). A new method for isolating large quantities of *Arabidopsis* trichomes for transcriptome, cell wall and other types of analysis. *Plant J.* 56, 483–492.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* 11, 266–277.

Moller, I., Marcus, S. E., Haeger, A., Verhertbruggen, Y., Verhoef, R., Schols, H., Ulvskov, P., Mikkelsen, J. D., Knox, J. P., and Willats, W. (2008). High-throughput screening of monoclonal antibodies against plant cell wall glycans by hierarchical clustering of their carbohydrate microarray profiles. *Glycoconj. J.* 25, 37–48.

Moore, P. J., Swords, K. M., Lynch, M. A., and Staehelin, L. A. (1991). Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J. Cell Biol.* 112, 589–602.

Nakamura, A., Furuta, H., Maeda, H., Nagamatsu, Y., and Yoshimoto, A. (2001). Analysis of structural components and molecular construction of soybean soluble polysaccharides by stepwise enzymatic degradation. *Biosci. Biotechnol. Biochem.* 65, 2249–2258.

Ngouemazong, D. E., Jolie, R. P., Cardinaels, R., Fraeye, I., Van Loey, A., Moldenaers, P., and Hendrickx, M. (2012). Stiffness of Ca^{2+}-pectin gels: combined effects of degree and pattern of methylesterification for various Ca^{2+} concentrations. *Carbohydr. Res.* 348, 69–76.

Nguyen, H. P., Chakravarthy, S., Velasquez, A. C., McLane, H. L., Zeng, L., Nakayashiki, H., Park, D. H., Collmer, A., and Martin, G. B. (2010). Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant Microbe Interact.* 23, 991–999.

Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H., and Hofte, H. (1998). A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J.* 17, 5563–5576.

Nishikawa, S., Zinkl, G. M., Swanson, R. J., Maruyama, D., and Preuss, D. (2005). Callose (beta-1,3 glucan) is essential for *Arabidopsis* pollen wall patterning but not tube growth. *BMC Plant Biol.* 5, 22. doi:10.1186/1471-2229-5-22

Paredez, A. R., Somerville, C. R., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495.

Park, Y. B., and Cosgrove, D. J. (2012a). Changes in cell wall biomechanical properties in the xyloglucan-deficient xxt1/xxt2 mutant of *Arabidopsis*. *Plant Physiol.* 158, 465–475.

Park, Y. B., and Cosgrove, D. J. (2012b). A revised architecture of primary cell walls based on biochemical changes induced by substrate-specific endoglucanases. *Plant Physiol.* 158, 1933–1943.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshnour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Pauly, M., Albersheim, P., Darvill, A., and York, W. S. (1999). Molecular domains of the cellulose/xyloglucan network in the cell walls of higher plants. *Plant J.* 20, 629–639.

Pauly, M., and Keegstra, K. (2008). Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant J.* 54, 559–568.

Peaucelle, A., Louvet, R., Johansen, J. N., Hofte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). *Arabidopsis* phyllotaxis is controlled by the methyl-esterification status of cell wall pectins. *Curr. Biol.* 18, 1943–1948.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C. R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15566–15571.

Prescher, J. A., Dube, D. H., and Bertozzi, C. R. (2004). Chemical remodeling of cell surfaces in living animals. *Nature* 430, 873–877.

Rabuka, D., Hubbard, S. C., Laughlin, S. T., Argade, S. P., and Bertozzi, C. R. (2006). A chemical reporter strategy to probe glycoprotein fucosylation. *J. Am. Chem. Soc.* 128, 12078–12079.

Refregier, G., Pelletier, S., Jaillard, D., and Hofte, H. (2004). Interaction between wall deposition and cell elongation in dark-grown hypocotyls cells in *Arabidopsis*. *Plant Physiol.* 135, 959–968.

Reiter, W. D., Chapple, C., and Somerville, C. R. (1997). Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J.* 12, 335–345.

Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002). A stepwise huisgen cycloaddition process: copper (I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed. Engl.* 41, 2596–2599.

Salic, A., and Mitchison, T. J. (2008). A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2415–2420.

Sawa, M., Hsu, T. L., Itoh, T., Sugiyama, M., Hanson, S. R., Vogt, P. K., and Wong, C. H. (2006). Glycoproteomic probes for fluorescent imaging of fucosylated glycans in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12371–12376.

Schmidt, M., Schwartzberg, A. M., Carroll, A., Chaibang, A., Adams, P. D., and Schuck, P. J. (2010). Raman imaging of cell wall polymers in *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* 395, 521–523.

Somerville, C. (2006). Cellulose synthesis in higher plants. *Annu. Rev. Cell Dev. Biol.* 22, 53–78.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., and Youngs, H. (2004). Towards a systems approach to understanding plant cell walls. *Science* 306, 2206–2211.

Sorensen, I., and Willats, W. G. (2011). Screening and characterization of plant cell walls using carbohydrate microarrays. *Methods Mol. Biol.* 715, 115–121.

Strasser, R., Altmann, F., Mach, L., Glossl, J., and Steinkellner, H. (2004). Generation of *Arabidopsis thaliana* plants with complex N-linked glycans lacking β-1,2-linked xylose and core α-1,3-linked fucose. *FEBS Lett.* 561, 132–136.

Sugimoto, K., Williamson, R. E., and Wasteneys, G. O. (2000). New techniques enable comparative analysis of microtubule orientation, wall texture, and growth rate in intact roots of *Arabidopsis*. *Plant Physiol.* 124, 1493–1506.

Taylor, N. G., Howells, R. M., Huttly, A. K., Vickers, K., and Turner, S. R. (2003). Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1450–1455.

Tornoe, C. W., Christensen, C., and Meldal, M. (2002). Peptidotriazoles on solid phase: [1,2,3]-triazoles by regioselective copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* 67, 3057–3064.

Vanzin, G. F., Madson, M., Carpita, N. C., Raikhel, N. V., Keegstra, K., and Reiter, W. D. (2002). The mur2 mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3340–3345.

Vincken, J. P., de Keizer, A., Beldman, G., and Voragen, A. G. (1995). Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiol.* 108, 1579–1585.

Vissenberg, K., Martinez-Vilchez, I. M., Verbelen, J. P., Miller, J. G., and Fry, S. C. (2000). In vivo colocalization of xyloglucan endotransglycosylase activity and its donor substrate in the elongation zone of *Arabidopsis* roots. *Plant Cell* 12, 1229–1237.

Western, T. L., Burn, J., Tan, W. L., Skinner, D. J., Martin-McCaffrey, L., Moffatt, B. A., and Haughn, G. W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiol.* 127, 998–1011.

Wu, Y., Williams, M., Bernard, S., Driouich, A., Showalter, A. M., and Faik,

A. (2010). Functional identification of two nonredundant *Arabidopsis* alpha (1,2) fucosyltransferases specific to arabinogalactan proteins. *J. Biol. Chem.* 285, 13638–13645.

Xie, B., Wang, X., Zhu, M., Zhang, Z., and Hong, Z. (2011). CalS7 encodes a callose synthase responsible for callose deposition in the phloem. *Plant J.* 65, 1–14.

Xu, W., Purugganan, M. M., Polisensky, D. H., Antosiewicz, D. M., Fry, S. C., and Braam, J. (1995). *Arabidopsis* TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *Plant Cell* 7, 1555–1567.

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Recent advances on the posttranslational modifications of EXTs and their roles in plant cell walls

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The genetic set up and the enzymes that define the O-glycosylation sites and transfer the activated sugars to cell wall glycoprotein Extensins (EXTs) have remained unknown for a long time. We are now beginning to see the emerging components of the molecular machinery that assembles these complex O-glycoproteins on the plant cell wall. Genes conferring the posttranslational modifications, i.e., proline hydroxylation and subsequent O-glycosylation, of the EXTs have been recently identified. In this review we summarize the enzymes that define the O-glycosylation sites on the O-glycoproteins, i.e., the prolyl 4-hydroxylases (P4Hs), the glycosyltransferases that transfer arabinose units (named arabinosyltransferases, AraTs), and the one responsible for transferring a single galactose (galactosyltransferase, GalT) on the protein EXT backbones. We discuss the effects of posttranslational modifications on the structure and function of extensins in plant cell walls.

Keywords: plant cell wall, O-glycosylation, proline hydroxylation, O-glycoproteins, extensins, cell expansion

INTRODUCTION

The major components of primary plant cell walls include a complex composite of networks of cellulose microfibrils, branched xyloglucans, and a diverse pectin matrix (Somerville et al., 2004; Cosgrove, 2005). In addition, a structural role has been clearly assigned to the hydroxyproline-rich O-glycoprotein extensins (EXTs) in building and maintaining the growing cell wall (Lamport and Northcote, 1960; Hall and Cannon, 2002; Held et al., 2004; Cannon et al., 2008; Lamport et al., 2011; Velasquez et al., 2011a,b). EXTs are plant cell wall O-glycoproteins that belong to the hydroxyproline-rich glycoproteins (HRGPs) superfamily with a modular highly repetitive sequence that includes the O-glycosylated Ser-$(Hyp)_{3–4}$ repeats and some of the crosslinking motifs Val-Tyr-Lys, Val-Tyr-Lys-Tyr-Lys, Tyr-Tyr-Tyr-Lys, or Tyr-X-Tyr, all of a hydrophobic nature (**Figure 1A**; Fry, 1982; Brady et al., 1998; Held et al., 2004; Cannon et al., 2008). EXTs can also occur in the context of natural, chimeric glycosylated polypeptides, e.g., EXT-arabinogalactan proteins (Lind et al., 1994), leucine-rich repeat (LRR) EXT proteins (Rubinstein et al., 1995), or lectin-EXTs (Kieliszewski and Lamport, 1994). Here in this review, we will highlight the major breakthroughs of the posttranslational modifications of EXTs and how these modifications affect the function of EXTs within the primary plant cell wall.

Although the protein sequences that define the O-glycosylation site on HRGPs are partially known (Kieliszewski and Lamport, 1994; Shpak et al., 1999, 2001), the glycosyltransferases that transfer the activated sugars to the protein backbone (arabinose and galactose) have remained a mystery for decades. Now we are beginning to perceive the emerging picture of the molecular machinery that assembles sugars into these complex O-glycoproteins which comprises an important part of the cell wall. Most of the genes involved in the posttranslational modification of EXTs that includes proline hydroxylation and subsequent O-glycosylation are now being identified (Egelund et al., 2007; Cannon et al., 2008; Gille et al., 2009; Velasquez et al., 2011a), increasing our understanding of plant cell wall EXTs synthesis and their roles in cell expansion during growth.

While several aspects of EXTs have been reviewed recently (Kieliszewski et al., 2010; Showalter et al., 2010; Lamport et al., 2011), the present review focuses specifically on the enzymes that define the O-glycosylation sites, namely the prolyl 4-hydroxylases (P4Hs) and the glycosyltransferases that transfer arabinosyl units (named arabinosyltransferases, AraTs) or a single galactosyl residue (galactosyltransferase, GalT) to the protein EXT backbones. Finally, the structure and functions of the O-glycoprotein EXTs are discussed in the context of cell wall expansion during growth.

STEPS TO BUILD MATURE, FUNCTIONAL EXTs

Structural O-glycoproteins EXTs that are secreted into plant cell walls are first shaped by several posttranslational modifications that include signal peptide processing in the ER, hydroxylation of peptidyl-proline residues into hydroxyproline (Hyp) in the ER-Golgi apparatus (Lamport and Northcote, 1960; Lamport, 1967; Yuasa et al., 2005; Velasquez et al., 2011a), O-glycosylation on Hyp and Ser by the action of several glycosyltranferases, mostly in the Golgi apparatus, although early steps could be taking place in the ER (**Figure 1B**; Shimma et al., 2011; Velasquez et al., 2011a), and finally, covalent intra- and inter-molecular crosslinking at the plant cell wall level (Fry, 1982; Held et al., 2004). All these

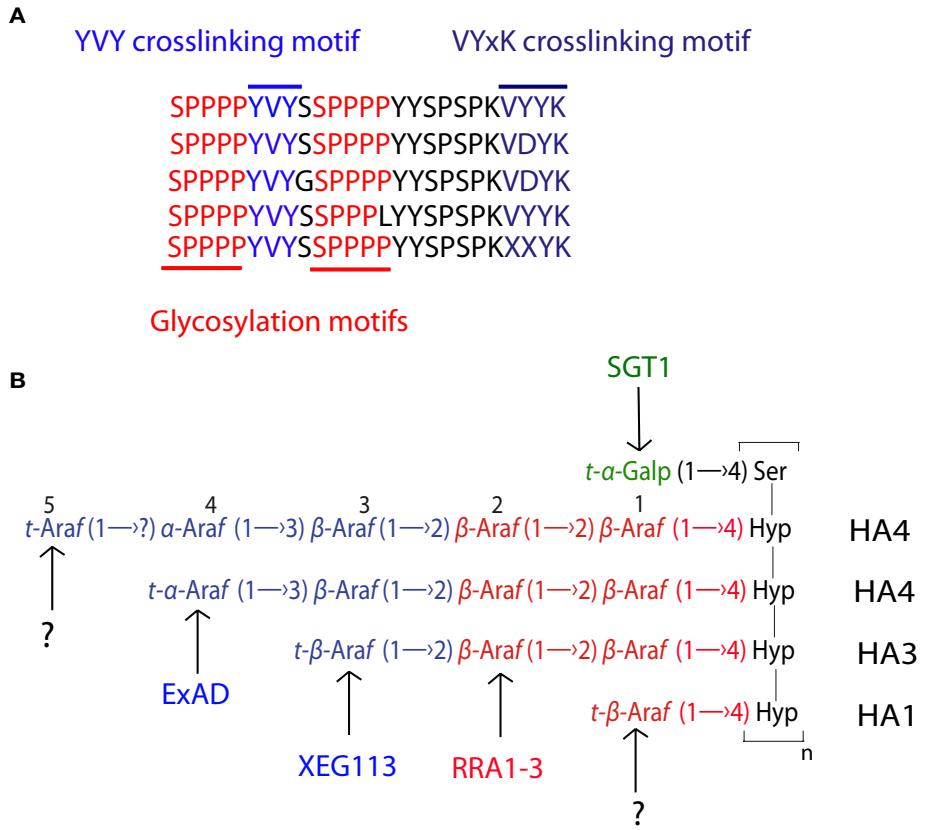


FIGURE 1 | (A) Predominant repetitive modular sequences in certain most common EXTs with isodityrosine (YVY) and other crosslinking sites (VYxK) as well as the Ser-$(Hyp)_4$, which is O-glycosylated. **(B)** O-glycans present in the Ser-$(Hyp)_4$ motif. In addition, the glycosyltransferases that transfer specific sugars on the EXT backbone are indicated in the figure.

posttranslational modifications of EXTs are crucial for the function of EXTs in the plant cell wall during cell development and growth (Hall and Cannon, 2002; Cannon et al., 2008; Velasquez et al., 2011a,b).

Two P4H families, the collagen P4Hs (C-P4Hs) and the hypoxia-inducible transcription factor (HIF)-associated P4Hs, are responsible for the hydroxylation of collagens and HIF, respectively (Myllyharju, 2003, 2008). The vertebrate C-P4Hs are $\alpha_2\beta_2$ tetramers, in which the α-subunits possess the enzymatic activity and the β-subunits are identical to the enzyme disulfide isomerase. The tetrameric assembly is required for stability and full activity. In contrast, plant P4Hs and also HIF-P4Hs are monomers (Hieta and Myllyharju, 2002; Myllyharju, 2003; Koski et al., 2007, 2009). Plant P4Hs are membrane-bound enzymes that catalyze the hydroxylation of peptidyl-proline to yield hydroxyproline by introducing an oxygen atom between the hydrogen and carbon atoms of the peptidyl-proline adding merely 16 atomic mass units (Lamport, 1963). From four possible isomers of hydroxyproline, the most common isomer in nature is (2*S*,4*R*)-4-hydroxyproline, herein after referred to as Hyp (*trans*-4-hydroxyproline) in reference to the *trans*-relationship between the –COOH and –OH substituents on the pyrrolidine ring (Hieta and Myllyharju, 2002; Myllyharju, 2003). In addition, the *cis*-hydroxyproline (2*S*,4*S*)-4-hydroxyproline is reported to be present in the toxic cyclic peptides from *Amanita* mushrooms (e.g., alpha-amanitin and phalloidin; Alexopoulos et al., 1996). The P4Hs belong to a family of 2-oxoglutarate-dependent dioxygenases that require 2-oxoglutarate and O_2 as co-substrates, Fe^{2+} as a cofactor and ascorbate to prevent the rapid inactivation by self-oxidation (Hieta and Myllyharju, 2002; Koski et al., 2007, 2009). From a mechanistic point of view, α-ketoglutarate is oxidatively decarboxylated to produce succinate and CO_2. The conversion of Pro to Hyp in the secretory pathway provides reactive hydroxyl groups for further modification such as O-glycosylation. In addition, the product of this reaction, poly-(4-hydroxyproline), has an even greater tendency than polyproline to adopt a helical polyproline II-type conformation (Lamport, 1977, 1980; Van Holst and Varner, 1984; Valentin et al., 2010).

In vitro and *in vivo* characterization of plant P4Hs has been carried out in *Arabidopsis thaliana* (Hieta and Myllyharju, 2002; Tiainen et al., 2005; Vlad et al., 2007; Asif et al., 2009; Velasquez et al., 2011a), *Nicotiana tabacum* (Yuasa et al., 2005), *Dianthus caryophyllus* (Vlad et al., 2010), and the green alga *Chlamydomonas reinhardtii* (Keskiaho et al., 2007). Only the activity of AtP4H1 and AtP4H2, out of the 13 P4Hs encoded in the *Arabidopsis* genome, have been fully characterized *in vitro*, and only CrP4H1 has been crystallized, without and with the $(Ser\text{-}Pro)_5$ peptide as a substrate (Koski et al., 2007, 2009). Both AtP4Hs hydroxylate proline-rich peptides of both extreme types: arabinogalactan proteins (AGPs)

and EXTs. However, AtP4H5 seems to be more specific as yeast two-hybrid assays and molecular modeling strongly suggest its preference for polyproline II peptide instead of the AGP-type substrate (Velasquez et al., 2011a). Moreover, three AtP4H T-DNA insertional mutants (*Atp4h2,5,13*) exhibited a clear short root hair phenotype and reduced root Hyp levels as well. Remarkably, overexpression of these P4Hs displayed extra long root hairs (Velasquez et al., 2011a,b) and much higher Hyp contents in roots when compared to WT Col-0 (Estevez et al., unpublished results). Interestingly, heterologous peptides that mimic collagen $(X–Pro–Gly)_n$ are also hydroxylated by some plant P4Hs, though inefficiently (Tanaka et al., 1981; Kaska et al., 1988).

Based on multiple pieces of evidence, it became clear that proline hydroxylation of HRGPs, and specifically EXTs, are essential for polarized cell expansion in root hairs. Therefore, it is tempting to imagine that homologous P4Hs and HRGPs will also be relevant in cell expansion in other rapidly expanding cell types such as pollen tubes or dark-grown hypocotyls. On the other hand, suppression of the gene encoding 1 out of 10 *Chlamydomonas reinhardtii* prolyl 4-hydroxylases (CrP4H1), which hydroxylates algal HRGP sequences, led to a defective cell wall (Keskiaho et al., 2007), suggesting a major unique structural role of certain HRGPs as targets of CrP4H1 in this unicellular green algae. In two other studies, a direct role of P4Hs in hypoxia stress and during different stages of plant growth and development is speculated, suggesting that P4Hs could be acting as putative oxygen sensors (Vlad et al., 2007; Asif et al., 2009). Nevertheless, further studies are needed to confirm this hypothesis.

The extent and type of O-glycosylation in HRGPs, and specifically in EXTs, can be predicted by the "Hyp contiguity" hypothesis, according to which O-glycosylation correlates with the primary sequence of the protein where location and context of Hyp residues are crucial (Kieliszewski and Lamport, 1994; Kieliszewski, 2001; Shpak et al., 1999, 2001). Generally, wherever Hyp residues are contiguous in the amino acid sequence, arabinosylation is predominant in EXTs, whereas arabinogalactans are rather added to clustered, non-contiguous Hyp residues in AGPs. However, it is worth mentioning that isolated Hyp units were also arabinogalactosylated in some cases (Zhao et al., 2002). Another clear exception to this hypothesis is exemplified by the small CLE-like peptides (e.g., Tob/Tom-HypSys, PSY1, CLV3, and CLE2) where single proline units are modified with β-linked-L-arabinofuranosides with identical linkages/stereochemistry of the innermost three arabinose units found in the EXTs (Ryan and Pearce, 2003; Ito et al., 2006; Kondo et al., 2006; Ohyama et al., 2009; Matsuzaki et al., 2010), suggesting that similar P4Hs and glycosyltransferases participate in their posttranslational modifications. It is important to note that Hyp O-glycosylation has not been found in animal cells, which makes HRGPs unique in their structure and functions for plant cells.

EXT O-glycosylation in the Ser-$(Hyp)_{3–4}$ repeats involves a series a glycosyltransferases that add a single galactose unit to serine and 4–5 arabinose units, in a linear fashion, to Hyp. The minimal set of activities necessary for synthesis of the entire glycomodule in EXTs (**Figure 1B**) may comprise: one Ser-α-D-galactosyltransferase acting exclusively on serine, one Hyp-β-L-arabinofuranosyltransferase, two β-(1 → 2)-L-arabinofuranosyl transferases, one α-(1 → 3)-L-arabinofuranosyltransferase and a last arabinosyltransferase adding the fifth arabinofuranose with a yet unknown regio-chemistry (Shimma et al., 2011; Velasquez et al., 2011a). Three glycosyltransferases involved in extensins maturation have been recently reported after characterizing EXT O-glycosylation in T-DNA mutant lines of the corresponding genes in *Arabidopsis* (Egelund et al., 2007; Gille et al., 2009; Shimma et al., 2011; Velasquez et al., 2011a). The arabinosyltransferase that would add the second β-(1 → 2)-arabinosyl unit in the *Hyp*-*O*-arabinosides is the proposed *R*educed *R*esidual *A*rabinose 3 (RRA3). The one likely to transfer the third β-(1 → 2)-arabinose was named as *X*ylo*E*ndo*G*lucanase (XEG113), both belonging to the GT77 Carbohydrate Active EnZymes (CAZY) family (Egelund et al., 2007; Gille et al., 2009; Velasquez et al., 2011a). Finally, the AraT that would transfer the fourth α-(1 → 3)-L-arabinose moiety was identified very recently as *E*xtensin *A*rabinose *D*eficient transferase (ExAD) within the GT47 family (Petersen et al. unpublished results). In addition, the galactosyltransferase that adds galactose on serine in the *Ser*-$(Hyp)_{3–4}$ repeats was recently reported as SGT1 (Shimma et al., 2011). To the present day, the arabinosyltransferase that adds the first and the last (fifth) arabinose remains unknown (**Figure 1B**). Remarkably, all the AraTs T-DNA mutants identified so far with deficient O-arabinosylation in EXT backbones displayed a clear root hair phenotype, suggesting that O-glycosylation on EXT is crucial on EXT function during cell expansion at least in this cell type and also in dark-grown hypocotyls for the mutant *xeg113-2* (Gille et al., 2009; Velasquez et al., 2011a,b). The evolutionary conservation from unicellular green algae to vascular plants of the Ser-$(Hyp)_{3–4}$ glycomodule implies a essential function of O-glycosylation in EXT shape and biological role.

IMPACT OF HYDROXYLATION AND O-GLYCOSYLATION ON EXT FUNCTION

It has been suggested that the O-glycans increase HRGP solubility, resistance to proteolytic degradation and thermal stability (Kieliszewski et al., 1989, 2010; Ferris et al., 2001; Shpak et al., 2001; Lamport et al., 2011). The structural and functional consequences of O-glycosylation in polyproline EXTs remained unclear although some evidence suggested that this posttranslational modification could provide conformational stability to these macromolecules (Lamport, 1980; Van Holst and Varner, 1984; Stafstrom and Staehelin, 1986). To understand the biological meaning of the lack of hydroxylation/O-glycosylation on EXTs, possible conformational changes induced by proline hydroxylation and subsequent O-arabinosylation on the minimal EXT repeat $Ser(Pro)_4$ and $Ser(Hyp)_4$ were tested by molecular dynamics modeling. In fact, O-glycosylation clearly appears to stabilize the helical conformation of the EXT minimal peptide whereas incomplete hydroxylation and O-glycosylation favors its flexible and disorganized conformation (Velasquez et al., 2011a) as it was previously suggested by circular dichroism on both short and repetitive EXT synthetic peptides and native EXTs (Van Holst and Varner, 1984; Shpak et al., 2001). In another recent study, it was found that O-glycosylation of Hyp residues causes a dramatic increase in the thermal stability of the polyproline type-II (PP-II) helix, which may be explained by a network of inter-glycan and glycan-peptide hydrogen bonds

(Owens et al., 2010). Analysis of the β-O-glycosylated polyproline model peptides indicates that Hyp O-glycosylation leads to a significant increase in conformational stability (Owens et al., 2010; Velasquez et al., 2011a). Since contiguous O-glycosylation seems to bring about a rise in conformational stability, similar increases in the conformational stability of EXTs are likely to occur. Based on this, we theorize that EXTs with incomplete hydroxylation/O-glycosylation have an enormous negative impact on EXTs overall shape, affecting EXTs interactions with the surrounding environment, including those with other EXTs in the network or with other modifying enzymes such as EXT-specific peroxidases (PER_{ext}; Velasquez et al., 2011a). The latter belong to the type-III apoplastic peroxidases that form a large multigene family present in all land plants (Passardi et al., 2004).

Based on the *in vitro* activity of several peroxidases (Everdeen et al., 1988; Brownleader et al., 1995; Schnabelrauch et al., 1996; Magliano and Casal, 1998; Jackson et al., 2001; Price et al., 2003), it is proposed that the *in vivo* crosslinking of EXTs might involve multiple PER_{ext}, although their molecular roles and specific targets remain unknown. Some of these enzymes could catalyze the polymerization of EXT monomers via di-isodityrosine and pulcherosine, a tetramer and trimer of tyrosine, respectively (Fry, 1982; Brady et al., 1996, 1998) to form a covalent cross-linked network at the plant cell wall level (Cannon et al., 2008). For example, tomato PER_{ext} exhibited a strong activity onto diverse types of extensins (Schnabelrauch et al., 1996). Moreover, PER_{ext} activity might be modulated *in vivo*, as shown by a study that used a natural inhibitor of extensin crosslinking isolated from a cell wall compartment containing extensins and peroxidases (Brownleader and Dey, 1993; Brownleader et al., 2000). The high number of genes encoding type-III- peroxidases, in addition to the biochemical variety of their substrates, is still one of the main obstacles for the assigning of their specific functions *in vivo*. However, in *Arabidopsis*, by means of co-expression analysis of well known root hair cell wall-associated genes, a few putative EXT peroxidases such as PER13 and PER73 were identified, suggesting that at least some of them are responsible for the crosslinking of EXTs specifically in the root hair cell walls (Velasquez et al., 2011a).

EXTENSIN FUNCTION

The known roles of extensins in cell wall assembly, cell shape and growth raise the question about the function for each individual extensin molecule (Hall and Cannon, 2002; Cannon et al., 2008; Velasquez et al., 2011a). Recently, it was reported that EXTs can form, at least *in vitro*, a tridimensional covalent network through Tyr linkages mediated by EXT peroxidases between individual EXT molecules and also via self-recognition and alignment of hydrophilic O-glycosylated Ser-$(Hyp)_{3–4}$ repeats and hydrophobic peptide-crosslinking modules (Cannon et al., 2008). Thus, the ordered EXT monomers assembly in the plant cell wall would involve a zipper-like endwise association via crosslinking at the ends of the molecules (Kieliszewski et al., 2010; Lamport et al., 2011). It is also proposed that the oligomeric extensin interacts with pectins by a simple acid-base reaction forming a supramolecular ionic structure in the nascent cell wall, which would serve as a template for further cell wall deposition (Cannon et al., 2008; Kieliszewski et al., 2010). This hypothesis was recently confirmed *in vitro* by using a layer-by-layer method to construct a multilayer thin film used to investigate the interactions between extensin and pectin. Multilayer formation was successfully produced, thereby evidencing the occurrence of interactions between EXTs and pectin (Valentin et al., 2010). In addition, covalent EXT-pectin crosslinks were also suggested (Qi et al., 1995; Nuñez et al., 2009).

Although the *Arabidopsis* genome encodes up to 63 putative extensins of which 20 would be defined as classical extensins, where most of them share both motifs for O-glycosylation and Try-mediated crosslinks (**Figure 1A**), 12 are shorter extensins and 31 are extensin-like chimeras and hybrid-extensins that could contain other protein domains (Cannon et al., 2008; Showalter et al., 2010; Lamport et al., 2011), only one extensin mutant, *root-*, *shoot-*, *hypocotyl-defective* (*rsh*) *ext3*, has been shown to have a nearly lethal phenotype (Hall and Cannon, 2002; Cannon et al., 2008). However, only two other single *ext* mutants, such as the *leucine-rich repeat extensins* 1 and 2 (*lrx1* and *lrx2*) with aberrant root hairs (Baumberger et al., 2001, 2003a,b; Ringli, 2010) and the *proline-rich extensin-like receptor kinase perk13* displaying longer root hairs (Humphrey et al., 2007), have been reported, thus suggesting a low degree of EXT genetic redundancy. In support of this notion, a number of root hair-relevant extensins have been found taking advantage of network analysis of co-expression with other cell wall genes known to be important for root hair growth, like expansins EXP7 and EXP18 (Won et al., 2009), proline-rich proteins PRP1 and PRP3 (Fowler et al., 1999; Bernhardt and Tierney, 2000) and leucine-rich extensin protein LRX1 (Baumberger et al., 2003a,b). Consistently, the same EXTs were identified by functional genomics on root hair mutants (Baumberger et al., 2003a,b; Jones et al., 2006; Deal and Henikoff, 2010). Through these two approaches, it was possible to identify several EXTs that seem to be important for root hair growth. This was later confirmed when T-DNA homozygous mutant lines (*ext6*, *ext7*, *ext10*, etc.) of these EXTs turned out to exhibit drastically shorter root hairs (Velasquez et al., 2011a,b). All these EXT-related mutants highlight an essential role of each individual EXT type on cell expansion and growth, specifically in root hairs. This suggests that location and function of each individual EXT type within the network at the cell wall are relevant to the whole EXT network assembly.

Finally, from an evolutionary perspective, highly organized but non-covalent EXT-like cell wall networks are ancient, as they occur in several unicellular and multicellular green algae (Sumper and Hallmann, 1998; Ferris et al., 2001; Bollig et al., 2007; Lee et al., 2007; Sørensen et al., 2011). In agreement, type-III peroxidase genes seem to be absent in green algae genomes (Passardi et al., 2004). Hence, a covalently cross-linked EXT network seems to have been a secondary development in evolution that led to strong plant cell walls, enabling terrestrial colonization, and turgor-driven growth in land plants.

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REFERENCES

Alexopoulos, C. J., Mims, C. W., and Blackwell, M. (1996). *Introductory Mycology*, 4th Edn. New York: John Wiley & Sons, 687–688.

Asif, M. H., Trivedi, P. K., Misra, P., and Nath, P. (2009). Prolyl-4-hydroxylase (AtP4H1) mediates and mimics low oxygen response in *Arabidopsis thaliana*. *Funct. Integr. Genomics* 9, 525–535.

Baumberger, N., Doesseger, B., Guyot, R., Diet, A., Parsons, R. L., Clark, M. A., Simmons, M. P., Bedinger, P., Goff, S. A., Ringli, C., and Keller, B. (2003a). Whole-genome comparison of leucine-rich repeat extensins in *Arabidopsis* and rice. A conserved family of cell wall proteins form a vegetative and a reproductive clade. *Plant Physiol.* 131, 1313–1326.

Baumberger, N., Steiner, M., Ryser, U., Keller, B., and Ringli, C. (2003b). Synergistic interaction of the two paralogous *Arabidopsis* genes LRX1 and LRX2 in cell wall formation during root hair development. *Plant J.* *35*, 71–81.

Baumberger, N., Ringli, C., and Keller, B. (2001). The chimeric leucine-rich repeat/extensin cell wall protein LRX1 is required for root hair morphogenesis in *Arabidopsis thaliana*. *Genes Dev.* 15, 1128–1139.

Bernhardt, C., and Tierney, M. L. (2000). Expression of AtPRP3, a proline-rich structural cell wall protein from *Arabidopsis* is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiol.* 122, 705–714.

Bollig, K., Lamshoft, M., Schweimer, K., Marner, F. J., Budzikiewicz, H., and Waffenschmidt, S. (2007). Structural analysis of linear hydroxyproline-bound O-glycans of *Chlamydomonas reinhardtii* – conservation of the inner core in *Chlamydomonas* and land plants. *Carbohydr. Res.* 342, 2557–2566.

Brady, J. D., Sadler, I. H., and Fry, S. C. (1996). Di-isodityrosine, a novel tetrametric derivative of tyrosine in plant cell wall proteins: a new potential cross-link. *Biochem. J.* 315(Pt 1), 323–327.

Brady, J. D., Sadler, I. H., and Fry, S. C. (1998). Pulcherosine, an oxidatively coupled trimer of tyrosine in plant cell walls: its role in cross-link formation. *Phytochemistry* 47, 349–353.

Brownleader, M. D., Ahmed, N., Trevan, M., Chaplin, M. F., and Dey, P. M. (1995). Purification and partial characterization of tomato extensin peroxidase. *Plant Physiol.* 109, 1115–1123.

Brownleader, M. D., and Dey, P. M. (1993). Purification of extension from cell walls of tomato (hybrid of *Lycopersicon esculentum* and *L. peruvianum*) cells in suspension culture. *Planta* 191, 457–469.

Brownleader, M. D., Hopkins, J., Mobasheri, A., Dey, P. M., Jackson, P., and Trevan, M. (2000). Role of extensin peroxidase in tomato (*Lycopersicon esculentum* Mill.) seedling growth. *Planta* 210, 668–676.

Cannon, M. C., Terneus, K., Hall, Q., Tan, L., Wang, Y., Wegenhart, B. L., Chen, L., Lamport, D. T. A., Chen, Y., and Kieliszewski, M. J. (2008). Self-assembly of the plant cell wall requires an extensin scaffold. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2226–2231.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 11, 850–861.

Deal, R. B., and Henikoff, S. (2010). A simple method for gene expression and chromatin profiling of individual cell types within a tissue. *Dev. Cell* 18, 1030–1040.

Egelund, J., Obel, N., Ulvskov, P., Geshi, N., Pauly, M., Bacic, A., and Larsen Petersen, B. (2007). Molecular characterization of two *Arabidopsis thaliana* glycosyltransferase mutants, rra1 and rra2, which have a reduced residual arabinose content in a polymer tightly associated with the cellulosic wall residue. *Plant Mol. Biol.* 64, 439–449.

Everdeen, D. S., Kiefer, S., Willard, J. J., Muldoon, E. P., Dey, P. M., Li, X.-B, and Lamport, D. T. A. (1988). Enzymic cross-linkage of monomeric extensin precursors in vitro. *Plant Physiol.* 87, 616–621.

Ferris, P. J., Woessner, J. P., Waffenschmidt, S., Kilz, S., Drees, J., and Goodenough, U. W. (2001). Glycosylated polyproline II rods with kinks as a structural motif in plant hydroxyproline-rich glycoproteins. *Biochemistry* 40, 2978–2987.

Fowler, T. J., Bernhardt, C., and Tierney, M. L. (1999). Characterization and expression of four proline-rich cell wall protein genes in *Arabidopsis* encoding two distinct subsets of multiple domain proteins. *Plant Physiol.* 121, 1081–1092.

Fry, S. C. (1982). Isodityrosine, a new cross-linking amino acid from plant cell wall glycoprotein. *Biochem. J.* 204, 449–455.

Gille, S., Hänsel, U., Ziemann, M., and Pauly, M. (2009). Identification of plant cell wall mutants by means of a forward chemical genetic approach using hydrolases. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14699–704.

Hall, Q., and Cannon, M. C. (2002). The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in *Arabidopsis*. *Plant Cell* 14, 1161–1172.

Held, M. A., Tan, L., Kamyab, A., Hare, M., Shpak, E., and Kieliszewski, M. J. (2004). Di-isodityrosine is the intermolecular cross-link of isodityrosine-rich extensin analogs cross-linked in vitro. *J. Biol. Chem.* *279*, 55474–55482.

Hieta, R., and Myllyharju, J. (2002). Cloning and characterization of a low molecular weight prolyl 4-hydroxylase from *Arabidopsis thaliana*. Effective hydroxylation of proline-rich, collagen-like, and hypoxia-inducible transcription factor alpha-like peptides. *J. Biol. Chem.* 277, 23965–23971.

Humphrey, T. V., Bonetta, D. T., and Goring, D. R. (2007). Sentinels at the wall: cell wall receptors and sensors. *New Phytol.* 176, 7–21.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., and Fukuda, H. (2006). Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 313, 842–845.

Jackson, P. A., Galinha, C. I., Pereira, C. S., Fortunato, A., Soares, N. C., Amâncio, S. B., and Pinto Ricardo, C. P. (2001). Rapid deposition of extensin during the elicitation of grapevine callus cultures is specifically catalyzed by a 40-kiloDalton peroxidase. *Plant Physiol.* 127, 1065–1076.

Jones, M. A., Raymond, M. J., and Smirnoff, N. (2006). Analysis of the root-hair morphogenesis transcriptome reveals the molecular identity of six genes with roles in root-hair development in *Arabidopsis*. *Plant J.* *45*, 83–100.

Kaska, D. D., Myllyla¨, R., Gu¨nzler, V., Gibor, A., and Kivirikko, K. I. (1988). Prolyl 4-hydroxylase from *Volvox carteri*. A low-*Mr* enzyme antigenically related to the a subunit of the vertebrate enzyme. *Biochem. J.* 256, 257–263.

Keskiaho, K., Hieta, R., Sormunen, R., and Myllyharju, J. (2007). *Chlamydomonas reinhardtii* has multiple prolyl 4-hydroxylases, one of which is essential for proper cell wall assembly. *Plant Cell* 19, 256–269.

Kieliszewski, M. J. (2001). The latest hype on Hyp-O-glycosylation codes. *Phytochemistry 57*, 319–323.

Kieliszewski, M. J., and Lamport, D. T. A. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant J. 5*, 157–172.

Kieliszewski, M. J., Lamport, D. T. A., Tan, L., and Cannon, M. C. (2010). "Hydroxyproline-rich glycoproteins: form and function," in *Annual Plant Reviews: Plant Polysaccharides, Biosynthesis and Bioengineering*, Vol. 41, ed. P. Ulvskov (Oxford: Wiley-Blackwell). doi: 10.1002/9781444391015.ch13

Kieliszewski, M. J., Leykam, J. F., and Lamport, D. T. A. (1989). Trypsin cleaves lysylproline in a hydroxyproline-rich glycoprotein from *Zea mays*. *Pept. Res.* 2, 246–248.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H., and Sakagam, Y. (2006). A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* 313, 845–848.

Koski, M. K., Hieta, R., Böllner, C., Kivirikko, K. I., Myllyharju, J., and Wierenga, R. K. (2007). The active site of an algal prolyl 4-hydroxylase has a large structural plasticity. The active site of an algal prolyl 4-hydroxylase has a large structural plasticity. *J. Biol. Chem.* 282, 37112–37123.

Koski, M. K., Hieta, R., Hirsilä, M., Rönkä, A., Myllyharju, J., and Wierenga, R. K. (2009). The crystal structure of an algal prolyl 4-hydroxylase complexed with a proline-rich peptide reveals a novel buried tripeptide binding motif. *J. Biol. Chem.* 284, 25290–25301.

Lamport, D. T., Kieliszewski, M. J., Chen, Y., and Cannon, M. C. (2011). Role of the extensin superfamily in primary cell wall architecture. *Plant Physiol. 156*, 11–19.

Lamport, D. T. A. (1963). Oxygen fixation into hydroxyproline of plant cell wall protein. *J. Biol. Chem.* 238, 1438–1440.

Lamport, D. T. A. (1967). Hydroxyproline-O-glycosidic linkage of the plant cell wall glycoprotein extensin. *Nature* 216, 1322–1324.

Lamport, D. T. A. (1977). "Structure, biosynthesis and significance of cell wall glycoproteins," in *Recent Advances in Phytochemistry*, eds F. A. Loewus and V. C. Runeckles (Plenum Publishing, New York), 79–115.

Lamport, D. T. A. (1980). *Structure, and Function of Plant Glycoproteins in the Biochemistry of Plants*, Vol. 3. New York: Academic Press, 501–540.

Lamport, D. T. A., and Northcote, D. H. (1960). Hydroxyproline in primary cell walls of higher plants. *Nature* 188, 665–666.

Lee, J. H., Waffenschmidt, S., Small, L., and Goodenough, U. (2007). Between-species analysis of short-repeat modules in cell wall and sex-related hydroxyproline-rich glycoproteins of *Chlamydomonas*. *Plant Physiol.* 144, 1813–1826.

Lind, J. L., Bacic, A., Clarke, A. E., and Anderson, M. A. (1994). A style-specific hydroxyproline-rich glycoprotein with properties of both extensins and arabinogalactan proteins. *Plant J.* 6, 491–502.

Magliano, T. M. A., and Casal, J. J. (1998). In vitro cross-linking of extensin precursors by mustard extracellular isoforms of peroxidase that respond either to phytochrome or to wounding. *J. Exp. Bot.* 49, 1491–1499.

Matsuzaki, Y., Ogawa-Ohnishi, M., Mori, A., and Matsubayashi, Y. (2010). Secreted peptide signals required for maintenance of root stem cell niche in *Arabidopsis*. *Science 329*, 1065–1067.

Myllyharju, J. (2003). Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* 22, 15–24.

Myllyharju, J. (2008). Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. *Ann. Med.* 40, 402–417.

Nuñez, A., Fishman, M. L., Fortis, L. L., Cooke, P. H., and Hotchkiss, A. T. Jr. (2009). Identification of extensin protein associated with sugar beet pectin. *J. Agric. Food Chem.* 57, 10951–10958.

Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M., and Matsubayashi, Y. (2009). A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. *Nat. Chem. Biol. 5*, 578–580.

Owens, N. W., Stetefeld, J., Lattová, E., and Schweizer, F. (2010). Contiguous *O*-galactosylation of 4(R)-hydroxy-l-proline residues forms very stable polyproline II helices. *J. Am. Chem. Soc. 132*, 5036–5042.

Passardi, F., Longet, D., Penel, C., and Dunand, C. (2004). The plant peroxidase multigenic family in rice and its evolution in green plants. *Phytochemistry* 65, 1879–1893.

Price, N. J., Pinheiro, C., Soares, C. M., Ashford, D. A., Ricardo, C. P., and Jackson, P. A. (2003). A biochemical and molecular characterization of LEP1, an extensin peroxidase from lupin. *J. Biol. Chem.* 278, 41389–41399.

Qi, X., Behrens, B. X., West, P. R., and Mort, A. J. (1995). Solubilization and partial characterization of extensin fragments from cell walls of cotton suspension cultures: evidence for a covalent cross-link between extensin and pectin. *Plant Physiol.* 108, 1691–1701.

Ringli, C. (2010). The hydroxyproline-rich glycoprotein domain of the *Arabidopsis* LRX1 requires Tyr for function but not for insolubilization in the cell wall. *Plant J. 63*, 662–669.

Rubinstein, A. L., Broadwater, A. H., Lowrey, K. B., and Bedinger, P. A. (1995). PEX1, a pollen-specific gene with an extensin-like domain. *Proc. Natl. Acad. Sci. U.S.A.* 92, 3086–3090.

Ryan, C. A., and Pearce, G. (2003). Systemic signaling in tomato plants for defense against herbivores: isolation and characterization of three novel defense-signaling glycopeptides hormones coded in a single precursor gene. *J. Biol. Chem.* 278, 30044–30050.

Schnabelrauch, L. S., Kieliszewski, M., Upham, B. L., Alizedeh, H., and Lamport, D. T. (1996). Isolation of pl 4.6 extensin peroxidase from tomato cell suspension cultures and identification of Val-Tyr-Lys as putative intermolecular cross-link site. *Plant J. 4*, 477–489.

Shimma, Y., Saito, F., Suyama, A., Oka, T., Yoko-o, T., Matsuoka, K., and Jigami, Y. (2011). "Identification of novel peptidyl serine O-galactosyltransferase gene family in plants," in *XVIII International Botanical Congress*, Melbourne, July 23–30, IBC2011 Abstract 116.

Showalter, A. M., Keppler, B., Lichtenberg, J., Gu, D., and Welch, L. R. (2010). A bioinformatics approach to the identification, classification, and analysis of hydroxyproline-rich glycoproteins. *Plant Physiol. 153*, 485–513.

Shpak, E., Barbar, E., Leykam, J. F., and Kieliszewski, M. J. (2001). Contiguous hydroxyproline residues direct hydroxyproline arabinosylation in *Nicotiana tabacum*. *J. Biol. Chem.* 276, 11272–11278.

Shpak, E., Leykam, J. F., and Kieliszewski, M. J. (1999). Synthetic genes for glycoprotein design and the elucidation of hydroxyproline-O-glycosylation codes. *Proc. Natl. Acad. Sci. U.S.A. 96*, 14736–14741.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., and Youngs, H. (2004). Toward a systems approach to understanding plant cell walls. *Science 306*, 2206–2211.

Sørensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F. C., O'Neill, M. A., Fei, Z. Z., Rose, J. K. C., Domozych, D. S., and Willats, W. G. T. (2011). The charophycean green algae provide insights into the early origins of plant cell walls. *Plant J. 68*, 201–211.

Stafstrom, J. P., and Staehelin, L. A. (1986). The role of carbohydrate in maintaining extensin in an extended conformation. *Plant Physiol. 81*, 242–246.

Sumper, M., and Hallmann, A. (1998). Biochemistry of the extracellular matrix of Volvox. *Int. Rev. Cytol. 180*, 51–85.

Tanaka, M., Sato, K., and Uchida, T. (1981). Plant prolyl hydroxylase recognizes poly(l-proline) II helix. *J. Biol. Chem.* 256, 11397–11400.

Tiainen, P., Myllyharju, J., and Koivunen, P. (2005). Characterization of a second *Arabidopsis thaliana* prolyl 4-hydroxylase with distinct substrate specificity. *J. Biol. Chem.* 280, 1142–1148.

Valentin, R., Cerclier, C., Geneix, N., Aguié-Béhin, V., Gaillard, C., Ralet, M. C., and Cathala, B. (2010). Elaboration of extensin-pectin thin film model of primary plant cell wall. *Langmuir* 26, 9891–9898.

Van Holst, G. J., and Varner, J. E. (1984). Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. *Plant Physiol.* 74, 247–251.

Velasquez, S. M., Ricardi, M. M., Gloazzo Dorosz, J., Fernandez, P. V., Nadra, A. D., Pol-Fachin, L., Egelund, J., Gille, S., Ciancia, M., Verli, H., Pauly, M., Bacic, A., Erik Olsen, C., Ulvskov, P., Larsen Petersen, B., Somerville, C., Iusem, N. D., and Estevez, J. M. . (2011a). O-glycosylated cell wall extensins are essential in root hair growth. *Science 33*, 1401–1403.

Velasquez, S. M., Iusem, N. D., and Estevez, J. M. Root hair sweet growth. (2011b). *Plant Signal. Behav.* 6, 1600–1602.

Vlad, F., Spano, T., Vlad, D., Bou Daher, F., Ouelhadj, A., and Kalaitzis, P. (2007). *Arabidopsis* prolyl 4-hydroxylases are differentially expressed in response to hypoxia, anoxia and mechanical wounding. *Physiol. Plant* 130, 471–483.

Vlad, F., Tiainen, P., Owen, C., Spano, T., Bou Daher, T., Oualid, F., Senol, N. O., Vlad, D., Myllyharju, J., and Kalaitzis, P. (2010). Characterization of two carnation petal prolyl 4 hydroxylases. *Physiol. Plant* 140, 199–207.

Won, S. K., Lee, Y. J., Yeon Lee, H., Kyung Heo, Y., Cho, M., and Hyung-Taeg, C. (2009). Cis-element- and transcriptome-based screening of root hair-specific genes and their functional characterization in *Arabidopsis*. *Plant Physiol. 150*, 1459–1473.

Yuasa, K., Toyooka, K., Fukuda, H., and Matsuoka, K. (2005). Membrane-anchored prolyl hydroxylase with an export signal from the endoplasmic reticulum. *Plant J.* 41, 81–94.

Zhao, Z. D., Tan, L., Showalter, A. M., Lamport, D. T. A., and Kieliszewski, M. J. (2002). Tomato LeAGP-1 arabinogalactan-protein purified from transgenic tobacco corroborates the Hyp contiguity hypothesis. *Plant J.* 31, 431–444.

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Cotton fiber: a powerful single-cell model for cell wall and cellulose research

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Cotton fibers are single-celled extensions of the seed epidermis. They can be isolated in pure form as they undergo staged differentiation including primary cell wall synthesis during elongation and nearly pure cellulose synthesis during secondary wall thickening. This combination of features supports clear interpretation of data about cell walls and cellulose synthesis in the context of high throughput modern experimental technologies. Prior contributions of cotton fiber to building fundamental knowledge about cell walls will be summarized and the dynamic changes in cell wall polymers throughout cotton fiber differentiation will be described. Recent successes in using stable cotton transformation to alter cotton fiber cell wall properties as well as cotton fiber quality will be discussed. Future prospects to perform experiments more rapidly through altering cotton fiber wall properties via virus-induced gene silencing will be evaluated.

Keywords: cellulose, cotton fiber, elongation, *Gossypium*, pectin, transition stage, virus-induced gene silencing, xyloglucan

IMPORTANCE OF COTTON FIBER CELL WALLS

Cotton fiber, a highly elongated and thickened single cell of the seed epidermis, is the world's most important natural textile fiber. This article will focus on allotetraploid *Gossypium hirsutum*, the source of most commercial cotton fiber, and fiber cell wall polymers that help to define cotton fiber quality. Cotton fibers initiate by swelling above the ovule surface near the day of flower opening (or anthesis). Days post anthesis (DPA) describes the temporal progression of fiber development, although genotype-by-environment interactions affect the rate of progression (see below). Fiber initials expand isodiametrically, which likely involves cell wall loosening, due to increased turgor pressure in selected epidermal cells. Over the next ∼50 days the cotton fiber undergoes: (a) extreme elongation (to >2.5 cm) via primary wall synthesis; (b) transitional wall thickening and primary wall remodeling; and (c) secondary wall thickening via deposition of nearly pure cellulose. Finally, ill-defined "maturation" and cell death processes occur before the boll opens to reveal the fluffy fiber within the cotton fruit (or boll; **Figure 1**). Typically four carpels (or locules) of one fruit contain about 32 seeds and ∼500,000 long cotton fibers (lint fiber; Bowman et al., 2001). A population of thick-walled but short fibers (fuzz fiber or linters) also exists; these are an important source of chemical cellulose for many industrial applications.

In addition to defining morphogenesis, cotton fiber cell walls define the industrially important fiber quality parameters (Wakelyn et al., 2007). The fiber length generated through primary wall synthesis is essential for spinning yarn. Fiber strength is contributed by a thin "winding" cell wall layer (see below) and secondary wall cellulose, which also allows the fiber to dye deeply and absorb water. The amount of secondary wall cellulose versus fiber perimeter determines the desirable intermediary extent of secondary wall thickening, which is required for the fiber to collapse into the kidney bean shape that facilitates the spinning of cotton yarn. Cellulose biophysical properties such as degree of crystallinity, fibril angle, and degree of polymerization affect the performance of the fiber. The mature cotton fiber contains >90% (w/w) crystalline cellulose, including its cellulosic secondary wall surrounded by a ∼200 nm cuticulated primary wall.

COTTON FIBER AS MODEL CELL

Cotton fiber is a powerful cell wall research model because it is an easily isolated single cell with distinct stages of cell wall synthesis. Other advantages include the ability to culture cotton ovules/fibers *in vitro* (Kim and Triplett, 2001). The expression of many genes in cotton fiber (Hovav et al., 2008) supports diverse cell wall-related developmental events. After ∼6 DPA, frozen cotton fibers can be isolated from the developing seed by gentle grinding. At earlier DPA, fiber enriched RNA can be isolated by beating frozen ovules/fiber with glass beads (Taliercio and Boykin, 2007). Since initial large-scale efforts began a decade ago (Arpat et al., 2004), many studies to characterize the fiber transcriptome have occurred. Even earlier, cotton fiber transcriptomics led to the identification of the first (putative) plant cellulose synthase gene (Pear et al., 1996), a finding that was then genetically confirmed in arabidopsis (Arioli et al., 1998). Use of biochemical, molecular, and microscopic techniques to analyze cotton fiber showed that sucrose synthase is able to provide substrate, as UDP-glucose, to cellulose synthesis (Amor et al., 1995). Cryogenic electron microscopy and biochemical experiments showed that sucrose synthase was present inside and outside the cotton fiber plasma membrane (Salnikov et al., 2003; Brill et al., 2011), which could be related to callose (β-1, 3-glucan)

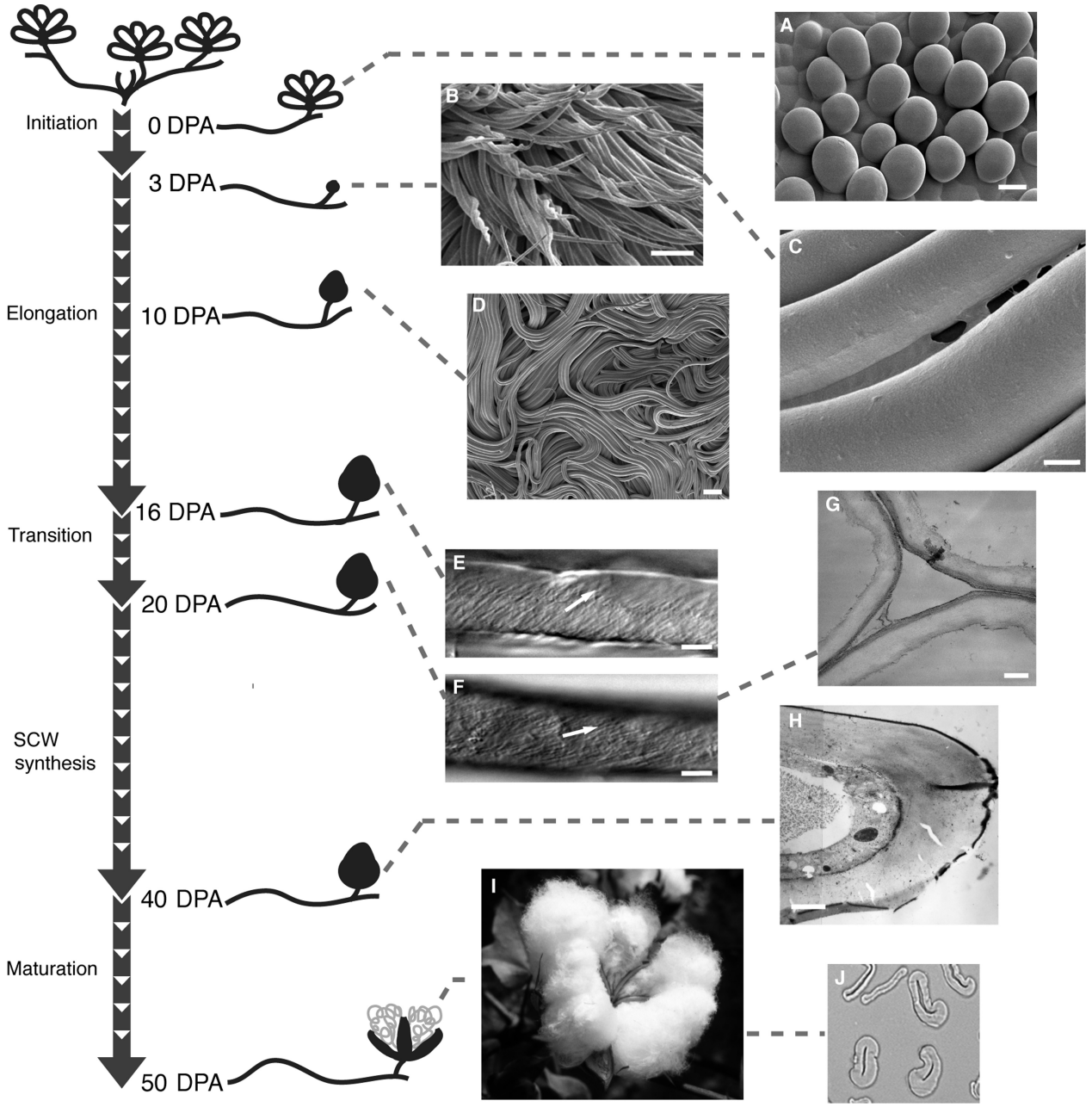


FIGURE 1 | A representation of the progression of cotton fiber development. The "stem" indicates the fastest developmental timeline when plants are grown under warm conditions. "Branches" show DPA with associated images of fiber. Cryo- field-emission SEM of **(A)** fiber initials on the ovule surface (bar = 10 μm); **(B)** twisting and elongating 3 DPA fibers (bar = 100 μm); **(C)** CFML stretched between two 3 DPA fibers (bar = 4 μm); **(D)** ordered bundles of fibers inside the boll (bar = 100 μm). Differential interference contrast micrographs indicating microfibril angle (as highlighted by arrows) in fiber at **(E)** 16 DPA and **(F)** 20 DPA (bars = 10 μm). TEM fiber cross-section show **(G)** an early stage of secondary wall thickening (bar = 300 nm); and **(H)** a more advanced stage of secondary wall deposition (bar = 1 μm). **(I)** Mature cotton boll and **(J)** cross-section of mature fiber viewed in the light microscope. SCW, secondary cell wall. Re-published with permission from The Cotton Foundation (http://www.cotton.org/foundation/index.cfm; Stiff and Haigler, in press).

and/or cellulose (β-1, 4-glucan) synthesis. Drug treatments of cotton fiber showed that cellulose fibril orientation, but not cellulose synthesis itself, depended on microtubules (Seagull, 1993), which was confirmed by live cell imaging in arabidopsis (Paredez et al., 2006). Biophysical analyses on cotton fiber revealed cellulose IV in primary walls (Chanzy et al., 1978), helped to define cellulose Iα and cellulose Iβ cellulose allomorphs (Atalla and Vanderhart, 1984), and demonstrated the higher molecular weight of secondary wall versus primary wall cellulose (Timpa and Triplett, 1993).

For secondary wall cellulose synthesis, cotton fibers adopted the genetic program of sclerenchyma cells of the plant body (e.g., xylem tracheary elements and interfascicular fibers) while shutting down the synthesis of the matrix polysaccharides and lignin (Haigler et al., 2009; Betancur et al., 2010). This finding was surprising given that cotton fibers are morphologically classified as trichomes. Nonetheless, the apparent orthologs of genes required for secondary wall synthesis in arabidopsis sclerenchyma cells are up-regulated at the onset of cotton fiber secondary wall deposition (Haigler et al., 2005, 2009; Al-Ghazi et al., 2009; Singh et al., 2009a; Hinchliffe et al., 2010). In contrast, genes encoding the secondary wall-related CESA isoforms are not up-regulated in arabidopsis leaf trichomes as shown by qRT–PCR, microarrays, analysis of CESA promoter::GUS reporter genes in transgenic arabidopsis, and observation of thick trichome walls in arabidopsis lines with non-functional secondary wall cellulose synthases (Betancur et al., 2010). This result is consistent with the contrast between the nearly pure cellulose in cotton fiber secondary walls versus a blend of traditionally defined primary and secondary wall components (including cellulose, pectin, mannan, and lignin) in mature trichome cell walls (Marks et al., 2008). Comparisons of developmentally regulated gene expression sets confirmed the traditional primary wall-related characteristics of arabidopsis leaf trichomes as contrasted with traditional secondary wall-related characteristics of cotton fiber at the onset of wall thickening (Betancur et al., 2010). The xylem-derived genetic program adopted by cotton fiber was present at the base of the land plant lineage with little change since that time (Haigler et al., 2009; Hinchliffe et al., 2010; Zhong et al., 2010), as also reflected in comparisons of gene expression within and between *Gossypium* species (Al-Ghazi et al., 2009; Rapp et al., 2010). Sclerenchyma cells, not leaf trichomes, provide the best analogies in the model plant arabidopsis for cell wall thickening in cotton fiber. This illustrates the flexible use of a "plant cell wall toolbox" to generate diverse cell walls particularly suited for their purpose (Betancur et al., 2010).

COTTON FIBER CELL WALL COMPOSITION

Cotton fiber cell wall composition changes dynamically throughout development. In young fiber between 0 and 2 DPA: (a) a pectin-rich, xyloglucan-depleted outer sheath develops; (b) an epitope is lost that is characteristic of (1–6)-β-D-galactan carrying arabinose (possibly contained *in situ* within arabinogalactan protein); and (c) an epitope appears that is characteristic of (1–4)-β-D-galactan, one of the possible side chains of rhamnogalacturonan I (Vaughn and Turley, 1999; Bowling et al., 2011). Also by 2 DPA, the synthesis of a cotton fiber middle lamella (CFML) begins. This thin adhesive outer primary cell wall layer is enriched in fucosylated- and non-fucosylated xyloglucan and homogalacturonan with no or relatively low esterification. The CFML serves to join adjacent fibers into tissue-like bundles that become highly organized and tightly packed within the confined boll space (Singh et al., 2009a). The inner cotton fiber primary wall contains ~22% crystalline cellulose fibrils surrounded by xyloglucan and pectin (Meinert and Delmer, 1977; Singh et al., 2009a). Glycome profiling of fiber wall extracts with numerous monoclonal antibodies recognizing plant polysaccharide epitopes (Pattathil et al., 2010) showed that xylan and acetylated mannan also exist in cotton fiber (Haigler et al., in preparation). Genes encoding expansin, a cell wall loosening protein, are expressed during cotton fiber elongation and associated with QTLs related to fiber length (Harmer et al., 2002; An et al., 2007). At the transition between primary and secondary wall deposition, transcriptionally-regulated cell wall degradative enzymes break down the CFML so that fibers are released as individuals – a phenomenon correlated with decreases in primary wall-related sugars, up-regulation of cell wall hydrolases, and reduction of pectin and xyloglucan molecular mass as secondary wall synthesis begins (Meinert and Delmer, 1977; Tokumoto et al., 2002, 2003; Guo et al., 2007; Singh et al., 2009a).

A distinct transition stage between primary and secondary wall deposition occurs in cotton fiber. During the transition, a unique "winding" cell wall layer, analogous to the S1 layer in wood fiber, is laid down over several days (Seagull, 1993). Fiber strength increases substantially at this time although wall thickening is minimal (Hsieh et al., 1995; Hinchliffe et al., 2011), presumably due to the differently oriented cellulose microfibrils in the winding layer compared to the primary wall. The winding layer may also fuse the primary and secondary cell walls together. Other developmental changes also occur at the transition stage: microtubules rearrange to support the changed direction of cellulose fibril deposition in the cell wall; respiration rate declines temporarily; concentrations of some metabolic sugars change; cellulose synthesis rate increases along with an increase to ~ 35% (w/w) in cellulose content; the CFML degrades; and the level of callose peaks within the cotton fiber cell wall. At the onset of secondary wall thickening, the rate of cellulose synthesis increases again and both microtubules and cellulose fibrils adopt a steep helix relative to the fiber axis (Meinert and Delmer, 1977; Maltby et al., 1979; Seagull, 1993; Martin and Haigler, 2004; Guo et al., 2007; Singh et al., 2009a; Abidi et al., 2010). The cotton fiber secondary wall ultimately becomes 3–6 μm thick.

CELL WALL-BASED CONTROLS OF COTTON FIBER MORPHOGENESIS AND QUALITY

Through comparing different cotton genotypes and/or experiments with cotton ovule/fiber culture, insights are emerging about how cell walls control cotton fiber morphogenesis and quality. As predicted given the positive correlation between pectin supply and cell expansion in plants (Boyer, 2009), signaling pathways (related to ethylene and C24:0 lignoceric acid) stimulate gene expression related to pectin biosynthesis as part of promoting fiber elongation (Qin et al., 2007; Pang et al., 2010). A family of recombinant inbred lines between *Gossypium* species with lower and higher fiber quality showed a correlation between expression of a pectin methylesterase gene and fiber quality (Al-Ghazi et al., 2009). Increasing the amount of de-esterified homogalacturonan reduced fiber elongation in transgenic cotton: up to 16% shorter fiber developed on plants with lower activity of pectate lyase, which can degrade de-esterified homogalacturonan (Wang et al., 2010a). In keeping with the above correlations, probing wild-type fiber cross-sections and cell wall extracts with antibodies provided evidence for increased de-esterification of homogalacturonan near the end of fiber elongation (Singh et al., 2009a). These data are

consistent with the possibility of developmentally programmed regulation of fiber growth through cell wall rigidification as de-esterified homogalacturonan become cross-linked by calcium, a phenomenon that is well known in plants (Cosgrove, 2005; Boyer, 2009).

Related to xyloglucan, the family of xyloglucan endo-transglycosylase/hydrolase (*XTH*) genes with expression peaks during elongation has been explored in cotton fiber (Michailidis et al., 2009; Lee et al., 2010). The XTH proteins have one or both abilities to degrade xyloglucan irreversibly (xyloglucan endo-transglycosylase or XET activity) or to cleave and transfer chain ends between molecules (xyloglucan endo-hydrolase or XEH activity; Eklöf and Brumer, 2010). Both activities could possibly increase the plasticity of the primary wall and promote fiber elongation (Cosgrove, 2005). Consistent with this prediction, transgenic cotton plants constitutively over-expressing *GhXTH1* showed a positive correlation between inheritance of the transgene and increased fiber length. The transgenic plants had about twofold higher XET activity and 15–20% longer fiber compared to wild-type cotton or null segregants under greenhouse or field conditions. Therefore, the transfer of xyloglucan chain ends between molecules was predicted to be a limiting factor for cotton fiber elongation (Lee et al., 2010). Similarly, XTH activity was lower in a short fiber mutant compared to wild-type cotton (Shao et al., 2011).

The timing of the transition to secondary wall deposition is: (a) affected by temperature and exogenous hormones *in vitro* (Roberts et al., 1992; Singh et al., 2009b); (b) differs between cultivars grown in the greenhouse (Abidi et al., 2010); and (c) correlates with differences in fiber bundle strength in two near-isogenic lines grown in the field (Hinchliffe et al., 2010). Field studies of the two near-isogenic lines showed that the time of onset of the transition stage was determined by the accumulation of a minimal number of heat units. Earlier entry into the transition stage resulted in higher fiber bundle strength, for reasons that remain to be discovered (Hinchliffe et al., 2011), and it is correlated with shorter fiber in transgenic cotton over-expressing *GhPFN2*, a profilin gene. Profilin is an actin bundling protein, and *GhPFN2* is normally highly expressed between 15 and 24 DPA (Wang et al., 2010b). Correlating with these results, transgenic cotton plants with down-regulated expression of *GhADF1*, encoding an actin-depolymerizing factor, had a heritable increase in fiber length (+5.6%) and thicker secondary walls (Wang et al., 2009).

Under salt stress, higher cellulose content occurred in transgenic cotton over-expressing mustard annexin compared to wild-type, and genes encoding sucrose phosphate synthase, sucrose synthase, and cellulose synthase were up-regulated in leaves and fiber (Divya et al., 2010). These results correlate with increased fiber cellulose content when transgenic cotton over-expressing spinach sucrose phosphate synthase was grown in the growth chamber under cool night conditions (Haigler et al., 2007).

VIRUS-INDUCED GENE SILENCING AS AN EFFICIENT TOOL FOR TESTING COTTON GENE FUNCTION

Knock-downs of gene expression through virus-induced gene silencing (VIGS; Robertson, 2004) can occur without stable transformation, which is inefficient in cotton (Wilkins et al., 2004). In VIGS experiments, a modified virus transiently down-regulates (silences) a plant messenger RNA (mRNA; Ruiz and Baulcombe, 1998). During viral infection, the plant produces small-interfering RNAs (siRNAs) complementary to the viral genome (Hamilton and Baulcombe, 1999). These siRNAs bind to homologous target mRNAs and ultimately prevent translation of the target gene (Brodersen et al., 2008). When a fragment of a plant gene is inserted into a VIGS vector, both the viral transcripts and the targeted plant transcript(s) are silenced (Kumagai et al., 1995). Down-regulation of the targeted gene will ideally lead to a phenotype that reveals the gene's function. For a possibly redundant gene family, a conserved fragment can be used to potentially knock-down the expression of several genes and increase the chances of observing a phenotype. VIGS has been successfully used in *Nicotiana benthamiana* to produce phenotypes by silencing cell wall biosynthetic genes (Burton et al., 2000; Hoffmann et al., 2004; Ahn et al., 2006; Zhu et al., 2010).

Two VIGS vectors have been developed for cotton: one from a DNA virus (Cotton leaf crumple virus, CLCrV; Tuttle et al., 2008) and another from a broad-host-range RNA virus (Tobacco rattle virus, TRV; Ye et al., 2010; Gao et al., 2011). These VIGS vectors are introduced into the plant by either agrobacterium (TRV and CLCrV) or particle bombardment (CLCrV only). Following inoculation, silencing initiates between 7 and 14 days post infection (dpi; TRV) or by 21 dpi (CLCrV). The extent of silencing from both CLCrV and TRV is inversely associated with growth temperature (Fu et al., 2006; Tuttle et al., 2008). Neither CLCrV-VIGS nor TRV-VIGS produced completely uniform silencing in all cultivars (Idris et al., 2010; Gao et al., 2011). Silencing a visible marker, such as PDS or GFP, along with the target gene may be necessary in some cases to facilitate phenotyping (Quadrana et al., 2011), and experiments are ongoing to employ this strategy in cotton fiber (Tuttle, 2011). There are two strategies for achieving VIGS in cotton fiber: (1) plants are inoculated early followed by translocation of the vector or siRNAs to the developing cotton fibers; or (2) isolated ovules are inoculated and cultured *in vitro*. Given the persistence of CLCrV-VIGS in cotton (Idris et al., 2010), the CLCrV vector is suitable for plant inoculation. For cultured ovules, only TRV-VIGS has been tested so far (Ye et al., 2010).

Consistent with the role of expansins as cell wall loosening agents (Cosgrove, 2005), down-regulation of *GhExp1* in 16 DPA cotton fiber resulted in a short fiber phenotype (Tuttle, 2011; Tuttle et al., in preparation). This experiment proves the efficacy of VIGS to test the function of cell wall-related genes expressed in cotton fiber. We are continuing to investigate whether VIGS is effective throughout cotton fiber development, given that fibers undergo symplastic isolation during the mid-phase of elongation (Ruan et al., 2001) so that the spread of silencing into the fiber may be blocked (Voinnet et al., 1998). If the VIGS vectors cannot replicate in fiber, silencing a target gene may depend on the translocation of siRNAs into fiber from adjacent cells in the seed coat.

FUTURE PROSPECTS

Virus-induced gene silencing technology will synergize with cotton genome sequencing (Chen et al., 2007; Paterson et al., 2010; see http://www.phytozome.net/cotton.php) to make cotton fiber

functional genomics more efficient. Cotton fiber transcriptomics provides many clues about important genes to test. Proteomics, metabolomics, and antibody probing of cell wall composition are in the early stages of revealing additional insights. Other areas that were not fully summarized here, such as investigation of cell wall-related signaling mechanisms and regulation of cotton fiber cellulose synthesis, also benefit from the clarity provided by staged development in single-celled cotton fiber. The rich history of *Gossypium* evolution and domestication, including living ancestral genotypes with different fiber developmental programs and properties (Wendel et al., 2009), allows insights about changes in cell wall properties under natural or human selection pressures. In summary, key elements are converging to make cotton fiber one of the most useful systems for cell wall and cellulose research. Further analysis of cotton fiber cell walls is relevant to improvement of this important textile fiber and to creating the next generation of crop plants for optimized production of biofuels and biomaterials.

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REFERENCES

Abidi, N., Hequet, E., and Cabrales, L. (2010). Changes in sugar composition and cellulose content during the secondary cell wall biogenesis in cotton fibers. *Cellulose* 17, 153–160.

Ahn, J., Verma, R., Kim, M., Lee, J., Kim, Y., Bang, J., Reiter, W., and Pai, H. (2006). Depletion of UDP-d-apiose/UDP-d-xylose synthases results in Rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. *J. Biol. Chem.* 281, 13708–13716.

Al-Ghazi, Y., Bourot, S., Arioli, T., Dennis, E. S., and Llewellyn, D. J. (2009). Transcript profiling during fiber development identifies pathways in secondary metabolism and cell wall structure that may contribute to cotton fiber quality. *Plant Cell Physiol.* 50, 1364–1381.

Amor, Y., Haigler, C. H., Johnson, S., Wainscott, M., and Delmer, D. P. (1995). A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9353–9357.

An, C., Saha, S., Jenkins, J. N., Scheffler, B. E., Wilkins, T. A., and Stelly, D. M. (2007). Transcriptome profiling, sequence characterization, and SNP-based chromosomal assignment of the EXPANSIN genes in cotton. *Mol. Genet. Genomics* 279, 539–553.

Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998). Molecular analysis of cellulose biosynthesis in Arabidopsis. *Science* 279, 717–720.

Arpat, A. B., Waugh, M., Sullivan, J. P., Gonzales, M., Frisch, D., Main, D., Wood, T., Leslie, A., Wing, R. A., and Wilkins, T. A. (2004). Functional genomics of cell elongation in developing cotton fibers. *Plant Mol. Biol.* 54, 911–929.

Atalla, R. H., and Vanderhart, D. L. (1984). Native cellulose: a composite of two distinct crystalline forms. *Science* 223, 283–285.

Betancur, L., Singh, B., Rapp, R. A., Wendel, J. F., Marks, M. D., Roberts, A. W., and Haigler, C. H. (2010). Phylogenetically distinct cellulose synthase genes support secondary wall thickening in Arabidopsis shoot trichomes and cotton fiber. *J. Integr. Plant Biol.* 52, 205–220.

Bowling, A. J., Vaughn, K. C., and Turley, R. B. (2011). Polysaccharides and glycoprotein distribution in the epidermis of cotton ovules during early fiber initiation and growth. *Protoplasma* 248, 579–590.

Bowman, D. T., van Esbroeck, G. A., Van't Hof, J., and Jividen, G. M. (2001). Ovule fiber cell numbers in modern upland cottons. *J. Cotton Sci.* 5, 81–83.

Boyer, J. S. (2009). Cell wall biosynthesis and the molecular mechanism of plant enlargement. *Funct. Plant Biol.* 36, 383–394.

Brill, E., van Thournout, M., White, R. G., Llewellyn, D., Campbell, P. M., Engelen, S., Ruan, Y.-L., Arioli, T., and Furbank, R. T. (2011). A novel isoform of sucrose synthase is targeted to the cell wall during secondary cell wall synthesis in cotton fibre. *Plant Physiol.* 157, 40–54.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y. Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. *Science* 320, 1185–1190.

Burton, R. A., Gibeaut, D. M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D. C., and Fincher, G. B. (2000). Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell* 12, 691–706.

Chanzy, H., Imada, K., and Vuong, R. (1978). Electron diffraction from the primary walls of cotton fibers. *Protoplasma* 94, 299–306.

Chen, Z. J., Scheffler, B. E., Dennis, E., Triplett, B., Zhang, T., Chen, X., Stelly, D. M., Rabinowicz, P. D., Town, C., Arioli, T., Brubaker, C., Cantrell, R., Lacape, J.-M., Ulloa, M., Chee, P., Gingle, A. R., Haigler, C. H., Percy, R., Saha, S., Wilkins, T., Wright, R. J., Deynze, A. V., Zhu, Y., Yu, S., Guo, W., Abdurakhmonov, I., Katageri, I., Rahman, M., Zafar, Y., Yu, J. Z., Kohel, R. J., Wendel, J., and Paterson, A. H. (2007). Towards sequencing cotton (*Gossypium*) genomes. *Plant Physiol.* 145, 1303–1310.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861.

Divya, K., Jami, S. K., and Kirti, P. B. (2010). Constitutive expression of mustard annexin, AnnBj1, enhances abiotic stress tolerance and fiber quality in cotton under stress. *Plant Mol. Biol.* 73, 293–308.

Eklöf, J. M., and Brumer, H. (2010). The XTH Gene Family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* 153, 456–466.

Fu, D. Q., Zhu, B. Z., Zhu, H. L., Zhang, H. X., Xie, Y. H., Jiang, W. B., Zhao, X. D., and Luo, K. B. (2006). Enhancement of virus-induced gene silencing in tomato by low temperature and low humidity. *Mol. Cells* 21, 153–160.

Gao, X., Wheeler, T., Li, Z., Kenerley, C. M., He, P., and Shan, L. (2011). Silencing GhNDR1 and GhMKK2 compromises cotton resistance to *Verticillium* wilt. *Plant J.* 66, 293–305.

Guo, G.-Y., Wang, L.-J., Chen, S.-P., Hu, W.-L., and Chen, X.-Y. (2007). Gene expression and metabolite profiles of cotton fiber during cell elongation and secondary cell wall synthesis. *Cell Res.* 17, 422–434.

Haigler, C. H., Zhang, D., and Wilkerson, C. G. (2005). Biotechnological improvement of cotton fibre. *Physiol. Plant.* 124:285–294.

Haigler, C. H., Singh, B., Zhang, D., Hwang, S., Wu, C., Cai, W. X., Hozain, M., Kang, W., Kiedaisch, B., Strauss, R. E., Hequet, E. F., Wyatt, B. G., Jividen, G. M., and Holaday, A. S. (2007). Transgenic cotton overproducing spinach sucrose phosphate synthase showed enhanced leaf sucrose synthesis and improved fiber quality under controlled environmental conditions. *Plant Mol. Biol.* 63, 815–832.

Haigler, C. H., Singh, B., Wang, G., and Zhang, D. (2009). "Genomics of cotton fiber secondary wall deposition and cellulose biogenesis," in *Genetics and Genomics of Cotton*, ed. A. H. Paterson (New York: Springer), 385–417.

Harmer, S. E., Orford, S. J., and Timmis, J. N. (2002). Characterisation of six *alpha-expansin* genes in *Gossypium hirsutum* (upland cotton). *Mol. Genet. Genomics* 268, 1–9.

Hamilton, A. J., and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952.

Hinchliffe, D. J., Meredith, W. R., Delhorn, C. D., Thibodeaux, D. P., and Fang, D. D. (2011). Elevated growing degree days influence transition stage timing during cotton fiber development resulting in increased fiber-bundle strength. *Crop Sci.* 51, 1683–1692.

Hinchliffe, D. J., Meredith. W. R., Yeater, K. M., Kim, H. J., Woodward, A. W., Chen, Z. J., and Triplett, B. A. (2010). Near-isogenic cotton germplasm lines that differ in fiber-bundle strength have temporal differences in fiber gene expression patterns as revealed by comparative high-throughput profiling. *Theor. Appl. Genet.* 120, 1347–1366.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre,

C., Pollet, B., and Legrand, M. (2004). Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. *Plant Cell* 16, 1446–1465.

Hovav, R., Udall, J. A., Hovav, E., Rapp, R., Flagel, L., and Wendel, J. F. (2008). A majority of cotton genes are expressed in single-celled fiber. *Planta* 227, 319–329.

Hsieh, Y.-L., Honik, D. D., and Hartzell, M. M. (1995). A developmental study of single fiber strength: greenhouse grown SJ-2 Acala cotton. *Text. Res. J.* 65, 101–112.

Idris, A. M., Tuttle, J. R., Robertson, D., Haigler, C. H., and Brown, J. K. (2010). Differential cotton leaf crumple virus-VIGS-mediated gene silencing and viral genome localization in different *Gossypium hirsutum* genetic backgrounds. *Physiol. Mol. Plant Pathol.* 75, 13–22.

Kim, H. J., and Triplett, B. A. (2001). Cotton fiber growth in planta and *in vitro*. Models for plant cell elongation and cell wall biogenesis. *Plant Physiol.* 127, 1361–1366.

Kumagai, M. H., Donson, J., della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. K. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. U.S.A.* 92, 1679–1683.

Lee, J., Burns, T. H., Light, G., Sun, Y., Fokar, M., Kasukabe, Y., Fujisawa, K., Maekawa, Y., and Allen, R. D. (2010). Xyloglucan endotransglycosylase/hydrolase genes in cotton and their role in fiber elongation. *Planta* 232, 1191–1205.

Maltby, D., Carpita, N. C., Montezinos, D., Kulow, Y., and Delmer, D. P. (1979). Beta-1, 3-glucan in developing cotton fibers – structure, localization, and relationship of synthesis to that of secondary wall cellulose. *Plant Physiol.* 63, 1158–114.

Marks, M. D., Betancur, L, Gilding, E., Chen, F., Bauer, S., Wenger, J., Dixon, R. A., and Haigler, C. H. (2008). A new method for isolating large quantities of Arabidopsis trichomes for transcriptome, cell wall and other types of analyses. *Plant J.* 56, 483–492.

Martin, L. K., and Haigler, C. H. (2004). Cool temperature hinders flux from glucose to sucrose during cellulose synthesis in secondary wall stage cotton fibers. *Cellulose* 11, 339–349.

Meinert, M. C., and Delmer, D. P. (1977). Changes in biochemical composition of the cell wall of the cotton fiber during development. *Plant Physiol.* 59, 1088–1097.

Michailidis, G., Argiriou, A., Darzentas, N., and A. Tsaftaris, A. (2009). Analysis of xyloglucan endotransglycosylase/hydrolase (XTH) genes from allotetraploid (*Gossypium hirsutum*) cotton and its diploid progenitors expressed during fiber elongation. *J. Plant Physiol.* 166, 403–416.

Pang, C. Y., Wang, H., Pang, Y., Xu, C., Jiao, Y., Qin, Y. M., Western, T. L., Yu, S. X., and Zhu, Y. X. (2010). Comparative proteomics indicates that biosynthesis of pectic precursors is important for cotton fiber and Arabidopsis root hair elongation. *Mol. Cell. Proteomics* 9, 2019–2033.

Paredez, A. R., Somerville, C. R., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495.

Paterson, A., Rong, J., Gingle, A., Chee, P., Dennis, E., Llewellyn, D., Dure, L., Haigler, C., Myers, G., Peterson, D., Rahman, M., Zafar, Y., Reddy, U., Saranga, Y., Stewart, J., Udall, J., Waghmare, V., Wendel, J., Wilkins, T., Wright, R., Zaki, E., Hafez, E., and Zhu, J. (2010). Sequencing and utilization of the *Gossypium* genomes. *Trop. Plant Biol.* 3, 71–74.

Pattathil, S. K., Avci, U., Baldwin, D., Alton, G. S., McGill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Pear, J. R., Kawagoe, Y., Schreckengost, W. E., Delmer, D. P., and Stalker, D. M. (1996). Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 93, 12637–12642.

Qin, Y. M., Hu, C. Y., Pang, Y., Kastaniotis, A. J., Hiltunen, J. K., and Zhu, Y. X. (2007). Saturated very-long-chain fatty acids promote cotton fiber and Arabidopsis cell elongation by activating ethylene biosynthesis. *Plant Cell* 19, 3692–3704.

Quadrana, L., Rodriguez, M. C., Lopez, M., Bermudez, L., Nunes-Nesi, A., Fernie, A. R., Descalzo, A., Asis, R., Rossi, M., Asurmendi, S., and Carrari, F. (2011). Coupling virus-induced gene silencing to exogenous green fluorescence protein expression provides a highly efficient system for functional genomics in Arabidopsis and across all stages of tomato fruit development. *Plant Physiol.* 156, 1278–1291.

Rapp, R. A., Haigler, C. H., Flagel, L., Hovav, R. H., Udall, J. A., and Wendel, J. F. (2010). Gene expression in developing fibers of Upland cotton (*Gossypium hirsutum* L.) was massively altered by domestication. *BMC Biol.* 8, 139. doi: 10.1186/1741-7007-8-139

Roberts, E. M., Nunna, R. R., Huang, J. Y., Trolinder, N. L., and Haigler, C. H. (1992). Effects of cycling temperatures on fiber metabolism in cultured cotton ovules. *Plant Physiol.* 100, 979–986

Robertson, D. (2004). VIGS vectors for gene silencing: many targets, many tools. *Annu. Rev. Plant. Biol.* 55, 495–519.

Ruan, Y. L., Llewellyn, D. J., and Furbank, R. T. (2001). The control of single-celled cotton fiber elongation by developmentally reversible gating of plasmodesmata and coordinated expression of sucrose and K+ transporters and expansin. *Plant Cell* 13, 47–60.

Ruiz, M., and Baulcombe, D. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10, 937–946.

Salnikov, V., Grimson, M. J., Seagull, R. W., and Haigler, C. H. (2003). Localization of sucrose synthase and callose in freeze substituted, secondary wall stage, cotton fibers. *Protoplasma* 221, 175–184.

Seagull, R. W. (1993). Cytoskeletal involvement in cotton fiber growth and development. *Micron* 24, 643–660.

Shao, M. Y., Wang, X. D., Ni, M., Bibi, N., Yuan, S. N., Malik, W., Zhang, H. P., Liu, Y. X., and Hua, S. J. (2011). Regulation of cotton fiber elongation by xyloglucan endotransglycosylase/hydrolase genes. *Genet. Mol. Res.* 10, 3771–3782.

Singh, B., Avci, U., Eichler Inwood, C. H., Grimson, M. J., Landgraf, J., Mohnen, D., Sorenson, I., Wilkerson, C. G., Willats, W. G. T., and Haigler, C. H. (2009a). A specialized outer layer of the primary cell wall joins elongating cotton fibers into tissue-like bundles. *Plant Physiol.* 150, 684–699.

Singh, B., Cheek, H. D., and Haigler, C. H. (2009b). A synthetic auxin (NAA) suppresses secondary wall cellulose synthesis and enhances elongation in cultured cotton fiber. *Plant Cell Rep.* 28, 1023–1032.

Stiff, M. R., and Haigler, C. H. (in press). "Recent advances in cotton fiber development," in *Cotton Flowering and Fruiting*, Cotton Physiology Book Series, eds, D. Oosterhuis and T. Cothren (Cordova TN: The Cotton Foundation).

Taliercio, E. W., and Boykin, D. (2007). Analysis of gene expression in cotton fiber initials. *BMC Plant Biol.* 7, 22. doi: 10.1186/1471-2229-7-22

Timpa, J. D., and Triplett, B. A. (1993). Analysis of cell-wall polymers during cotton fiber development. *Planta* 189, 101–108.

Tokumoto, H., Wakabayashi, K., Kamisaka, S., and Hoson, T. (2002). Changes in the sugar composition and molecular mass distribution of matrix polysaccharides during cotton fiber development. *Plant Cell Physiol.* 43, 411–418.

Tokumoto, H., Wakabayashi, K., Kamisaka, S., and Hoson, T. (2003). Xyloglucan breakdown during cotton fiber development. *J. Plant Physiol.* 160, 1411–1414.

Tuttle, J. R. (2011). *Development of a Virus-induced Gene Silencing System for Cotton and its Application for Functional Genomics in Fiber*. Doctoral Dissertation, 201 p. Available at: http://www.lib.ncsu.edu/resolver/1840.16/7024

Tuttle, J. R., Idris, A. M., Brown, J. K., Haigler, C. H., and Robertson, D. (2008). Geminivirus-mediated gene silencing from cotton leaf crumple virus is enhanced by low temperature in cotton. *Plant Physiol.* 148, 41–50.

Vaughn, K. C., and Turley, R. B. (1999). The primary walls of cotton fibers contain an ensheathing pectin layer. *Protoplasma* 209, 226–237.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95, 177–187.

Wakelyn, P. J., Bertoniere, N. R., French, A. D., Thibodeaux, D. P., Triplett, B. A., Rousselle, M. A., Goynes, W. R. Jr., Edwards, J. V., Hunter, L., McAlister, D. D., and Gamble, G. R. (2007). *Cotton Fiber Chemistry and Technology*. Boca Raton, FL: CRC Press, 162 p.

Wang, H., Guo, Y., Lv, F., Zhu, H., Wu, S., Jiang, Y., Li, F., Zhou, B., Guo, W., and Zhang, T. (2010a). The essential role of GhPEL gene, encoding a pectate lyase, in cell wall loosening by depolymerization of the de-esterified pectin during fiber elongation in cotton. *Plant Mol. Biol.* 72, 397–406.

Wang, J., Wang, H., Zhao, P., Han, L., Jiao, G., Zheng, Y., Huang, S., and Xia, G. (2010b). Overexpression of a profilin (GhPFN2) promotes the progression of developmental phases in

cotton fibers. *Plant Cell Physiol.* 51, 1276–1290.

Wang, H., Wang, J., Gao, P., Jiao, G., Zhao, P., Li, Y., Wang, G., and Xia, G. (2009). Down-regulation of GhADF1 gene expression affects cotton fibre properties. *Plant Biotechnol. J.* 7, 13–23.

Wendel, J. F., Brubaker, C., Alvarez, I., Cronn, R., and Stewart, J. M. D. (2009). "Evolution and natural history of the cotton genus," in *Genetics and Genomics of Cotton*, ed. A. H. Paterson (New York: Springer).

Wilkins, T. A., Mishra, R., and Trolinder, N. L. (2004). Agrobacterium-mediated transformation and regeneration of cotton. *J. Food Agric. Envt.* 2, 179–187.

Ye, J., Chua, N. H., Qu, J., Gene, Y. F., and Bu, Y. P. (2010). *Virus Induced Gene Silencing (VIGS) for Functional Analysis of Genes in Cotton*. Patent: WO/2010/144058, Publication Date: 16.12.2010, 1–92.

Zhong, R., Lee, C., and Ye, Z.-H. (2010). Evolutionary conservation of the transcriptional network regulating secondary wall biosynthesis. *Trends Plant Sci.* 15, 625–632.

Zhu, X., Pattathil, S., Mazumder, K., Brehm, A., Hahn, M. G., Dinesh-Kumar, S. P., and Joshi, C. P. (2010). Virus-induced gene silencing offers a functional genomics platform for studying plant cell wall formation. *Mol. Plant* 3, 818–833.

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The CELLULOSE SYNTHASE-LIKE A and CELLULOSE SYNTHASE-LIKE C families: recent advances and future perspectives

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The CELLULOSE SYNTHASE (CESA) superfamily of proteins contains several sub-families of closely related CELLULOSE SYNTHASE-LIKE (CSL) sequences. Among these, the CSLA and CSLC families are closely related to each other and are the most evolutionarily divergent from the CESA family. Significant progress has been made with the functional characterization of *CSLA* and *CSLC* genes, which have been shown to encode enzymes with 1,4-β-glycan synthase activities involved in the biosynthesis of mannan and possibly xyloglucan backbones, respectively. This review examines recent work on the *CSLA* and *CSLC* families from evolutionary, molecular, and biochemical perspectives. We pose a series of questions, whose answers likely will provide further insight about the specific functions of members of the CSLA and CSLC families and about plant polysaccharide biosynthesis is general.

Keywords: CELLULOSE SYNTHASE-LIKE, mannan, xyloglucan, CSLA, CSLC, plant cell wall

INTRODUCTION

Plant cell walls are complex composites that consist mainly of carbohydrates, including cellulose, hemicelluloses, and pectins (Somerville et al., 2004; Lerouxel et al., 2006; Sandhu et al., 2009; Doblin et al., 2010; Liepman et al., 2010; Scheller and Ulvskov, 2010; Carpita, 2011). The carbohydrates present in plant cell walls vary in structure and composition among plants (Carpita, 2011) and even within different cells and tissues of a single plant (Lee et al., 2011). This structural heterogeneity among carbohydrates is a key factor underlying the functional diversity of plant cell walls (Pauly and Keegstra, 2010). Plant cell walls also represent the most abundant source of renewable biomass, and provide materials with a multitude of human uses and other important roles in the biosphere (Pauly and Keegstra, 2010; Lee et al., 2011).

Due to the complexity of monosaccharide composition and glycosidic linkages present in plant cell walls, it is predicted that hundreds of enzymes are involved in cell wall carbohydrate biosynthesis (Keegstra and Raikhel, 2001; Scheible and Pauly, 2004). This estimate substantially increases when including other cell wall-related proteins (Girke et al., 2004; McCann and Carpita, 2008). Spurred by genome sequencing and the availability of powerful comparative and functional genomic tools, the cell wall research community has made significant progress over the last decade in identifying and determining the function of numerous enzymes involved in the synthesis of plant cell wall carbohydrates (Farrokhi et al., 2006; Lerouxel et al., 2006; Penning et al., 2009; Sandhu et al., 2009; Doblin et al., 2010; Liepman et al., 2010). Among these are members of the CELLULOSE SYNTHASE (CESA) superfamily of proteins (CAZy GT2; Cantarel et al., 2009). The CESA superfamily includes bona fide CESA proteins involved in cellulose synthesis (Youngs et al., 2007; Endler and Persson, 2011), as well as CELLULOSE SYNTHASE-LIKE (CSL) proteins (Richmond and Somerville, 2000; Hazen et al., 2002; Fincher, 2009) that have been implicated in the synthesis of various β-glycan polymers.

This minireview focuses upon the CSLA and CSLC subgroups, the most divergent CSL subgroups relative to the CESA proteins (Richmond and Somerville, 2000; Youngs et al., 2007). Because members of the CSLA and CSLC subgroups are thought to have evolved through duplication and diversification from a common ancestral gene (Yin et al., 2009; Del Bem and Vincentz, 2010), they share some structural and physicochemical features (Youngs et al., 2007), however they differ in membrane topology and in enzymatic function (Davis et al., 2010). A number of *CSLA* genes have been shown to encode mannan synthase enzymes that polymerize the 1,4-β-linked backbone of mannans and glucomannans (Dhugga et al., 2004; Liepman et al., 2005, 2007; Suzuki et al., 2006; Gille et al., 2011). The CSLC proteins have been implicated in the synthesis of 1,4-β-glucan backbone of xyloglucans (Cocuron et al., 2007) and possibly other polysaccharides (Dwivany et al., 2009). Due to space limitations, we are unable to provide a comprehensive review of these topics; instead we will focus upon some important unanswered questions about the CSLA and CSLC families.

ARE ALL CSLA PROTEINS INVOLVED IN MANNAN SYNTHESIS?

CSLA genes appear to be present in all land plants, and ancestral genes with characteristics similar to *CSLA* and *CSLC* sequences have been identified in a number of green algal genomes, in which they are thought to represent a homolog of the progenitor gene from which *CSLA* and *CSLC* genes evolved (Del Bem and

Vincentz, 2010). It has been hypothesized that these *CSLA*/*CSLC*-like sequences encode mannan synthases (Yin et al., 2009; Popper et al., 2011), however experimental evidence is needed to test this hypothesis. Heterologous expression of recombinant CSLA proteins has proven particularly effective for determining their enzymatic functions. The involvement of CSLA family members from diverse plant species in the synthesis of 1,4-β-mannan and glucomannan backbones has been demonstrated by a number of studies (**Table 1**; Dhugga et al., 2004; Liepman et al., 2005, 2007; Suzuki et al., 2006; Goubet et al., 2009; Gille et al., 2011). Studies of recombinant CSLA proteins have further demonstrated that expression of a single CSLA protein in a heterologous host is sufficient to impart enzymatic activity, and that the incorporation of mannose and glucose into glucomannan chains is mediated by a single enzyme (Liepman et al., 2005, 2007; Suzuki et al., 2006; Gille et al., 2011). Since recombinant CSLA proteins from a variety of plants exhibit mannan synthase activity, it is possible that all CSLA proteins are involved in the synthesis of mannans. An alternative possibility is that certain CSLA proteins may catalyze the synthesis of other polysaccharides; in particular a clade of CSLA proteins present only in monocots may have divergent function (Dhugga et al., 2004; Liepman et al., 2007; Del Bem and Vincentz, 2010; Dhugga, 2011). Efforts to characterize members of this clade will provide more information about the biosynthetic capabilities of CSLA proteins. Detailed biochemical studies of CSLA proteins from plants producing mannans of different structures also are needed in order to define whether structural features of these enzymes govern mannan product structure.

WHAT ARE THE PHYSIOLOGICAL FUNCTIONS OF MANNANS?

Within plants and algae, mannans are structurally and functionally diverse, and they serve well-known roles as structural elements and as energy reserves (Moreira and Filho, 2008). In angiosperms, mannans are cross-linking glycans that are present at low levels in primary cell walls (Zablackis et al., 1995; Schroder et al., 2009; Marcus et al., 2010), and in greater abundance in secondary cell walls (Handford et al., 2003; Goubet et al., 2009). In gymnosperms, mannans are the most abundant hemicellulosic polysaccharide present in wood (Maeda et al., 2000; Pauly and Keegstra, 2010). Mannans also are very abundant in the primary cell walls of ferns, where they appear to be the dominant cross-linking glycan of the recently defined Type III cell wall (Silva et al., 2011). A variety of plants store energy in the form of mannans in their endosperm tissue, including members of the Palmae, Liliaceae, Iridaceae, and Leguminosae families (Meier and Reid, 1982; Buckeridge, 2010). Glucomannans also are used for energy storage in corms of plants within the genus *Amorphophallus*. The AkCSLA3 protein, involved in the synthesis of glucomannan stored in the corms of Konjac, recently has been characterized along with many other sequences encoding proteins involved in other aspects of glucomannan biosynthesis (Gille et al., 2011).

In addition to carbohydrate storage and structure, mannans serve a variety of other functions. In fern roots, mannans are deposited as constituents of cell wall appositions as a defense mechanism to limit microbial ingress (Leroux et al., 2011). Mannans impart hardness to seeds of some plants, such as tomato

Table 1 | Biochemical attributes of recombinant CSLA and CSLC proteins.

Species*/protein name	Gene identifier [GenBank ID (Phytozome** ID)]	Enzymatic function(s)***	Reference
AkCSLA3	HQ833588	Mannan synthase, glucomannan synthase	Gille et al. (2011)
AtCSLA1	NM_117760 (At4g16590)	Mannan synthase, glucomannan synthase	Liepman et al. (2007)
AtCSLA2	NM_122180 (At5g22740)	Mannan synthase, glucomannan synthase	Liepman et al. (2005)
AtCSLA7	NM_129120 (At2g35650)	Mannan synthase	Liepman et al. (2005)
AtCSLA9	NM_120457 (At5g03760)	Mannan synthase, glucomannan synthase, glucan synthase (GDP)	Liepman et al. (2005)
CtMANS	AY372247	Mannan synthase	Dhugga et al. (2004)
OsCSLA1	NM_001052699 (Os02g09930)	Mannan synthase, glucomannan synthase	Liepman et al. (2007)
PpCSLA1	DQ417756 (Pp1s65_194V6)	Mannan synthase, glucomannan synthase	Liepman et al. (2007)
PpCSLA2	DQ417757 (Pp1s36_62V6)	Mannan synthase, glucomannan synthase	Liepman et al. (2007)
PtCSLA1	XM_002311936 (POPTR_0008s02650)	Mannan synthase, glucomannan synthase	Suzuki et al. (2006)
PtCSLA3	XM_002326203 (POPTR_0006s11810)	Mannan synthase	Suzuki et al. (2006)
PtaCSLA1	DQ641986	Mannan synthase, glucomannan synthase	Liepman et al. (2007)
AtCSLC4	NM_113737 (At3g28180)	Glucan synthase (UDP)	Cocuron et al. (2007)
TmCSLC	Not present in databases	Glucan synthase (UDP)	Cocuron et al. (2007)

**Prefixes: Ak, Amorphophallus konjac; At, Arabidopsis thaliana; Ct, Cyamopsis tetragonoloba; Os, Oryza sativa; Pp, Physcomitrella patens; Pt, Populus trichocarpa; Pta, Pinus taeda; Tm, Tropaeolum majus.*

***Phytozome (www.phytozome.net; Goodstein et al., 2012) identifiers provided, where available.*

****Mannan synthase, GDP-mannose-dependent 1,4-β-mannan synthase activity; glucomannan synthase, GDP-glucose-dependent 1,4-β-glucomannan synthase activity; glucan synthase (GDP), GDP-glucose-dependent 1,4-β-glucan synthase activity; glucan synthase (UDP), UDP-glucose-dependent 1,4-β-glucan synthase activity.*

and lettuce, thereby protecting the embryo and controlling radicle protrusion (Schroder et al., 2009; Buckeridge, 2010). In tomato fruits, mannans also have roles in cell adhesion (Ordaz-Ortiz et al., 2009), and recent immunological studies have revealed a much wider distribution of mannans in cell walls than previously appreciated (Marcus et al., 2010). Pre-treatment of tissue sections with pectate lyase revealed homogalacturonan-masked mannan epitopes (Marcus et al., 2010). Mannose is an abundant constituent of *Arabidopsis* trichomes and the *CSLA9* gene encoding a glucomannan synthase is among the top one hundred most abundant transcripts present in trichomes (Marks et al., 2008). Mannan epitopes appear to be present throughout trichome cell walls and at trichome bases, indicating that mannans may be involved in trichome to leaf adherence (**Figure 1**). Mannans also are involved in pollen tube growth, as this process is perturbed in *Arabidopsis csla7* mutant plants (Goubet et al., 2003).

A number of studies also implicate mannans within plant developmental signaling pathways. For example, *Arabidopsis csla7* mutant embryos exhibit defective embryogenesis, arresting at the globular stage (Goubet et al., 2003), and *csla9* mutants have reduced numbers of lateral roots (Zhu et al., 2003). Complementation of the *csla7* mutant phenotype has been achieved by overexpression of *CSLA9*, demonstrating that the CSLA7 and CSLA9 proteins likely make structurally interchangeable mannans *in vivo* (Goubet et al., 2009). Interestingly, aborted embryos and developmental asynchrony were documented in siliques of transgenic *Arabidopsis* plants overexpressing various *CSLA* genes, indicating that mannan abundance influences the progression of embryogenesis (Goubet et al., 2009). A number of other studies have documented growth and developmental responses of plants and cultured plant cells to the application of galactoglucomannan oligosaccharides (GGMOs). For example, GGMOs enhance cell population density and alter the protoxylem:metaxylem ratio of xylogenic cultures of Zinnia (Benova-Kakosova et al., 2006). Treatment of pea stem segments with GGMOs also inhibits auxin-stimulated elongation growth (Auxtova-Samajova et al., 1996), possibly through the action of recently discovered mannan transglycosylases (Schroder et al., 2006, 2009). Additional studies are needed to provide more insight about the biological significance of GGMOs and the mechanism of their action.

Efforts to understand the physiological roles of mannans likely have been hindered by functional redundancy, since *CSLA* genes are members of multiple-gene families in many plants (Richmond and Somerville, 2001; Hazen et al., 2002; Liepman et al., 2007; Roberts and Bushoven, 2007). In *Arabidopsis*, *csla* single mutants have been identified for each of the nine *CSLA* genes (**Table 2**). Aside from the *csla7* mutant, none of these *csla* single mutants exhibited notable phenotypic abnormalities (Goubet et al., 2009). It seems likely that at least some of the remaining uncharacterized *Arabidopsis* CSLA proteins also are mannan synthases, however the significant degree of overlap among the expression patterns of these sequences (Hamann et al., 2004; Liepman et al., 2007) probably masks defects resulting from their loss of function in *csla* single mutants, necessitating the analysis of higher order mutants. One such mutant, the *Arabidopsis csla2/csla3/csla9* triple mutant, lacks detectable glucomannan in stems. This glucomannan deficiency did not impact stem strength, indicating that mannans are not required for stem strength in *Arabidopsis*, or that a compensatory mechanism may exist in their absence (Goubet et al., 2009).

ARE DIFFERENT FORMS OF MANNANS SYNTHESIZED BY DIFFERENT CSL SUBCLASSES?

Recent studies have shed interesting new light on mannan synthesis, by implicating members of the CSLD family in this process (Verhertbruggen et al., 2011; Yin et al., 2011). Analysis

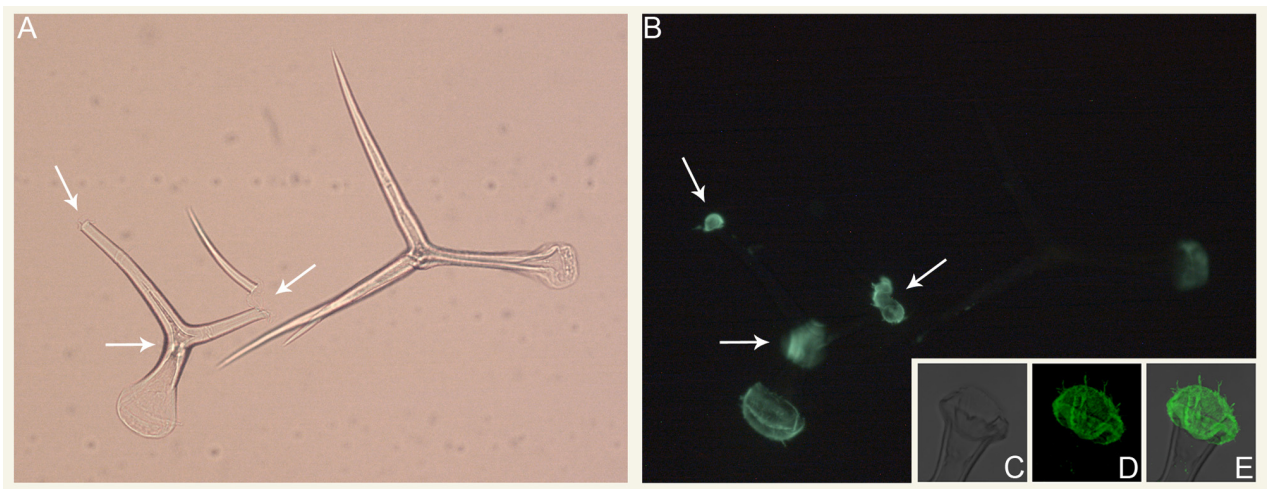


FIGURE 1 | Immunolocalization of mannans in isolated *Arabidopsis* trichomes. (A) Bright field and **(B)** epifluorescence micrographs of intact and broken trichomes isolated using the procedure of Marks et al. (2008). Arrows denote the locations of trichome branch breakpoints. **(C–E)** Confocal average projections of the base region of an isolated *Arabidopsis* trichome. For **(B,D,E)**, the LM21 and LM22 antibodies (Marcus et al., 2010) were used for mannan immunolocalization.

Table 2 | Analyses of *csla* mutants.

Mutant name	Gene identifier	Mutant phenotype	Reference
atcsla1	At4g16590	No apparent mutant phenotype	Goubet et al. (2009)
atcsla2	At5g22740	No apparent mutant phenotype	Goubet et al. (2009)
atcsla2	At5g22740	Reduction of phase II xylem in secondary thickened hypocotyl tissue	Ubeda-Tomas et al. (2007)
atcsla3	At1g23480	No apparent mutant phenotype	Goubet et al. (2009)
atcsla7	At2g35650	Defective embryogenesis, impaired pollen tube growth	Goubet et al. (2003)
rat4 (atcsla9)	At5g03760	Reduction of lateral root formation and rate of growth, decreased efficiency of root-mediated transformation by *Agrobacterium tumefaciens*	Zhu et al. (2003)
atcsla9	At5g03760	~81% reduction of mannose quantity in inflorescence stems	Goubet et al. (2009)
atcsla10	At1g24070	No apparent mutant phenotype	Goubet et al. (2009)
atcsla11	At5g16190	No apparent mutant phenotype	Goubet et al. (2009)
atcsla14	At3g56000	No apparent mutant phenotype	Goubet et al. (2009)
atcsla15	At4g13410	No apparent mutant phenotype	Goubet et al. (2009)
atcsla2/3	At5g22740/At1g23480	Slight reduction of mannan quantity in inflorescence stems	Goubet et al. (2009)
atcsla2/9	At5g22740/At5g03760	Reduction of mannose quantity to trace level in inflorescence stems	Goubet et al. (2009)
atcsla3/9	At1g23480/At5g03760	Reduction of mannose quantity to trace level in inflorescence stems	Goubet et al. (2009)
atcsla2/3/9	At5g22740/At1g23480/At5g03760	Reduction of mannose quantity below detection level in inflorescence stems	Goubet et al. (2009)

of a collection of single mutants revealed that mutations within the *CSLD2* and *CSLD3* genes result in abnormal root hair morphology. These observations are consistent with other studies that indicate that CSLD proteins are important in tip-growing cells (Doblin et al., 2001; Favery et al., 2001; Wang et al., 2001; Kim et al., 2007; Bernal et al., 2008; Galway et al., 2011; Park et al., 2011). The *csld2* and *csld3* single mutants, along with all possible double and triple mutant combinations of *csld2*, *csld3*, and *csld5* also exhibited abnormal mannan immunolocalization patterns in root hairs. Inflorescence stem development and mannan patterning therein of the *csld2*/*csld3*/*csld5* triple mutant also was disrupted. To complement these loss of function studies, the CSLD2, CSLD3, and CSLD5 proteins were transiently expressed in tobacco leaves. Elevated mannan synthase activity was observed in microsomal membrane fractions prepared from leaf tissue of tobacco plants expressing either CSLD5 or both CSLD2 and CSLD3, but not in lines expressing CSLD2 or CSLD3 individually (Yin et al., 2011).

The CSLD proteins long have been suspected to be involved in the synthesis of a β-glucan polymer (Doblin et al., 2001), so the implication of CSLD proteins in mannan synthesis comes as a surprise for several reasons. First, among the CSL proteins, sequences of the CSLD proteins are the most similar to the CESA proteins, which synthesize cellulose (Youngs et al., 2007). Furthermore, genetic complementation of the *csld3* mutant phenotype has been achieved using a chimaeric protein consisting of the CSLD3 protein containing the CESA6 catalytic domain (Park et al., 2011). Additionally, the *csla2*/*csla3*/*csla9* triple mutant lacks detectable glucomannan in the inflorescence stem (Goubet et al., 2009), indicating that the quantity of mannan synthesized by CSLD proteins must be small. It also is not clear why the patterns of mannans synthesized by CSLA proteins are aberrant in the *csld2*/*csld3*/*csld5* triple mutant. In light of the lack of consensus about the function(s) of CSLD proteins, additional studies are needed to clarify their functions.

DO THE CSLCs ENCODE XYLOGLUCAN GLUCAN SYNTHASES?

Like the *CSLA*s, *CSLC* sequences have been found in many extant species of Viridiplantae, spanning several divisions, including Magnoliophyta, Lycopodiophyta, Bryophyta, and Charophyta (Del Bem and Vincentz, 2010). Within angiosperms, *CSLCs* have been found in all species surveyed thus far (Yin et al., 2009; Del Bem and Vincentz, 2010), including three species that have been used as models to investigate CSLC function: nasturtium (*TmCSLC*), *Arabidopsis* (*AtCSLC4*, *AtCSLC5*, *AtCSLC6*, *AtCSLC8*, *AtCSLC12*) and barley (*HvCSLC1*, *HvCSLC2*, *HvCSLC3*, *HvCSLC4*, and possibly *HvCSLC5*).

The first evidence that xyloglucan glucan synthase (XGS) might be encoded by a member of the CSLC family was provided by Cocuron et al. (2007) using a comparative genomics approach. Using nasturtium (*Tropaeolum majus*) seeds, which utilize xyloglucan as the primary seed storage polysaccharide (Gidley et al., 1991), transcriptional profiling was used to identify genes preferentially expressed during the stage of seed development when xyloglucan deposition occurs. The only *CSL* gene transcripts detected by this analysis were those of the *TmCSLC* gene, a homolog of *Arabidopsis CSLC4* (*AtCSLC4*). Transgenic *Pichia pastoris* cells expressing the TmCSLC or AtCSLC4 protein produced soluble 1,4-β-glucans with a low degree of polymerization

(DP4–DP6), indicating that these two proteins have glucan synthase activity (**Table 1**). While efforts to coexpress, in *P. pastoris,* the *Arabidopsis* CSLC4 and a xyloglucan xylosyltransferase (AtXXT1) protein did not result in the synthesis of xyloglucan (likely due, at least in part, to the absence of UDP-xylose metabolism in *P. pastoris*; De Schutter et al., 2009), several additional lines of evidence support the hypothesis that AtCSLC4 is an XGS. *P. pastoris* cultures expressing AtCSLC4 and AtXXT1 together produced insoluble 1,4-β-glucans with a higher degree of polymerization than glucans synthesized by expression of AtCSLC4 alone, possibly indicating that the AtCSLC4 and AtXXT1 proteins act in a cooperative manner to produce xyloglucan. Additionally, in *Arabidopsis* there is a strong correlation between the expression patterns of the *AtCSLC4* and *AtXXT1* genes, suggesting that these two proteins participate in related processes. Finally, AtCSLC4 is a Golgi-localized protein, and xyloglucan synthesis is known to take place within this organelle (Ray et al., 1969; Ray, 1980; White et al., 1993). Although Cocuron et al. (2007) show that CSLC4 has glucan synthase activity and present a reasonable argument that CSLC4 is an XGS, it is unknown if other CSLC members have glucan synthase activity and whether mutation of one or more *CSLC* genes would affect xyloglucan content or structure.

In contrast to using an experimental system in which a significant portion of the hemicellulose present is xyloglucan, Dwivany et al. (2009) studied CSLCs of barley (*Hordeum vulgare* L.), where xyloglucan is a minor cell wall component (Sakurai and Masuda, 1978; Kato et al., 1981; Fincher, 1993). The authors identified and characterized four barley *CSLCs*, *HvCSLC1–4* (a fifth, HvCSLC5 also was identified but not characterized). Phylogenetic analysis of CSLC family members from several eudicots and monocots, *Physcomitrella patens*, *Selaginella moellendorffii*, and *Chara globularis* indicates that the CSLC family contains four clades: HvCSLC1, 2, 4, and AtCSLC12 belonging to clade 1, AtCSLC4, 5, 8, and HvCSLC3 belonging to clade 2, *Physcomitrella* and *Selaginella* CSLCs comprise clade 3, and AtCSLC6 belongs to clade 4. In addition to taxonomic relationships having an effect on tree structure (i.e., clade 3), the authors hypothesize that when considered in conjunction with the biochemical and molecular evidence (discussed below), the structure of the phylogenetic tree may show functional specialization, with members of clades 2 and 4 having GS and XGS activities, respectively. Based on results of transcriptional profiling of barley organs and tissues, coexpression analysis of HvCSLCs and putative barley xyloglucan xylosyltransferases (*HvGT1*–5), and its high sequence identity and similarity to AtCSLC4, Dwivany et al. (2009) concluded that HvCSLC3 is probably involved in xyloglucan biosynthesis. Alternatively, in barley suspension cultured cells, which the authors show have transient, low levels of xyloglucan and barely detectable *HvCLSC3* transcripts, the CSLC proteins immunolocalize to the plasma membrane in immuno-EM and membrane fractionation experiments. Based on results from these experiments and the molecular characterization at the gene and transcript levels, Dwivany et al. (2009) conclude that there is insufficient evidence to assign functions to HvCSLC1 and HvCSLC4. However, the authors propose that HvCSLC2 likely is not involved in xyloglucan biosynthesis, and instead suggest that it may be involved in cellulose biosynthesis. Although the conclusions reached by Dwivany et al. (2009) are plausible, additional evidence from heterologous expression and mutant genetic studies would strengthen their arguments.

Key to identifying the function(s) of the CSLC family members is to determine how polysaccharide content and structure is affected in plants with mutant *CSLC* genes. To this end, there is an abundance of *Arabidopsis* T-DNA insertion lines available from several sources, and mutants for all members of the CSLC family are present within these mutant collections. Furthermore, reverse genetics resources currently are being developed in the model grass species *Brachypodium distachyon* (Thole et al., 2010, 2012). Because of the likelihood that genetic redundancy exists among the five members of the *Arabidopsis CSLC* family, it probably will be necessary to generate mutant lines harboring multiple mutant *CSLC* genes to determine whether members of the *Arabidopsis* CSLC family are involved in xyloglucan biosynthesis. While it is possible that mutants with severe reductions of xyloglucan content could prove lethal, the existence of the *xxt1/xxt2* mutant, which lacks detectable xyloglucan and grows normally under laboratory conditions (Cavalier et al., 2008), shows that it is possible to develop viable xyloglucan mutants. Therefore, if members of the CSLC family are involved in xyloglucan biosynthesis it should be possible to develop *Arabidopsis* lines harboring multiple mutant *CSLC* genes.

In addition to genetic redundancy and lethality, determining the effects upon polysaccharide content in *cslc* mutants could pose a significant challenge if members of the CSLC family are involved in cellulose biosynthesis. One potential difficulty would be the inability to distinguish between cellulose synthesized by CSLCs versus CESAs. Another would be determining if changes in amorphous or crystalline cellulose are due directly to the mutation or are the result of a secondary response to the mutation. Keeping these challenge in mind, detailed studies of such mutants, coupled with biochemical studies of recombinant CSLC proteins ultimately are expected to provide the evidence needed to conclusively define the function(s) of many CSLC family members.

CONCLUDING REMARKS

Within the last decade, our understanding of the functions of CSLA and CSLC proteins has markedly improved. However, many important questions remain relating to the evolution and functions of these related sequences: What is the architecture of the transcriptional network controlling expression of these genes? What proteins are present in the carbohydrate synthesizing enzyme complexes likely to contain these proteins, and how are these complexes regulated? What factors influence the processes of polysaccharide synthesis (initiation, elongation, and termination)? What are the physiological roles and factors influencing the structures of polysaccharides synthesized by CSLA and CSLC proteins? A body of research shows that the CSLA and CSLC families can be successfully studied using heterologous expression and forward and reverse genetics. By leveraging these powerful tools it should be possible to gain significant insights into the specific functions of the CSLA

and CSLC proteins and the synthesis of plant polysaccharides in general.

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REFERENCES

Auxtova-Samajova, O., Liskova, D., Kakoniova, D., Kubackova, M., Karacsonyi, S., and Bilisics, L. (1996). Inhibition of auxin stimulated short-term elongation growth of pea steam segments by galactoglucomannan-derived oligosaccharides. *J. Plant Physiol.* 147, 611–613.

Benova-Kakosova, A., Digonnet, C., Goubet, F., Ranocha, P., Jauneau, A., Pesquet, E., Barbier, O., Zhang, Z., Capek, P., Dupree, P., Liskova, D., and Goffner, D. (2006). Galactoglucomannans increase cell population density and alter the protoxylem/metaxylem tracheary element ratio in xylogenic cultures of zinnia. *Plant Physiol.* 142, 696–709.

Bernal, A. J., Yoo, C. M., Mutwil, M., Jensen, J. K., Hou, G., Blaukopf, C., Sorensen, I., Blancaflor, E. B., Scheller, H. V., and Willats, W. G. (2008). Functional analysis of the *cellulose synthase-like* genes *CSLD1*, *CSLD2*, and *CSLD4* in tip-growing *Arabidopsis* cells. *Plant Physiol.* 148, 1238–1253.

Buckeridge, M. S. (2010). Seed cell wall storage polysaccharides: models to understand cell wall biosynthesis and degradation. *Plant Physiol.* 154, 1017–1023.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell.* 20, 1519–1537.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233–D238.

Carpita, N. C. (2011). Progress in the biological synthesis of the plant cell wall: new ideas for improving biomass for bioenergy. *Curr. Opin. Biotechnol.* doi: 10.1016/j.copbio.2011.12.003 [Epub ahead of print].

Cocuron, J. C., Lerouxel, O., Drakakaki, G., Alonso, A. P., Liepman, A. H., Keegstra, K., Raikhel, N., and Wilkerson, C. G. (2007). A gene from the cellulose synthase-like C family encodes a β-1,4 glucan synthase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8550–8555.

Davis, J., Brandizzi, F., Liepman, A. H., and Keegstra, K. (2010). *Arabidopsis* mannan synthase CSLA9 and glucan synthase CSLC4 have opposite orientations in the Golgi membrane. *Plant J.* 64, 1028–1037.

Del Bem, L. E., and Vincentz, M. G. (2010). Evolution of xyloglucan-related genes in green plants. *BMC Evol. Biol.* 10, 341. doi: 10.1186/1471-2148-10-341

De Schutter, K., Lin, Y.-C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouze, P., Van de Peer, Y., and Callewaert, N. (2009). Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nat. Biotech.* 27, 561.

Dhugga, K. S. (2011). Biosynthesis of non-cellulosic polysaccharides of plant cell walls. *Phytochemistry* 74, 8–19.

Dhugga, K. S., Barreiro, R., Whitten, B., Stecca, K., Hazebroek, J., Randhawa, G. S., Dolan, M., Kinney, A. J., Tomes, D., Nichols, S., and Anderson, P. (2004). Guar seed β-mannan synthase is a member of the cellulose synthase super gene family. *Science* 303, 363–366.

Doblin, M., Pettolino, F., and Bacic, A. (2010). Plant cell walls: the skeleton of the plant world. *Funct. Plant Biol.* 37, 357–381.

Doblin, M. S., De Melis, L., Newbigin, E., Bacic, A., and Read, S. M. (2001). Pollen tubes of *Nicotiana alata* express two genes from different beta-glucan synthase families. *Plant Physiol.* 125, 2040–2052.

Dwivany, F. M., Yulia, D., Burton, R. A., Shirley, N. J., Wilson, S. M., Fincher, G. B., Bacic, A., Newbigin, E., and Doblin, M. S. (2009). The CELLULOSE-SYNTHASE LIKE C (CSLC) family of barley includes members that are integral membrane proteins targeted to the plasma membrane. *Mol. Plant* 2, 1025–1039.

Endler, A., and Persson, S. (2011). Cellulose synthases and synthesis in *Arabidopsis*. *Mol. Plant* 4, 199–211.

Farrokhi, N., Burton, R. A., Brownfield, L., Hrmova, M., Wilson, S. M., Bacic, A., and Fincher, G. B. (2006). Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes. *Plant Biotechnol. J.* 4, 145–167.

Favery, B., Ryan, E., Foreman, J., Linstead, P., Boudonck, K., Steer, M., Shaw, P., and Dolan, L. (2001). *KOJAK* encodes a cellulose synthase-like protein required for root hair cell morphogenesis in *Arabidopsis*. *Genes Dev.* 15, 79–89.

Fincher, G. B. (1993). "Cell wall metabolism in barley," in *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology*, ed. P. R. Shewry (Wallingford: CAB International), 413–437.

Fincher, G. B. (2009). Revolutionary times in our understanding of cell wall biosynthesis and remodeling in the grasses. *Plant Physiol.* 149, 27–37.

Galway, M. E., Eng, R. C., Schiefelbein, J. W., and Wasteneys, G. O. (2011). Root hair-specific disruption of cellulose and xyloglucan in AtCSLD3 mutants, and factors affecting the post-rupture resumption of mutant root hair growth. *Planta* 233, 985–999.

Gidley, M. J., Lillford, P. J., Rowlands, D. W., Lang, P., Dentini, M., Crescenzi, V., Edwards, M., Fanutti, C., and Reid, J. S. (1991). Structure and solution properties of tamarind-seed polysaccharide. *Carbohydr. Res.* 214, 299–314.

Gille, S., Cheng, K., Skinner, M. E., Liepman, A. H., Wilkerson, C. G., and Pauly, M. (2011). Deep sequencing of voodoo lily (*Amorphophallus konjac*): an approach to identify relevant genes involved in the synthesis of the hemicellulose glucomannan. *Planta* 234, 515–526.

Girke, T., Lauricha, J., Tran, H., Keegstra, K., and Raikhel, N. (2004). The Cell Wall Navigator database. A systems-based approach to organism-unrestricted mining of protein families involved in cell wall metabolism. *Plant Physiol.* 136, 3003–3008.

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., and Rokhsar, D. S. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40, D1178–D1186.

Goubet, F., Barton, C. J., Mortimer, J. C., Yu, X., Zhang, Z., Miles, G. P., Richens, J., Liepman, A. H., Seffen, K., and Dupree, P. (2009). Cell wall glucomannan in *Arabidopsis* is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. *Plant J.* 60, 527–538.

Goubet, F., Misrahi, A., Park, S. K., Zhang, Z., Twell, D., and Dupree, P. (2003). AtCslA7, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in *Arabidopsis*. *Plant Physiol.* 131, 547–557.

Hamann, T., Osborne, E., Youngs, H., Misson, J., Nussaume, L., and Somerville, C. (2004). Global expression analysis of *CESA* and *CSL* genes in *Arabidopsis*. *Cellulose* 11, 279–286.

Handford, M. G., Baldwin, T. C., Goubet, F., Prime, T. A., Miles, J., Yu, X., and Dupree, P. (2003). Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. *Planta* 218, 27–36.

Hazen, S. P., Scott-Craig, J. S., and Walton, J. D. (2002). *Cellulose synthase-like* (*CSL*) genes of rice. *Plant Physiol.* 128, 336–340.

Kato, Y., Iki, K., and Matsuda, K. (1981). Cell-wall polysaccharides of immature barley plants. II. Characterization of a xyloglucan. *Agric. Biol. Chem.* 45, 2745–2753.

Keegstra, K., and Raikhel, N. V. (2001). Plant Glycosyltransferases. *Curr. Opin. Plant Biol.* 4, 219–224.

Kim, C. M., Park, S. H., Je, B. I., Park, S. H., Park, S. J., Piao, H. L., Eun, M. Y., Dolan, L., and Han, C. D. (2007). *OsCSLD1*, a *Cellulose Synthase-Like D1* gene, is required for root hair morphogenesis in rice (*Oryza sativa* L.). *Plant Physiol.* 143, 1220–1230.

Lee, K. J., Marcus, S. E., and Knox, J. P. (2011). Cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219.

Leroux, O., Leroux, F., Bagniewska-Zadworna, A., Knox, J. P., Claeys, M., Bals, S., and Viane, R. L. (2011). Ultrastructure and composition of cell wall appositions in the roots of *Asplenium* (Polypodiales). *Micron* 42, 863–870.

Lerouxel, O., Cavalier, D. M., Liepman, A. H., and Keegstra, K. (2006). Biosynthesis of plant cell wall polysaccharides – a complex process. *Curr. Opin. Plant Biol.* 9, 621–630.

Liepman, A. H., Nairn, C. J., Willats, W. G., Sorensen, I., Roberts, A. W., and Keegstra, K. (2007). Functional genomic analysis supports conservation of function among *CELLULOSE SYNTHASE-LIKE A* gene family members and suggests diverse roles of mannans in plants. *Plant Physiol.* 143, 1881–1893.

Liepman, A. H., Wightman, R., Geshi, N., Turner, S. R., and Scheller, H. V. (2010). *Arabidopsis* – a powerful model system for plant cell wall research. *Plant J.* 61, 1107–1121.

Liepman, A. H., Wilkerson, C. G., and Keegstra, K. (2005). Expression of cellulose synthase-like (*Csl*) genes in insect cells reveals that *CslA* family members encode mannan synthases. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2221–2226.

Maeda, Y., Awano, T., Takabe, K., and Fujita, M. (2000). Immunolocalization of glucomannans in the cell wall of differentiating tracheids in *Chamaecyparis obtusa. Protoplasma* 213, 148–156.

Marcus, S. E., Blake, A. W., Benians, T. A., Lee, K. J., Poyser, C., Donaldson, L., Leroux, O., Rogowski, A., Petersen, H. L., Boraston, A., Gilbert, H. J., Willats, W. G., and Knox, J. P. (2010). Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *Plant J.* 64, 191–203.

Marks, M. D., Betancur, L., Gilding, E., Chen, F., Bauer, S., Wenger, J. P., Dixon, R. A., and Haigler, C. H. (2008). A new method for isolating large quantities of *Arabidopsis* trichomes for transcriptome, cell wall and other types of analyses. *Plant J.* 56, 483–492.

McCann, M. C., and Carpita, N. C. (2008). Designing the deconstruction of plant cell walls. *Curr. Opin. Plant Biol.* 11, 314–320.

Meier, H., and Reid, J. S. G. (1982). "Reserve polysaccharides other than starch in higher plants," in *Encyclopedia of Plant Physiology*, eds. F. A. Loewus, and W. Tanner (Berlin: Springer), 418–471.

Moreira, L. R., and Filho, E. X. (2008). An overview of mannan structure and mannan-degrading enzyme systems. *Appl. Microbiol. Biotechnol.* 79, 165–178.

Ordaz-Ortiz, J. J., Marcus, S. E., and Knox, J. P. (2009). Cell wall microstructure analysis implicates hemicellulose polysaccharides in cell adhesion in tomato fruit pericarp parenchyma. *Mol. Plant* 2, 910–921.

Park, S., Szumlanski, A. L., Gu, F., Guo, F., and Nielsen, E. (2011). A role for CSLD3 during cell-wall synthesis in apical plasma membranes of tip-growing root-hair cells. *Nat. Cell Biol.* 13, 973–980.

Pauly, M., and Keegstra, K. (2010). Plant cell wall polymers as precursors for biofuels. *Curr. Opin. Plant Biol.* 13, 305–312.

Penning, B. W., Hunter, C. T. III., Tayengwa, R., Eveland, A. L., Dugard, C. K., Olek, A. T., Vermerris, W., Koch, K. E., McCarty, D. R., Davis, M. F., Thomas, S. R., McCann, M. C., and Carpita, N. C. (2009). Genetic resources for maize cell wall biology. *Plant Physiol.* 151, 1703–1728.

Popper, Z. A., Michel, G., Herve, C., Domozych, D. S., Willats, W. G., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant Biol.* 62, 567–590.

Ray, P. M. (1980). Cooperative action of β-glucan synthetase and UDP-xylose xylosyl transferase of Golgi membranes in the synthesis of xyloglucan-like polysaccharide. *Biochim. Biophys. Acta* 629, 431–444.

Ray, P. M., Shininger, T. L., and Ray, M. M. (1969). Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes. *Proc. Natl. Acad. Sci. U.S.A.* 64, 605–612.

Richmond, T., and Somerville, C. R. (2001). Integrative approaches to determining Csl function. *Plant Mol. Biol.* 47, 131–143.

Richmond, T. A., and Somerville, C. R. (2000). The cellulose synthase superfamily. *Plant Physiol.* 124, 495–498.

Roberts, A. W., and Bushoven, J. T. (2007). The *cellulose synthase* (*CESA*) gene superfamily of the moss *Physcomitrella patens. Plant Mol. Biol.* 63, 207–219.

Sakurai, N., and Masuda, Y. (1978). Auxin-induced changes in barley coleoptile cell wall composition. *Plant Cell Physiol.* 19, 1217–1223.

Sandhu, A. P., Randhawa, G. S., and Dhugga, K. S. (2009). Plant cell wall matrix polysaccharide biosynthesis. *Mol. Plant* 2, 840–850.

Scheible, W.-R., and Pauly, M. (2004). Glycosyltransferases and cell wall biosynthesis: novel players and insights. *Curr. Opin. Plant Biol.* 7, 285–295.

Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289.

Schroder, R., Atkinson, R. G., and Redgwell, R. J. (2009). Re-interpreting the role of endo-beta-mannanases as mannan endotransglycosylase/hydrolases in the plant cell wall. *Ann. Bot.* 104, 197–204.

Schroder, R., Wegrzyn, T. F., Sharma, N. N., and Atkinson, R. G. (2006). LeMAN4 endo-β-mannanase from ripe tomato fruit can act as a mannan transglycosylase or hydrolase. *Planta* 224, 1091–1102.

Silva, G. B., Ionashiro, M., Carrara, T. B., Crivellari, A. C., Tine, M. A., Prado, J., Carpita, N. C., and Buckeridge, M. S. (2011). Cell wall polysaccharides from fern leaves: evidence for a mannan-rich Type III cell wall in *Adiantum raddianum. Phytochemistry* 72, 2352–2360.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Perrson, S., Raab, T., Vorwerk, S., and Youngs, H. (2004). Toward a systems approach to understanding plant cell walls. *Science* 306, 2206–2211.

Suzuki, S., Li, L., Sun, Y. H., and Chiang, V. L. (2006). The cellulose synthase gene superfamily and biochemical functions of xylem-specific cellulose synthase-like genes in *Populus trichocarpa. Plant Physiol.* 142, 1233–1245.

Thole, V., Peraldi, A., Worland, B., Nicholson, P., Doonan, J. H., and Vain, P. (2012). T-DNA mutagenesis in *Brachypodium distachyon. J. Exp. Bot.* 63, 567–576.

Thole, V., Worland, B., Wright, J., Bevan, M. W., and Vain, P. (2010). Distribution and characterization of more than 1000 T-DNA tags in the genome of *Brachypodium distachyon* community standard line Bd21. *Plant Biotechnol. J.* 8, 734–747.

Ubeda-Tomas, S., Edvardsson, E., Eland, C., Singh, S. K., Zadik, D., Aspeborg, H., Gorzsas, A., Teeri, T. T., Sundberg, B., Persson, P., Bennett, M., and Marchant, A. (2007). Genomic-assisted identification of genes involved in secondary growth in *Arabidopsis* utilising transcript profiling of poplar wood-forming tissues. *Physiol. Plant.* 129, 415–428.

Verhertbruggen, Y., Yin, L., Oikawa, A., and Scheller, H. V. (2011). Mannan synthase activity in the CSLD family. *Plant Signal. Behav.* 6, 1620–1623.

Wang, X., Cnops, G., Vanderhaeghen, R., De Block, S., Van Montagu, M., and Van Lijsebettens, M. (2001). *AtCSLD3*, a cellulose synthase-like gene important for root hair growth in *Arabidopsis. Plant Physiol.* 126, 575–586.

White, A. R., Xin, Y., and Pezeshk, V. (1993). Xyloglucan glucosyltransferase in Golgi membranes from *Pisum sativum* (pea). *Biochem. J.* 294, 231–238.

Yin, L., Verhertbruggen, Y., Oikawa, A., Manisseri, C., Knierim, B., Prak, L., Jensen, J. K., Knox, J. P., Auer, M., Willats, W. G., and Scheller, H. V. (2011). The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal *Arabidopsis* development. *Mol. Plant* 4, 1024–1037.

Yin, Y., Huang, J., and Xu, Y. (2009). The cellulose synthase superfamily in fully sequenced plants and algae. *BMC Plant Biol.* 9, 99. doi: 10.1186/1471-2229-9-99

Youngs, H. L., Hamann, T., Osborne, E., and Somerville, C. R. (2007). "The cellulose synthase superfamily," in *Cellulose: Molecular and Structural Biology*, eds M. Brown and I. M. Saxena (Berlin: Springer), 35–49.

Zablackis, E., Huang, J., Darvill, A. G., and Albersheim, P. (1995). Characterization of the cell wall polysaccharides of *Arabidopsis thaliana* leaves. *Plant Physiol.* 107, 1129–1138.

Zhu, Y., Nam, J., Carpita, N., Matthysse, A. G., and Gelvin, S. B. (2003). *Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene. *Plant Physiol.* 133, 1000–1010.

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Cell wall mechanics and growth control in plants: the role of pectins revisited

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How is the extensibility of growing plant cell walls regulated? In the past, most studies have focused on the role of the cellulose/xyloglucan network and the enigmatic wall-loosening agents expansins. Here we review first how in the closest relatives of the land plants, the Charophycean algae, cell wall synthesis is coupled to cell wall extensibility by a chemical Ca^{2+}-exchange mechanism between Ca^{2+}–pectate complexes. We next discuss evidence for the existence in terrestrial plants of a similar "primitive" Ca^{2+}–pectate-based growth control mechanism in parallel to the more recent, land plant-specific, expansin-dependent process.

Keywords: pectin, pectin methylesterase, wall extensibility, *Chara corallina*

Plant cell growth reflects the balance between the extensibility of the cell wall and the forces exerted on the wall by the turgor pressure. Although growth in principle can be controlled by changing either parameter, in most documented cases growth changes reflect changes in cell wall extensibility (Cosgrove, 2005). The paradoxical properties of cell walls, which combine extensibility with extreme strength, can be easily understood by keeping in mind that wall strength is determined by the number and strength of load-bearing bonds and by assuming that a wall relaxation mechanism exists that can break these bonds and transfer the load to new bonds (**Figure 1**). Wall relaxation and cell expansion can occur, in principle, with or without a change in wall strength, depending on whether or not this chemorheological process causes a net change in the number or strength of the load-bearing bonds.

THE NATURE OF THE LOAD-BEARING BONDS IN PRIMARY CELL WALLS

Typical primary cell walls (i.e., the walls of growing cells) consist of strong cellulose microfibrils, hemicelluloses [primarily xyloglucans (XGs) in non-Commelinoid species], pectins, and structural proteins. The current "textbook view" of the control of cell wall extensibility (Carpita and McCann, 2000) is largely based on a series of seminal extensometer experiments, which showed that acid-promoted wall extension under a constant tension, also referred to as creep, can be reproduced on isolated walls. These studies led to the purification of a creep-promoting agent, called expansin (McQueen-Mason et al., 1992). Expansins are encoded by large gene families, the expression of whose members is frequently correlated with growth. All attempts so far have failed to detect enzymatic activity for expansins. Interestingly, the proteins can also promote creep of paper, essentially pure cellulose, without detectable cellulose hydrolysis. From this it was concluded that expansins have a "lubricating" activity perhaps by severing hydrogen bonds between glucan chains (cellulose and/or XG; Cosgrove, 2000). XG chains strongly bind to microfibrils and in principle are long enough to bridge microfibrils in the wall. Cross-links have been observed in certain cell wall preparations (McCann et al., 1993). This led to the so called "tethered network" model in which the main load-bearing network consists of cellulose–XGs, the extensibility of which is controlled by expansin and perhaps wall-bound XG endotransglycosylase (XET) activity (Fry et al., 1992). The latter can stitch XG fragments together and, if a transfer from load-bearing to relaxed XG chains occurs, this process also has the potential to promote creep.

Within this view, pectins form a matrix around the cellulose–XG network but are thought not to have a major load-bearing role (Cosgrove, 1999). Pectins are block co-polymers; homogalacturonan (HG), a main pectic polymer, consists of a-1,4-linked galacturonic acids. HG is secreted in a highly methylesterified form and selectively de-methylesterified by pectin methylesterases (PME; Pelloux et al., 2007). After de-methylesterification, pectate can form Ca^{2+}–pectate cross-linked complexes, referred to as "eggboxes" (**Figure 2B**; Grant et al., 1973). These cross-links, together with rhamnogalacturonan II (RGII)-boron diester bonds, are thought to indirectly affect the cellulose–XG network by influencing the wall porosity and hence the accessibility of primary wall relaxation proteins to their substrate (Cosgrove, 1999). The more Ca^{2+}–pectate present, the denser the gel and the more inextensible the cell wall is expected to be.

Recent observations necessitate a reconsideration of the "tethered network" model. Firstly, 3D-solid state nuclear magnetic resonance (NMR) studies suggest that at best only a small proportion of the XG is bound to cellulose (Dick-Perez et al., 2011).

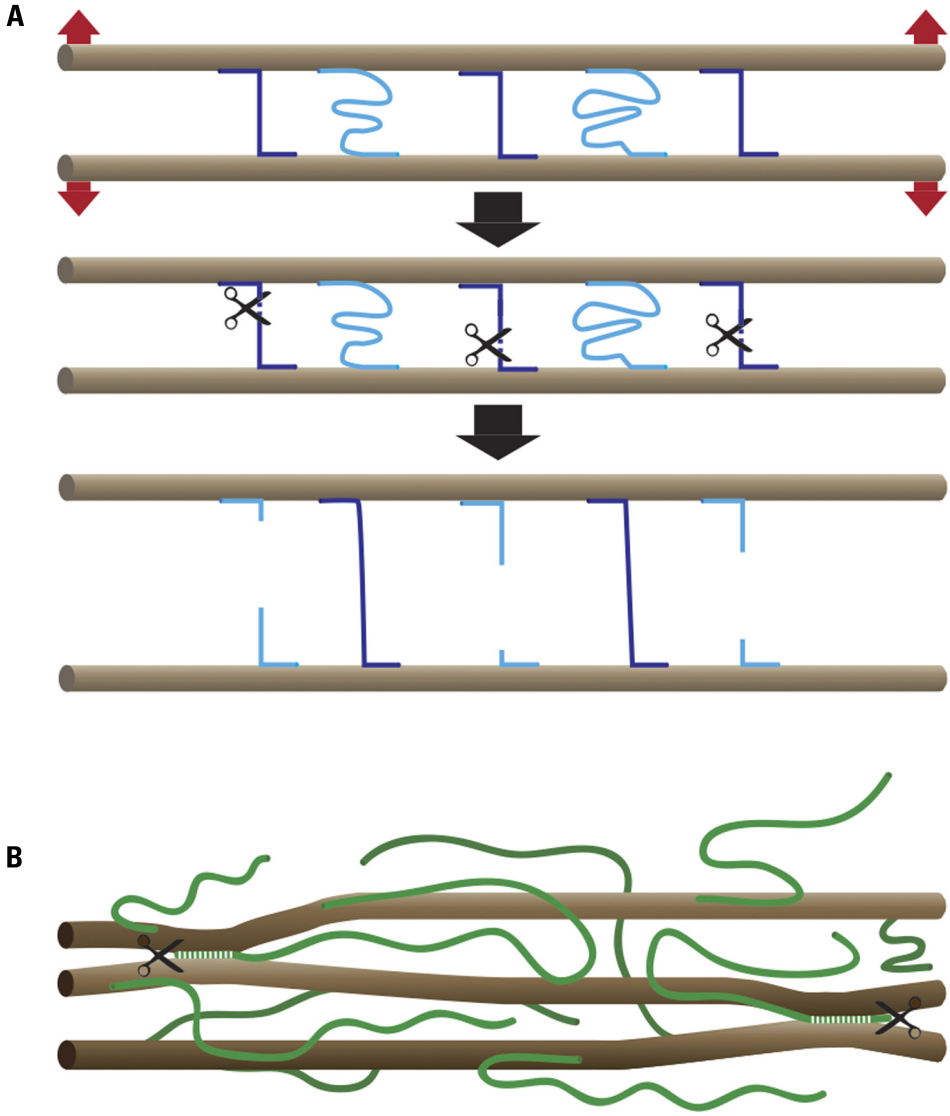


FIGURE 1 | Chemorheological control of wall extensibility. (A) Principle: microfibrils (brown), which in most cases form parallel arrays, are cross-linked by load-bearing (violet) and relaxed (light blue) bonds. The number and strength of the load-bearing bonds determines cell wall strength. Wall extensibility is controlled by chemorheological mechanisms that remove load-bearing bonds. The cell wall relaxes and undergoes turgor-driven mechanical deformation until previously relaxed bonds become load-bearing. **(B)** Cartoon of cell wall architecture showing microfibrils (brown) and XG chains (green). A small portion of the XG is intertwined or complexed with cellulose, thus sticking the microfibrils together at these points. The endoglucanase Cel12A as well as expansin may act on these relatively inaccessible XG–cellulose interaction domains.

Secondly, extensometer assays on isolated cucumber cell walls show that creep is promoted by a specific class of dual-specificity β-1,4-endo-glucanases (EGases) and not by XG- or cellulose-specific EGases (Park and Cosgrove, 2012). Based on these studies, the authors propose a model for the cell wall architecture (**Figure 1B**), in which minor and relatively inaccessible XG fractions are intertwined or complexed with cellulose and thus form a small number of load-bearing connections between microfibrils. The authors propose that these connections also may be the target not only of the dual-specificity EGases but also of expansins. Why would there be so few cellulose–XG interaction sites, given the high affinity of XG for cellulose (Hanus and Mazeau, 2006)? An interesting possibility is that pectin competes with XG for interaction with nascent cellulose chains, perhaps through the binding of the arabinan and galactan side chains to the cellulose (Zykwinska et al., 2005). In this case the secreted pectin/XG ratio as well as the pectin structure (the density and length of side chains) would determine the extent of the cellulose–XG interactions. Thirdly, a double mutant (*xylosyltransferase1/2* or *xt1xt2*) without detectable XG (Cavalier et al., 2008) shows a surprisingly subtle phenotype: mutant plants are slightly smaller than the wild type, but otherwise nearly normal in their development. Stress–strain assays on cell walls from petioles showed a 53% higher elastic compliance and a 164% higher plastic compliance for XG-less walls as compared to

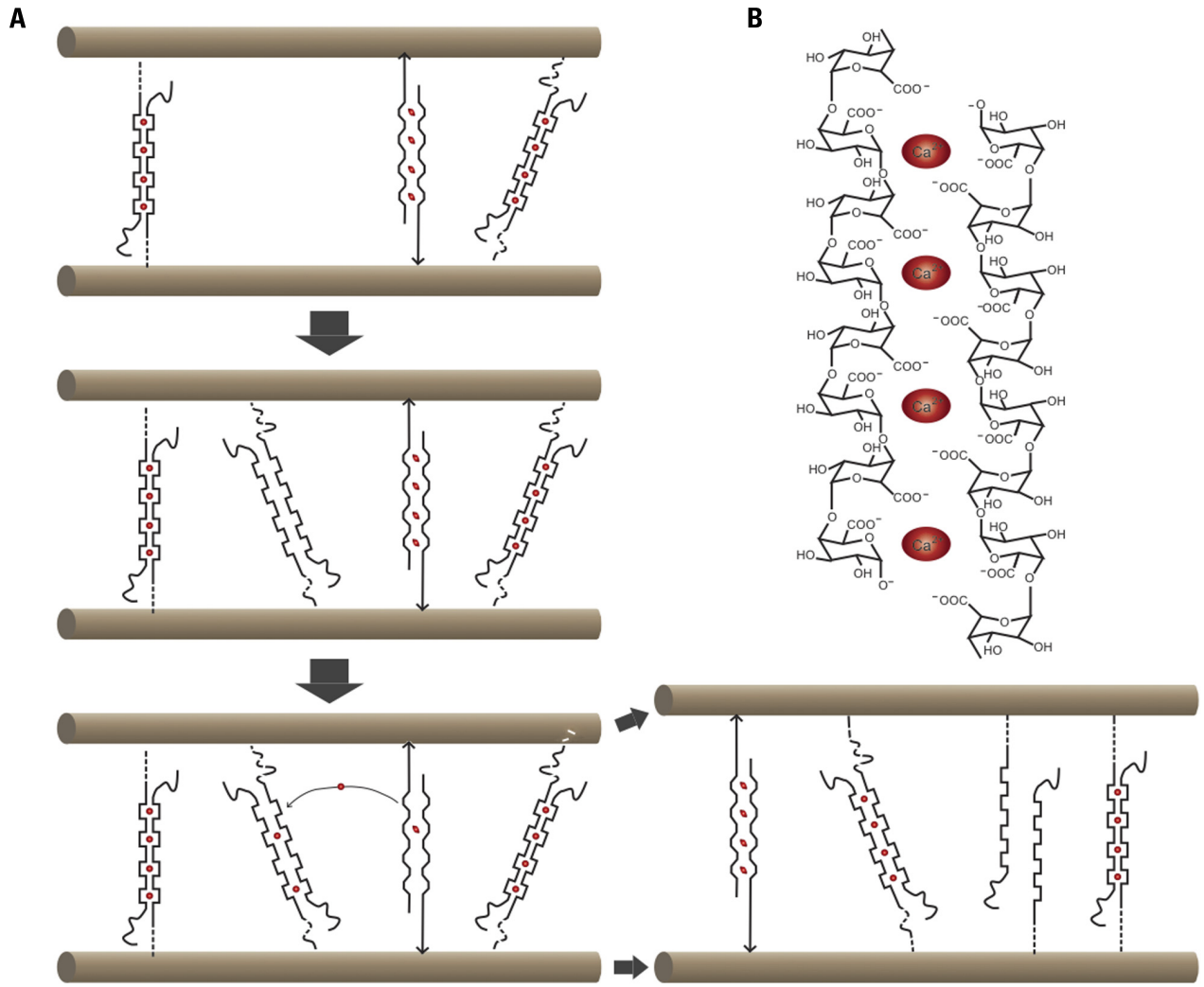


FIGURE 2 | Cartoon summarizing Ca^{2+}–pectate-dependent growth control in the alga *Chara corallina*. (A) In this species, cellulose microfibrils are parallel and oriented transversely to the elongation axis. Ca^{2+} (red ovals)-pectate complexes are load-bearing. One stretched and two relaxed Ca^{2+}–pectate complexes are shown. Newly deposited pectate will chelate Ca^{2+} preferentially out of the stretched complexes, thus leading to wall relaxation and turgor-driven mechanical deformation until other complexes become load-bearing. **(B)** Cartoon of an "eggbox" consisting of two antiparallel poly-galacturonic acid chains complexed by Ca^{2+}.

wild type walls, confirming that XGs have a wall-strengthening role (Park and Cosgrove, 2011). In contrast, *xt1xt2* cell walls showed strongly reduced acid-promoted/expansin-dependent creep, consistent with a reduced effectiveness of expansin in the absence of XG. Surprisingly, treatments that specifically act on pectate or xylans promoted creep more strongly in the mutant than in the wild type, showing that Ca^{2+}–pectate and, to a lesser extent, xylans are load-bearing in the mutant (Park and Cosgrove, 2011). These findings are reminiscent of the results on tomato cell cultures continually exposed to the cellulose inhibitor dichlorobenil (DCB); the walls of DCB habituated cells strikingly lacked cellulose and XG, and were held together by a strong pectate network, reinforced by phenolic ester and/or phenolic ether bonds (Shedletzky et al., 1990). In conclusion, in the absence of the cellulose–XG network, pectate cross-links play a major load-bearing role.

Another set of recent observations on the *Arabidopsis* shoot apical meristem underscore the importance of pectin metabolism in the control of wall extensibility also during normal development (Peaucelle et al., 2008, 2011). Microindentation using atomic force microscopy (AFM) showed that the appearance of organ primordia at the periphery of the meristem was preceded by an increased elastic compliance of the cell walls at that position. This could be attributed to the de-methylesterification of HG. Indeed, inhibition of PME activity by the ectopic expression of a PME inhibitor (PMEI) led to a global stiffening of the walls throughout the meristem and totally prevented the formation of primordia, whereas ectopic PME expression reduced the cell wall stiffness and caused the formation of ectopic primordia (Peaucelle et al., 2008).

This raises the following questions: (1) To what extent do pectin cross-links also have a load-bearing role in normal cell

walls? (2) How can we explain the association of HG de-methylesterification with a *decrease* in cell wall stiffness rather than the expected increase in stiffness? and more in general (3) What is the relevance of the changes in cell wall stiffness for the observed growth changes?

AN EVOLUTIONARY PERSPECTIVE ON GROWTH CONTROL

To provide an answer to these questions it is worthwhile to consider the growth mechanisms revealed in freshwater algae of the Charophyceae family, the closest relatives of land plants. *Chara corallina*, with its giant cells of up to several centimeters long, is ideally suited for the study of cell expansion. *Chara* cells can be easily impaled with a pipette, allowing the composition of the cytosol and the turgor pressure to be controlled. In addition, the cytoplasm can be removed entirely to study the expansion behavior of isolated walls (Proseus et al., 2000). The cell wall of *Chara* has a composition similar to that of higher plants with cellulose, pectin (primarily non-methyl-esterified HG), and minor amounts of XG (Sorensen et al., 2011). The results of a series of imaginative experiments on this species by the Boyer laboratory yielded an elegant model for wall expansion (**Figure 2**; for a recent review, see Boyer, 2009). Ca^{2+}–pectate cross-links are the main load-bearing bonds, and wall relaxation is coupled to pectate deposition through a simple non-enzymatic mechanism: Newly deposited pectate chelates Ca^{2+} away from existing Ca^{2+}–pectate, preferentially from the load-bearing bonds that are distorted by the wall tension. The loss of these Ca^{2+}–pectate bonds causes the cell wall to relax and irreversible wall extension occurs. The new Ca^{2+}–pectate then binds to the wall and the extension decelerates. New pectate is deposited and the cycle repeats itself.

A similar coupling mechanism may underlie growth control in tip-growing cells of land plants as shown by the mathematical modeling of growing pollen tubes (Rojas et al., 2011). An elegant model was developed based on the idea, similar to that proposed for *Chara*, that newly generated pectate causes turnover of load-bearing Ca^{2+}–pectate cross-links, thereby facilitating turgor-driven mechanical deformation. In this model, pectate production is the resultant of the deposition and de-methylesterification of pectin. Interestingly, the validity of the model was supported by its ability to precisely reproduce the morphologies of pollen tubes; to predict the growth oscillations observed in rapidly growing pollen tubes and the observed phase relationships between variables such as wall thickness, cell morphology, and growth rate in oscillatory cells (Rojas et al., 2011).

WHAT ABOUT DIFFUSELY GROWING CELLS IN LAND PLANTS?

A number of older studies have also provided evidence for a role of pectin cross-links in growth control of non-tip-growing cells of land plants: high concentrations of Ca^{2+} inhibit growth and acid-promoted extensibility of isolated cell wall preparations (Tagawa and Bonner, 1957), whereas chelation of Ca^{2+} strongly promotes cell wall extensibility and growth (Weinstein et al., 1956). It was concluded that, like in *Chara*, Ca^{2+}–pectate cross-links have a load-bearing role and that the exchange of pectate-bound Ca^{2+} for H^+ may explain at least in part the acid-promoted increase in cell wall extensibility (Virk and Cleland, 1988). An elegant study based on stress–strain experiments on glycerinated hollow cylinders prepared from elongating regions of soybean hypocotyls addressed this issue in more detail (Nakahori et al., 1991; Ezaki et al., 2005). It was shown that a pH shift in the physiological range from 6 to 5 simultaneously caused a strong increase in the extensibility, and a decrease in the yield threshold of the cell wall. Interestingly, high Ca^{2+} concentrations reverted this increase in extensibility but did not affect the yield threshold. A Ca^{2+} chelator perfectly mimicked at pH 6 the acid-induced increase in wall extensibility (Ezaki et al., 2005). The reduction in the yield threshold in the pH 6–5 range was almost entirely due to a heat-sensitive component (possibly the protein "yieldin"; Okamoto-Nakazato, 2002). Instead, the acid-induced extensibility increase was comprised of a heat-sensitive (presumably expansin) and a heat-insensitive, Ca^{2+}–pectate-dependent component. Pectin can also play a load-bearing role in mature cells after growth cessation as shown in cucumber hypocotyls (Zhao et al., 2008). Indeed, during maturation these cells lose the ability to grow and to respond to expansin. Interestingly, expansin-dependent extension could be restored in heat-inactivated walls of non-growing hypocotyl cells upon treatment with fungal pectinases or chelation of Ca^{2+} (Zhao et al., 2008).

Together these experiments can be interpreted as follows: the cell walls of the land plants studied have at least two integrated load-bearing components, Ca^{2+}–pectate and cellulose–XG. Growth control involves an "ancient" process elaborated upon the Ca^{2+}–pectate exchange mechanism, inherited from the Charophycean ancestors, combined with a more "recent" land plant-specific, expansin-based process, which "amplifies" the Ca^{2+}–pectate mechanism and allows faster growth. In this context it is interesting to note that the cell expansion rate of land plants can be 5–20× faster than that of *Chara* (Boyer, 2009).

The Ca-pectate growth-controlling mechanism is a system that, in the absence of appropriate compensation mechanisms, would be very sensitive to environmental fluctuations in Ca^{2+} or other divalent or monovalent cations. The latter would compete with Ca^{2+} for pectate binding and hence potentially interfere with cell wall integrity. The large amounts of HG (e.g., 23% of the cell walls in *Arabidopsis* leaves), combined with a precise regulation of the degree of methylesterification provides an important buffering capacity to compensate for changes in cation concentrations for instance during salt or drought stress. Growth control would require that the amount of pectate is precisely tuned, for instance through a mechanism that detects the presence of pectate in the cell wall and that controls the deposition of pectin, PME activity and/or pectate turnover. Pectate-binding receptor-like kinases, such as wall-associated kinases (WAKs; Kohorn, 2001) might play such a sensing role.

PERSPECTIVES

The combination of recent developments in live-cell imaging, spectroscopy, the mechanical measurement of cell walls, mathematical modeling, and the use of biomimetic systems should open the way for the study of a number of previously intractable processes involved in growth control in plants: (1) *The study of the deposition, de-methylesterification, and turnover of pectin in vivo.* A promising new tool based on the *in vivo* fluorescent labeling of

polysaccharides using click chemistry has been developed recently. The technique already allowed pectin deposition and turnover to be visualized in living root cells (Anderson et al., 2012). (2) *The quantification at different growth stages of wall-bound Ca^{2+} in different layers of the cell wall.* A promising technique is secondary ion mass spectroscopy (SIMS; Follet-Gueye et al., 1998). (3) *The study of the cell wall architecture and the interactions between polymers.* This can be done on intact cell walls using 3D-solid state NMR (Dick-Perez et al., 2011) or on reconstructed polymer composites with a large panel of physical techniques (Cerclier et al., 2010; Valentin et al., 2010). (4) *The dissection of feedback signaling pathways involved in the mechanical homeostasis of the cell wall.* This can be addressed using forward genetics combined with the identification and functional analysis of pectate-binding plasma membrane receptors. (5) *The study of growth-associated changes in wall mechanics of individual cells using micro- or nano-indentation methods.* For instance, high time-resolution microindentation studies on pollen tubes with oscillating growth patterns showed that increases in cell wall elasticity (presumably corresponding to cell wall relaxation cycles) preceded increases in growth rate (Zerzour et al., 2009). The observation of changes in cell wall mechanics over longer time scales may or may not be relevant for growth control. For instance, auxin- or brassinosteroid promoted growth acceleration appears to be initiated by the hydration of the cell wall (Caesar et al., 2011). This highly relevant process may simultaneously cause an increase in the elasticity and the extensibility of the wall. Similarly, oxidative cross-linking of proteins or phenolics at the end of growth phases or during stresses, simultaneously causes wall stiffening and growth arrest (Monshausen and Gilroy, 2009). On the other hand, the chemorheological process that causes wall relaxation by replacing existing bonds for bonds at new positions in principle can occur irrespective of the number and strength of the cross-links that determine the stiffness of the cell wall. For instance, growth inhibition by blue light occurred in the absence of detectable changes in the viscoelastic properties of the cell wall (Cosgrove, 1988). Finally (6) *mathematical modeling* will be essential to establish explicit frameworks for the experimental evaluation of relevant parameters (Rojas et al., 2011; Dyson et al., 2012; Huang et al., 2012).

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REFERENCES

Anderson, C. T., Wallace, I. S., and Somerville, C. R. (2012). Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in *Arabidopsis* cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1329–1334.

Boyer, J. S. (2009). Cell wall biosynthesis and the molecular mechanism of plant enlargement. *Funct. Plant Biol.* 36, 383–394.

Caesar, K., Elgass, K., Chen, Z., Huppenberger, P., Witthoft, J., Schleifenbaum, F., Blatt, M. R., Oecking, C., and Harter, K. (2011). A fast brassinolide-regulated response pathway in the plasma membrane of *Arabidopsis thaliana*. *Plant J.* 66, 528–540.

Carpita, N., and McCann, M. (2000). "The cell wall," in *Biochemistry and Molecular Biology of Plants*, eds B. Buchanan, W. Gruissem, and R. Jones (Rockville, MD: American Society of Plant Physiologists), 52–108.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Cerclier, C., Cousin, F., Bizot, H., Moreau, C., and Cathala, B. (2010). Elaboration of spin-coated cellulose–xyloglucan multilayered thin films. *Langmuir* 26, 17248–17255.

Cosgrove, D. J. (1988). Mechanism of rapid suppression of cell expansion in cucumber hypocotyls after blue-light irradiation. *Planta* 176, 109–116.

Cosgrove, D. J. (1999). Enzymes and other agents that enhance cell wall extensibility. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 391–417.

Cosgrove, D. J. (2000). Loosening of plant cell walls by expansins. *Nature* 407, 321–326.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861.

Dick-Perez, M., Zhang, Y., Hayes, J., Salazar, A., Zabotina, O. A., and Hong, M. (2011). Structure and interactions of plant cell-wall polysaccharides by 2D and 3D Magic-Angle-Spinning Solid-State NMR. *Biochemistry* 50, 989–1000.

Dyson, R. J., Band, L. R., and Jensen, O. E. (2012). A model of crosslink kinetics in the expanding plant cell wall: yield stress and enzyme action. *J. Theor. Biol.* doi: 10.1016/j.jtbi.2012.04.035 [Epub ahead of print].

Ezaki, N., Kido, N., Takahashi, K., and Katou, K. (2005). The role of wall Ca^{2+} in the regulation of wall extensibility during the acid-induced extension of soybean hypocotyl cell walls. *Plant Cell Physiol.* 46, 1831–1838.

Follet-Gueye, M. L., Verdus, M. C., Demarty, M., Thellier, M., and Ripoll, C. (1998). Cambium pre-activation in beech correlates with a strong temporary increase of calcium in cambium and phloem but not in xylem cells. *Cell Calcium* 24, 205–211.

Fry, S. C., Smith, R. C., Renwick, K. F., Martin, D. J., Hodge, S. K., and Matthews, K. J. (1992). Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* 282, 821–828.

Grant, G. T., Morris, E. R., Rees, D., Smith, P. J. C., and Thom, D. (1973). Biological interactions between polysaccharides and divalent cations: the "egg-box" model. *FEBS Lett.* 32, 195–198.

Hanus, J., and Mazeau, K. (2006). The xyloglucan–cellulose assembly at the atomic scale. *Biopolymers* 82, 59–73.

Huang, R., Becker, A. A., and Jones, I. A. (2012). Modelling cell wall growth using a fibre-reinforced hyperelastic–viscoplastic constitutive law. *J. Mech. Phys. Solids* 60, 750–783.

Kohorn, B. D. (2001). WAKs; cell wall associated kinases. *Curr. Opin. Cell Biol.* 13, 529–533.

McCann, M. C., Stacey, N. J., Wilson, R., and Roberts, K. (1993). Orientation of macromolecules in the walls of elongating carrot cells. *J. Cell Sci.* 106, 1347–1356.

McQueen-Mason, S., Durachko, D. M., and Cosgrove, D. J. (1992). Two endogenous proteins that induce cell wall extension in plant. *Plant Cell* 4, 1425–1433.

Monshausen, G. B., and Gilroy, S. (2009). Feeling green: mechanosensing in plants. *Trends Cell Biol.* 19, 228–235.

Nakahori, K., Katou, K., and Okamoto, H. (1991). Auxin changes both the extensibility and the yield threshold of the cell wall of *Vigna* hypocotyls. *Plant Cell Physiol.* 32, 121–129.

Okamoto-Nakazato, A. (2002). A brief note on the study of yieldin, a wall-bound protein that regulates the yield threshold of the cell wall. *J. Plant Res.* 115, 309–313.

Park, Y. B., and Cosgrove, D. J. (2011). Changes in cell wall biomechanical properties in the xyloglucan-deficient xxt1/xxt2 mutant of *Arabidopsis*. *Plant Physiol.* 158, 465–475.

Park, Y. B., and Cosgrove, D. J. (2012). A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. *Plant Physiol.* 158, 1933–1943.

Peaucelle, A., Braybrook, S. A., Le Guillou, L., Bron, E., Kuhlemeier, C., and Hofte, H. (2011). Pectin-induced changes in cell wall mechanics underlie organ initiation in *Arabidopsis*. *Curr. Biol.* 21, 1720–1726.

Peaucelle, A., Louvet, R., Johansen, J. N., Hofte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). *Arabidopsis* phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. *Curr. Biol.* 18, 1943–1948.

Pelloux, J., Rusterucci, C., and Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends Plant Sci.* 12, 267–277.

Proseus, T. E., Zhu, G. L., and Boyer, J. S. (2000). Turgor, temperature and the growth of plant cells: using *Chara corallina* as a model system. *J. Exp. Bot.* 51, 1481–1494.

Rojas, E. R., Hotton, S., and Dumais, J. (2011). Chemically mediated mechanical expansion of the pollen tube cell wall. *Biophys. J.* 101, 1844–1853.

Shedletzky, E., Shmuel, M., Delmer, D. P., and Lamport, D. T. (1990). Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose–xyloglucan network. *Plant Physiol.* 94, 980–987.

Sorensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F., O'Neill, M. A., Fei, Z., Rose, J. K., Domozych, D. S., and Willats, W. G. (2011). The Charophycean green algae provide insights into the early origins of plant cell walls. *Plant J.* 68, 201–211.

Tagawa, T., and Bonner, J. (1957). Mechanical properties of the *Avena* coleoptile as related to auxin and to ionic interactions. *Plant Physiol* 32, 207–212.

Valentin, R., Cerclier, C., Geneix, N., Aguie-Beghin, V., Gaillard, C., Ralet, M. C., and Cathala, B. (2010). Elaboration of extensin-pectin thin film model of primary plant cell wall. *Langmuir* 26, 9891–9898.

Virk, S. S., and Cleland, R. (1988). Calcium and the mechanical properties of soybean hypocotyl cell walls: possible role of calcium and protons in cell-wall loosening. *Planta* 176, 60–67.

Weinstein, L. H., Meiss, A. N., Uhler, R. L., and Purvis, E. R. (1956). Growth-promoting effects of ethylene-diamine tetra-acetic acid. *Nature* 178, 1188.

Zerzour, R., Kroeger, J., and Geitmann, A. (2009). Polar growth in pollen tubes is associated with spatially confined dynamic changes in cell mechanical properties. *Dev. Biol.* 334, 437–446.

Zhao, Q., Yuan, S., Wang, X., Zhang, Y., Zhu, H., and Lu, C. (2008). Restoration of mature etiolated cucumber hypocotyl cell wall susceptibility to expansin by pretreatment with fungal pectinases and EGTA in vitro. *Plant Physiol.* 147, 1874–1885.

Zykwinska, A. W., Ralet, M. C., Garnier, C. D., and Thibault, J. F. (2005). Evidence for in vitro binding of pectin side chains to cellulose. *Plant Physiol.* 139, 397–407.

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Accelerating forward genetics for cell wall deconstruction

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The elucidation of the genes involved in cell wall synthesis and assembly remains one of the biggest challenges of cell wall biology. Although traditional genetic approaches, using simple yet elegant screens, have identified components of the cell wall, many unknowns remain. Exhausting the genetic toolbox by performing sensitized screens, adopting chemical genetics or combining these with improved cell wall imaging, hold the promise of new gene discovery and function. With the recent introduction of next-generation sequencing technologies, it is now possible to quickly and efficiently map and clone genes of interest in record time. The combination of a classical genetics approach and cutting edge technology will propel cell wall biology in plants forward into the future.

Keywords: cell wall, forward genetics, genetic screen, next-generation mapping, *Arabidopsis*

CLASSICAL FORWARD GENETICS

Technological advances have changed how biological systems are studied by making it easier to collect vast amounts of data, and have even created scenarios where entire genomes can be sequenced in relatively short time frames. Progress in understanding complex networks, and the functionality of their components has, however, been far slower. For example, an estimated 15% of plant genes in *Arabidopsis thaliana* might be involved in the biosynthesis and metabolism of the plant cell wall and only a small subset have been characterized (Carpita et al., 2001; Liepman et al., 2010). The task of identifying the relevant genes in cell wall assembly and maintenance is a daunting one. However, deep sequencing platforms like Illumina's Genome Analyzer or ABI's SOLiD have changed the genetic horizon once again and hold the promise of narrowing the knowledge gap at a quicker pace. In model systems like *Arabidopsis*, this technology shrinks the time frame of positionally cloning a gene from months to days. For emerging models, it means that gene mapping is within reach; creating opportunities for expanding the use of genetics in these systems. The scope of this review is to look back at the successful application of forward genetics in *Arabidopsis* cell wall biology and suggest that, in light of the new sequencing platforms, it is worthwhile to intensify efforts using forward genetic approaches to understand cell wall biology.

Forward genetics is extremely powerful because of its exploratory nature; with no bias or *a priori* knowledge required about the pathways involved in a process. Forward genetics also has the advantage that it often utilizes mutagens that induce a wide spectrum of mutations, which can be used to generate an allelic series. For example, ethyl methanesulfonate (EMS) mutagenesis causes point mutations that can lead to amorphic, hypomorphic, hypermorphic, neomorphic, as well as conditional mutations; potentially obviating the pitfall of genetic redundancy or lethality. This variety of mutations far exceeds what is possible with biological mutagens, like T-DNA or transposons, which typically lead to amorphic alleles. Point mutations can also be informative with respect to the domain structure of the affected gene product. However, some of the limitations of forward genetics are that the screening process can be slow and laborious, and identifying the mutation using traditional positional cloning techniques can be tedious and time-consuming.

Despite the drawbacks, many cell wall-related genes have been identified using forward genetic screens using simple phenotypes such as hypocotyl length or root swelling. Although many morphological screens had a developmental rather than cell wall-specific scope, they have provided unequivocal evidence of the involvement of a number of genes in cell wall synthesis (see Hématy and Höfte, 2007 for review). In retrospect it might not be surprising that the genes identified through these screens, which focused on reduced cell elongation or anisotropy, are enriched in components related to cellulose synthesis. Indeed, one of the first genes identified from these screens was a cellulose synthase (CESA; Arioli et al., 1998). However, proteins not predicted to be involved in cellulose biosynthesis (Nicol et al., 1998; Zhong et al., 2000; Schindelman et al., 2001; Pagant et al., 2002) were also identified using this approach. While morphological screens might seem too generalized, they are relatively straightforward and can be scaled to saturation until an allelic series is generated. Since the screens can be done on Petri plates, the only real equipment that might be needed is a dissecting microscope, making them accessible to most labs. Furthermore, because they cast a large net, unintended targets might be identified that would be excluded by screens that are more focused. Although their precise function is still not known, it is unlikely that many of the non-CESA genes mentioned above would have been isolated if not for these screens. When dealing with complex, developmentally regulated structures like the cell wall it is foreseeable that we will continue to identify many more unexpected players. It seems, that in the case of this genetic fishing expedition, the by catch is a good thing.

Important players of cellulose biosynthesis have also been identified by more focused mutant screens. These have depended on

the use of specific inhibitors of cellulose biosynthesis or on ultrastructural changes of the wall. For example, resistance to the cellulose biosynthesis inhibitor (CBI), isoxaben, identified CESAs (CESA3 and 6), which are part of the cellulose synthase complex responsible for deposition of cellulose in primary walls (Scheible et al., 2001; Desprez et al., 2002). The advantage of using chemicals such as isoxaben is that a change in response to the chemical is clear-cut, and if resistance mutants are desired, then it is a simple matter to select for them on plates. An alternative screening method, which identified CESA genes involved in secondary wall-cellulose synthesis, was accomplished by screening sectioned stems for a collapsed xylem phenotype (Turner and Somerville, 1997; Taylor et al., 1999, 2000). Apart from CESAs this screen has also identified genes involved in lignin synthesis (Jones et al., 2001); again highlighting the power of genetic screens to identify a variety of cell wall constituents. Although screens like this one are more laborious than those based on inhibitor response, they obviously identify a different spectrum of genes that available inhibitors do not currently target.

A further refinement of cell wall-directed screening was achieved by Reiter et al. (1997) who performed a screen characterizing the monosaccharide composition of the cell wall mutants by gas-chromatography. This classic and laborious screen was performed on 5,200 mutagenized *Arabidopsis* plants resulting in 11 *mur* complementation groups, of which 5 have been cloned. *mur* mutants with deficiencies in a single sugar were due to mutations in a dehydratase (Bonin et al., 1997), an epimerase (Burget et al., 2003), and transferases (Vanzin et al., 2002; Madson et al., 2003), whereas *mur* mutants deficient in numerous monosaccharides are due to mutations in *CESA7* (Bosca et al., 2006) and *SAC9*, a phosphoinositide phosphatase (Austin et al., 2011). A similar screening protocol was utilized by Gardner et al. (2002) to identify variation in stem cell wall composition following hydrolysis with Driselase or trifluoroacetic acid. Brute-force screens like these have also been used to identify lignin mutants, where phenylpropanoid-deficient mutants were identified using thin-layer chromatography (Chapple et al., 1992). Additional brute-force approaches might be implemented by taking advantage of the analytical techniques used to determine the cell wall composition like oligosaccharide mass profiling method (Lerouxel et al., 2002; Obel et al., 2009), comprehensive microarray polymer profiling (Moller et al., 2007) and Fourier transform infrared microspectroscopy (Mouille et al., 2003). Although more time consuming, the power of these screens is that they have the potential to identify a very small subset of genes, which are directly related to cell wall synthesis or structure.

IDENTIFYING GENE INTERACTIONS

Once a mutant of interest has been identified from a forward genetic screen, secondary screening using this mutant background allows further dissection of the affected pathway. Due to the large number of gene families in *Arabidopsis*, many single gene mutations do not result in an observable phenotype (Cutler and McCourt, 2005). When screens are performed on a sensitized background, however, genes with overlapping functions can be unmasked. So, enhancer mutations might uncover a partially redundant pathway, while suppressor mutations may uncover interacting proteins or alternate pathways that become activated by the second-site suppressor. Overall, modifier screens are a powerful and, in plants, mostly underutilized method for the discovery of gene interaction.

There are very few examples of modifier screens in plant cell wall research (Diet et al., 2006; Hématy et al., 2007). One notable example, which demonstrates the utility of such a screen, was the identification of *theseus1* (*the1*; Hématy et al., 2007), a semidominant suppressor of the cellulose-deficient mutant *procuste1-1* (*prc1-1*); a mutant affected in *CESA6* (Fagard et al., 2000). Mutants defective in *prc1* display cellulose deficiency and develop short hypocotyls when grown in the dark, whereas, *the1*; *prc1-1* double mutants suppress the short hypocotyl phenotype but not the cellulose deficiency. THE1 belongs to the *Catharanthus roseus* protein-kinase-1-like family that plays a role in monitoring the integrity of the cell wall (Hématy et al., 2007). Although much is known about maintaining cell wall integrity in yeast, in which stress-sensing proteins act to redistribute the cell wall machinery to repair cell wall damage (Levin, 2011), the identification of THE1 is the first example of a potential cell wall integrity sensor in plants. The next step is to characterize the signaling pathway in which THE1 functions; the identification of the upstream activators or ligand(s) of THE1 and the downstream genes in the control of cell elongation and the defense against pathogens. In this respect, recent studies have begun to identify the role of the plant hormones, such as brassinosteroids, in the transcriptional regulation of THE1 and other related receptor-like kinases (Guo et al., 2009).

CHEMICAL GENETICS TO PROBE THE CELL WALL

The central principle of chemical genetics is the use of small, chemically diverse molecules to specifically target cellular gene products. In a manner analogous to mutation, the molecules are used to probe biological pathways by the activity of a target protein. Combinatorial chemistry has made it possible to generate large synthetic compound libraries, which can be used to distinguish multiple protein targets in a single pathway and so can be very specific. One major advantage of chemical genetics over classical genetics is that application of the chemicals is both tuneable and reversible. Chemicals can be targeted with spatiotemporal precision against a selected spectrum of proteins (in contrast to targeting one gene at a time), applied in defined dosage to distinct cells, organs or developmental stages. Since small molecules can be used to inactivate a family of proteins in a single step, they are ideal for functional characterization of genes with redundant functions, or can result in the selection of conditional mutants addressing loss-of-function lethality. When used in combination with classical genetics, a high-throughput screen might identify mutants that display an altered phenotype such as hypersensitivity or resistance to the chemical. Mutations conferring resistance/hypersensitivity can either affect the target of the chemical or upstream and downstream components of its target of the affected biological pathway (Raikhel and Pirrung, 2005; Norambuena et al., 2009).

Successful chemical genetic screens have been used in identifying components of the auxin signaling pathway (Armstrong et al., 2004), endomembrane system and gravitropism components (Surpin et al., 2005; Rosado et al., 2011), cytoskeleton-cellulose

synthesis interaction in plants (DeBolt et al., 2007; Yoneda et al., 2007, 2010), and abscisic acid (ABA) receptors (Park et al., 2009). The use of chemical genetic screens has not been saturated in cell wall research as it has in other organisms. However, they offer a promising tool that, when used in combination with classical genetics, can be extremely powerful. An example of this is the use of chemical and classical genetics to isolate ABA receptors (Park et al., 2009). Here the authors were able to identify an elusive receptor by utilizing a molecule that occupied different chemical space from the endogenous hormone. Similar scenarios might be imagined for cell wall structural or regulatory proteins. An interesting twist on the principle of chemical genetics has been to use an enzyme rather than a small molecule to direct a forward genetic screen (Gille et al., 2009). In this approach the authors used a hydrolase to identify mutants with altered response to the enzyme. The success of this screen highlights how cell wall perturbing molecules, whether small molecules, toxins or enzymes, can be used to identify novel cell wall-related genes by straightforward screening methods.

GENE DISCOVERY USING NEXT-GENERATION MAPPING

The whole genome sequence of model systems like *Arabidopsis* greatly shortened the time required to clone a gene from years to months, not only because it provided a reference genome but also because it eliminated the need to identify polymorphisms (Lukowitz et al., 2000). Despite this advance, mapping genes remained a protracted, labor-intensive and challenging endeavor. However, next generation sequencing technologies have shortened the time required to go from phenotype to gene down to days rather than months. Approaches to gene mapping using whole genome sequencing have been widely used to map causative mutations in various model organisms such as yeast (Smith et al., 2008; Irvine et al., 2009), bacteria (Srivatsan et al., 2008), *C. elegans* (Sarin et al., 2008; Zuryn et al., 2010), *Drosophila* (Blumenstiel et al., 2009), and *Arabidopsis* (Schneeberger et al., 2009; Hong et al., 2010; Austin et al., 2011). Many more examples of this approach will certainly emerge and with each successive study it is expected that the tools for mapping using whole genome sequencing (or next-generation mapping, NGM) will continue to improve. The cost of NGM is also expected to decrease; even though it is already very cost effective considering the cost of reagents and personnel hours that are required for traditional mapping projects.

There are two basic approaches that have been used to positionally clone genes by NGM. The first, more straightforward approach is to sequence the mutated genome and compare it to a reference, unmutagenized genome (Sarin et al., 2008). Because chemical mutagens like EMS tend to induce many mutations in the genome (Kurowska et al., 2011) the challenge is to identify the causative mutation out of this high level of sequence variation. One strategy to mitigate this problem is to roughly map the mutation before sequencing, so that once the mutant sequence is available then only a region of the genome is scrutinized. This scenario can be further improved if multiple alleles at the same locus are available so that they can be sequenced in parallel along with the reference. Since multiple alleles are generally desirable anyway, this aspect of cloning does not increase the work required to undertake NGM. The approach that has been used so far in *Arabidopsis* (Schneeberger et al., 2009; Hong et al., 2010), but also in *C. elegans* (Doitsidou et al., 2010), is based on bulked segregant analysis (Michelmore et al., 1991), whereby a mutant is crossed to a divergent wild-type to generate F2 progeny, which are then selected for their mutant phenotype and whose DNA is pooled and analyzed by whole genome sequencing. Refinement of this approach has shown that it is possible to accomplish this with no prior mapping information and using only a small F2 population to map candidate causative mutations (Austin et al., 2011). An important upshot of this work has been the development of a web-based, bioinformatic tool that is freely available to the scientific community, which can identify causative mutations in *Arabidopsis* (and other model organisms) once data is uploaded onto the web interface (Austin et al., 2011). This means that non-experts have the ability to take advantage of NGM and can easily perform their analysis in a matter of a few hours.

The impact of NGM in model genetic systems will be widespread. The speed of NGM means that multiple mutant pools can be handled simultaneously; making it easier to approach mutational saturation of a process and determine the relevant pathways involved in that process. Furthermore, researchers will now be free to spend their time designing new and inventive screens and understanding gene interaction, rather than in the doldrums of mapping. As an added bonus, even mutants obtained from time-consuming screens, with subtle or unwieldy phenotypes will be more easily accessed by NGM. This will include mutations that are very sensitive to genetic background, which are especially useful in bypassing genetic redundancy and might have been difficult to map previously. In addition, second-site modifiers or, taking it one step further, polygenic traits, will be more tractable through NGM, since candidates can be identified directly. Finally, one of the most exciting prospects of NGM is that mapping mutations in emerging model systems is also very close at hand. This means that the diversity of cell walls in different species (Popper et al., 2011) might be explored more deeply than it has been in the past.

In the context of the cell wall, there is still a lot of opportunity to expand the mutant collection. For example, it will be worthwhile to revisit many of the same screens that have been successful in the past, since many were not done to saturation. However, because of the nature of the wall simple screens based solely on morphology or growth characteristics will be limited. One lesson from previous screens that is clear is that these will continue to uncover components of the cellulose biosynthetic machinery. Therefore, cell wall-directed screens will ideally incorporate some additional tools. For example, morphology based screens could be extended by utilizing any number of the available cell wall probes like monoclonal antibodies, fluorescent tags, carbohydrate binding modules, or specific dyes. These probes could be used on tissues or specific cell types depending on the motivation behind the screen. Combining chemical genetics with classical genetics also has the potential to be very productive. Efforts could be devoted, for example, toward identifying inhibitors that target specific cell wall constituents. Since inhibitors can tackle functional redundancy, it would provide an alternative approach to reverse genetics in identifying relevant enzyme and regulatory networks for cell wall synthesis.

Once identified, it would be possible to use these inhibitors to then isolate their targets through resistance phenotypes. Whatever the approach, the current mutant collection has an underrepresentation of regulators for cell wall synthesis, assembly or integrity, and future efforts could strive to focus on identifying these. The benefit of having at least a few regulators in hand is that it will provide a framework for understanding cell wall dynamics.

It is an exciting time for cell wall geneticists with new emerging fields and tools for research exploration and discovery. Retooling what has been proven so successful in the past, the use of classical genetics for basic research, and integrating technology in innovative and novel ways, will be critical in moving forward at an unprecedented pace in uncovering the components involved in the cell wall.

REFERENCES

Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998). Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* 279, 717–720.

Armstrong, J. I., Yuan, S., Dale, J. M., Tanner, V. N., and Theologis, A. (2004). Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14978–14983.

Austin, R. S., Vidaurre, D., Stamatiou, G., Breit, R., Provart, N. J., Bonetta, D., Zhang, J., Fung, P., Gong, Y., Wang, P. W., McCourt, P., and Guttman, D. S. (2011). Next-generation mapping of *Arabidopsis* genes. *Plant J.* 67, 715–725.

Blumenstiel, J. P., Noll, A. C., Griffiths, J. A., Perera, A. G., Walton, K. N., Gilliland, W. D., Hawley, R. S., and Staehling-Hampton, K. (2009). Identification of EMS-induced mutations in *Drosophila melanogaster* by whole-genome sequencing. *Genetics* 182, 25–32.

Bonin, C. P., Potter, I., Vanzin, G. F., and Reiter, W. D. (1997). The MUR1 gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de novo synthesis of GDP-L-fucose. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2085–2090.

Bosca, S., Barton, C. J., Taylor, N. G., Ryden, P., Neumetzler, L., Pauly, M., Roberts, K., and Seifert, G. J. (2006). Interactions between MUR10/CesA7-dependent secondary cellulose biosynthesis and primary cell wall structure. *Plant Physiol.* 142, 1353–1363.

Burget, E. G., Verma, R., Mølhøj, M., and Reiter, W. D. (2003). The biosynthesis of L-arabinose in plants: molecular cloning and characterization of a Golgi-localized UDP-D-xylose 4-epimerase encoded by the MUR4 gene of *Arabidopsis*. *Plant Cell* 15, 523–531.

Carpita, N. C., Defernez, M., Findlay, K., Wells, B., Shoue, D. A., Catchpole, G., Wilson, R. H., and McCann, M. C. (2001). Cell wall architecture of the elongating maize coleoptile. *Plant Physiol.* 127, 551–565.

Chapple, C. C., Vogt, T., Ellis, B. E., and Somerville, C. R. (1992). An *Arabidopsis* mutant defective in the general phenylpropanoid pathway. *Plant Cell* 4, 1413–1424.

Cutler, S., and McCourt, P. (2005). Dude, where's my phenotype? Dealing with redundancy in signaling networks. *Plant Physiol.* 138, 558–559.

DeBolt, S., Gutierrez, R., Ehrhardt, D. W., Melo, C. V., Ross, L., Cutler, S. R., Somerville, C., and Bonetta, D. (2007). Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5854–5859.

Desprez, T., Vernhettes, S., Fagard, M., Refrégier, G., Desnos, T., Aletti, E., Py, N., Pelletier, S., and Höfte, H. (2002). Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiol.* 128, 482–490.

Diet, A., Link, B., Seifert, G. J., Schellenberg, B., Wagner, U., Pauly, M., Reiter, W. D., and Ringli, C. (2006). The *Arabidopsis* root hair cell wall formation mutant lrx1 is suppressed by mutations in the RHM1 gene encoding a UDP-L-rhamnose synthase. *Plant Cell* 18, 1630–1641.

Doitsidou, M., Poole, R. J., Sarin, S., Bigelow, H., and Hobert, O. (2010). *C. elegans* mutant identification with a one-step whole-genome-sequencing and SNP mapping strategy. *PLoS ONE* 5, e15435. doi: 10.1371/journal.pone.0015435

Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., McCann, M., Rayon, C., Vernhettes, S., and Höfte, H. (2000). PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. *Plant Cell* 12, 2409–2424.

Gardner, S. L., Burrell, M. M., and Fry, S. C. (2002). Screening of *Arabidopsis thaliana* stems for variation in cell wall polysaccharides. *Phytochemistry* 60, 241–254.

Gille, S., Hänsel, U., Ziemann, M., and Pauly, M. (2009). Identification of plant cell wall mutants by means of a forward chemical genetic approach using hydrolases. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14699–14704.

Guo, H., Li, L., Ye, H., Yu, X., Algreen, A., and Yin, Y. (2009). Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7648–7653.

Hématy, K., and Höfte, H. (2007). "Cellulose and cell elongation," in *The Expanding Cell*, eds J. P. Verbelen and K. Vissenberg (Berlin: Springer Verlag), 33–56.

Hématy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J. P., and Höfte, H. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931.

Hong, S., Song, H.-R., Lutz, K., Kerstetter, R. A., Michael, T. P., and McClung, C. R. (2010). Type II protein arginine methyltransferase 5 (PRMT5) is required for circadian period determination in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21211–21216.

Irvine, D. V., Goto, D. B., Vaughn, M. W., Nakaseko, Y., McCombie, W. R., Yanagida, M., and Martienssen, R. (2009). Mapping epigenetic mutations in fission yeast using whole-genome next-generation sequencing. *Genome Res.* 19, 1077–1083.

Jones, L., Ennos, A. R., and Turner, S. R. (2001). Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of *Arabidopsis*. *Plant J.* 26, 205–216.

Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I., and Maluszynski, M. (2011). TILLING: a shortcut in functional genomics. *J. Appl. Genet.* 52, 371–390.

Lerouxel, O., Choo, T. S., Séveno, M., Usadel, B., Faye, L., Lerouge, P., and Pauly, M. (2002). Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. *Plant Physiol.* 130, 1754–1763.

Levin, D. E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. *Genetics* 189, 1145–1175.

Liepman, A. H., Wightman, R., Geshi, N., Turner, S. R., and Scheller, H. V. (2010). *Arabidopsis* – a powerful model system for plant cell wall research. *Plant J.* 61, 1107–1121.

Lukowitz, W., Gillmor, C. S., and Scheible, W. R. (2000). Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. *Plant Physiol.* 123, 795–805.

Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G. F., Caplan, J., Shoue, D. A., Carpita, N. C., and Reiter, W. D. (2003). The MUR3 gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *Plant Cell* 15, 1662–1670.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. U.S.A.* 88, 9828–9832.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A., and Willats, W. G. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Mouille, G., Robin, S., Lecomte, M., Pagant, S., and Höfte, H. (2003). Classification and identification of *Arabidopsis* cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *Plant J.* 35, 393–404.

Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H., and Höfte, H. (1998). A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J.* 17, 5563–5576.

Norambuena, L., Raikhel, N. V., and Hicks, G. R. (2009). Chemical genomics approaches in plant biology. *Methods Mol. Biol.* 553, 345–354.

Obel, N., Erben, V., Schwarz, T., Kühnel, S., Fodor, A., and Pauly, M. (2009). Microanalysis of plant cell wall polysaccharides. *Mol. Plant* 2, 922–932.

Pagant, S., Bichet, A., Sugimoto, K., Lerouxel, O., Desprez, T., McCann, M., Lerouge, P., Vernhettes, S., and Höfte, H. (2002). KOBITO1 encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in *Arabidopsis*. *Plant Cell* 14, 2001–2013.

Park, S. Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T. F., Alfred, S. E., Bonetta, D., Finkelstein, R., Provart, N. J., Desveaux, D., Rodriguez, P. L., McCourt, P., Zhu, J. K., Schroeder, J. I., Volkman, B. F., and Cutler, S. R. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324, 1068–1071.

Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant Biol.* 62, 567–590.

Raikhel, N., and Pirrung, M. (2005). Adding precision tools to the plant biologists' toolbox with chemical genomics. *Plant Physiol.* 138, 563–564.

Reiter, W. D., Chapple, C., and Somerville, C. R. (1997). Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J.* 12, 335–345.

Rosado, A., Hicks, G. R., Norambuena, L., Rogachev, I., Meir, S., Pourcel, L., Zouhar, J., Brown, M. Q., Boirsdore, M. P., Puckrin, R. S., Cutler, S. R., Rojo, E., Aharoni, A., and Raikhel, N. V. (2011). Sortin1-hypersensitive mutants link vacuolar-trafficking defects and flavonoid metabolism in *Arabidopsis* vegetative tissues. *Chem. Biol.* 18, 187–197.

Sarin, S., Prabhu, S., O'Meara, M. M., Pe'er, I., and Hobert, O. (2008). *Caenorhabditis elegans* mutant allele identification by whole-genome sequencing. *Nat. Methods* 5, 865–867.

Scheible, W. R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001). Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis* Ixr1 mutants. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10079–10084.

Schindelman, G., Morikami, A., Jung, J., Baskin, T. I., Carpita, N. C., Derbyshire, P., McCann, M. C., and Benfey, P. N. (2001). COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in *Arabidopsis*. *Genes Dev.* 15, 1115–1127.

Schneeberger, K., Ossowski, S., Lanz, C., Juul, T., Petersen, A. H., Nielsen, K. L., Jørgensen, J. E., Weigel, D., and Andersen, S. U. (2009). SHOREmap: simultaneous mapping and mutation identification by deep sequencing. *Nat. Methods* 6, 550–551.

Smith, D. R., Quinlan, A. R., Peckham, H. E., Makowsky, K., Tao, W., Woolf, B., Shen, L., Donahue, W. F., Tusneem, N., Stromberg, M. P., Stewart, D. A., Zhang, L., Ranade, S. S., Warner, J. B., Lee, C. C., Coleman, B. E., Zhang, Z., McLaughlin, S. F., Malek, J. A., Sorenson, J. M., Blanchard, A. P., Chapman, J., Hillman, D., Chen, F., Rokhsar, D. S., McKernan, K. J., Jeffries, T. W., Marth, G. T., and Richardson, P. M. (2008). Rapid whole-genome mutational profiling using next-generation sequencing technologies. *Genome Res.* 18, 1638–1642.

Srivatsan, A., Han, Y., Peng, J., Tehranchi, A. K., Gibbs, R., Wang, J. D., and Chen, R. (2008). High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS Genet.* 4, e1000139. doi: 10.1371/journal.pgen.1000139

Surpin, M., Rojas-Pierce, M., Carter, C., Hicks, G. R., Vasquez, J., and Raikhel, N. V. (2005). The power of chemical genomics to study the link between endomembrane system components and the gravitropic response. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4902–4907.

Taylor, N. G., Laurie, S., and Turner, S. R. (2000). Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in *Arabidopsis*. *Plant Cell* 12, 2529–2540.

Taylor, N. G., Scheible, W. R., Cutler, S., Somerville, C. R., and Turner, S. R. (1999). The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. *Plant Cell* 11, 769–780.

Turner, S. R., and Somerville, C. R. (1997). Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell* 9, 689–701.

Vanzin, G. F., Madson, M., Carpita, N. C., Raikhel, N. V., Keegstra, K., and Reiter, W. D. (2002). The mur2 mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3340–3345.

Yoneda, A., Higaki, T., Kutsuna, N., Kondo, Y., Osada, H., Hasezawa, S., and Matsui, M. (2007). Chemical genetics screening identifies a novel inhibitor of parallel alignment of cortical microtubules and cellulose microfibrils. *Plant Cell Physiol.* 48, 1393–1403.

Yoneda, A., Ito, T., Higaki, T., Kutsuna, N., Saito, T., Ishimizu, T., Osada, H., Hasezawa, S., Matsui, M., and Demura, T. (2010). Cobtorin target analysis reveals that pectin functions in the deposition of cellulose microfibrils in parallel with cortical microtubules. *Plant J.* 64, 657–667.

Zhong, R., Ripperger, A., and Ye, Z. H. (2000). Ectopic deposition of lignin in the pith of stems of two *Arabidopsis* mutants. *Plant Physiol.* 123, 59–70.

Zuryn, S., Le Gras, S., Jamet, K., and Jarriault, S. (2010). A strategy for direct mapping and identification of mutations by whole-genome sequencing. *Genetics* 186, 427–430.

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The secreted plant *N*-glycoproteome and associated secretory pathways

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N-Glycosylation is a common form of eukaryotic protein post-translational modification, and one that is particularly prevalent in plant cell wall proteins. Large scale and detailed characterization of *N*-glycoproteins therefore has considerable potential in better understanding the composition and functions of the cell wall proteome, as well as those proteins that reside in other compartments of the secretory pathway. While there have been numerous studies of mammalian and yeast *N*-glycoproteins, less is known about the population complexity, biosynthesis, structural variation, and trafficking of their plant counterparts. However, technical developments in the analysis of glycoproteins and the structures the glycans that they bear, as well as valuable comparative analyses with non-plant systems, are providing new insights into features that are common among eukaryotes and those that are specific to plants, some of which may reflect the unique nature of the plant cell wall. In this review we present an overview of the current knowledge of plant *N*-glycoprotein synthesis and trafficking, with particular reference to those that are cell wall localized.

Keywords: glycoprotein, glycoproteome, secretory pathway, *N*-glycan, protein sorting

INTRODUCTION

A common feature of plant proteins that are resident in the cell wall and other compartments of the secretory pathway is glycosylation; a complex form of post-translational modification (PTM) that has been detected in a spectrum of taxonomic groups, including eubacteria and archaea (Lechner and Wieland, 1989; Messner, 1997). Decades of research, primarily focused on yeast and mammalian glycoproteins, have demonstrated that glycosylation is important in numerous biological processes and affects protein characteristics such as folding, enzyme activity, trafficking, localization and ligand interactions (Spiro, 2002; Helenius and Aebi, 2004). However, while glycosylation is particularly prevalent and often extensive in secreted plant proteins, the detailed structures, dynamics, and in most cases functional significance, of their glycans are generally obscure.

Protein glycosylation may be divided into two principal types. The most studied is *N*-glycosylation, which involves the attachment of the *N*-acetylglucosamine (GlcNAc) of an oligosaccharide moiety by an amide bond to an asparagine residue (Asn) that is generally referred to as belonging to a consensus sequence N-X-(S/T; also written as N-!P-[S/T]), where X can be any amino acid except proline (Pless and Lennarz, 1977). Alternative non-canonical consensus sequences, such as N-X-C, have been suggested for small subset of *N*-glycosylation events (Zielinska et al., 2010). The second kind of glycosylation is *O*-glycosylation, which in plants occurs on serine (S or Ser), threonine (T or Thr), and hydroxyproline (Hyp) residues (Showalter, 2001; Gomord et al., 2010; Velasquez et al., 2011), with no apparent single common core structure or consensus protein sequence. Plant protein *O*-glycosylation will not be specifically discussed here, but has been the subject of several recent reviews (Gomord et al., 2010; Mohnen and Tierney, 2011; Taylor et al., 2011); rather this article will focus on plant *N*-glycoproteins.

The glycan structures that are attached to nascent proteins typically reflect the actions of an array of often competing glycosidases and glycosyl transferases in the secretory pathway. While the presence of the consensus sequence is generally required for *N*-linked glycosylation, the occupation of a potential site is not mandatory. Therefore, a glycoprotein may contain a number of potentially *N*-glycosylated sites, each of which may or may not be glycosylated (An et al., 2009). This results in a population of decorated proteins with a high degree of microheterogeneity at specific sites, making structural characterization extremely difficult. This challenge is compounded by the large number of possible stereo- and regio-isomers (An et al., 2009; Marino et al., 2010). Moreover, the glycan structures and frequencies cannot be reliably predicted at the level of primary DNA or protein sequences and there is not yet a single analytical platform for their accurate or high-throughput identification (Ruiz-May et al., this issue).

Despite these major obstacles, recent reports have described the characterization of hundreds of glycoproteins from several bacterial (Nothaft and Szymanski, 2010) and animal species

Abbreviations: Asn, asparagine; BFA, brefeldin A; CAH, carbonic anhydrase; CNX, calnexin; Con A, concanavalin A; CSC, cellulose synthase complex; CT, cytosolic tail; CTR, calreticulin; ECD, electron capture dissociation; ER, endoplasmic reticulum; ERES, ER export/exit sites; Fuc, α-1,3-fucose; GA, Golgi apparatus; GlcNAc, *N*-acetylglucosamine; Hyp, hydroxyproline; Le^a, Lewis a; Man, α-mannose; PM, plasma membrane; PSV, protein storage vacuole; PTM, post-translational modification; S or Ser, serine; SCAMP2, Secretory carrier membrane protein 2; T or Thr, threonine; TGN, *trans*-Golgi network; TMD, transmembrane domain; Xyl, β-1,2-xylose.

(Bunkenborg et al., 2004; Liu et al., 2005b; Kaji et al., 2007; Gundry et al., 2009; Lee et al., 2009; Wollscheid et al., 2009; Zielinska et al., 2010). In contrast, there have been no similar published studies of plant glycoproteomes on this scale. Rather, the emphasis to date has typically been on characterizing the *N*-glycosylation of individual proteins (Jamet et al., 2008), often in the context of using plants as a factories to produce recombinant proteins for human therapy and diagnostics (Chrispeels and Faye, 1996; Gomord et al., 2010). The purpose of this review is to provide a summary of advances in understanding plant *N*-glycoprotein synthesis and trafficking, with a specific focus on secreted (i.e., cell wall resident) proteins. Such studies are laying the foundation for a more advanced understanding of the plant *N*-glycoproteome, and consequently the identity, function and targeting of cell wall proteins.

BIOSYNTHESIS OF *N*-GLYCOPROTEINS

The yeast *Saccharomyces cerevisiae* has served as a model for the elucidation of *N*-glycan biosynthesis in eukaryotes and substantial numbers of the associated genes have been annotated (Burda and Aebi, 1999; Kelleher and Gilmore, 2006; O'Reilly et al., 2006). Considerably fewer have been identified and characterized in plants, but based on existing examples it appears that there is general conservation of the glycosylation machinery (see examples listed in **Table 1**). There follows an overview of recent developments and questions in the field of plant glycoprotein biosynthesis and trafficking in the context of other experimental model systems.

CYTOPLASMIC FACE

As with all eukaryotes, protein glycosylation in plants is initiated in the endoplasmic reticulum (ER). However, the biosynthesis of the *N*-glycan precursor begins on the cytosolic side of the ER (**Figure 1**; **Table 1**), starting with the transfer of *N*-acetylglucosamine-phosphate from soluble UDP-GlcNAc to the lipid membrane bound dolichyl monophosphate (Dol-P), forming *N*-acetylglucosamine-pyrophosphatidyldolichol (GlcNAc-PP-Dol). Dol-P is one of the rate-limiting factors in *N*-linked protein glycosylation in yeast and mammalian cells (Burda and Aebi, 1999; Jones et al., 2005) and while this is likely also to be the case in plants, it has not yet been demonstrated. However, mutational defects in the biosynthesis of Dol-P in *Arabidopsis* have been shown cause multiple physiological effects, such as impaired plasma membrane integrity resulting in electrolyte leakage, reduced cellular turgor and stomatal conductance, and increased drought resistance (Zhang et al., 2008). One GlcNAc and five mannose (Man) residues are subsequently transferred from UDP-GlcNAc, or GDP-Man, respectively, in a controlled, stepwise manner to GlcNAc-PP-Dol, producing the $Man_5GlcNAc_2$-PP-Dol branched heptasaccharide intermediate (Helenius and Aebi, 2002).

LUMEN OF THE ENDOPLASMIC RETICULUM

The second phase of synthesis involves the translocation of the $Man_5GlcNAc_2$-PP-Dol moiety across the ER membrane to the luminal leaflet (**Figure 1**; **Table 1**). This is thought to be catalyzed by a flippase, although the identity of this protein has not yet been confirmed in eukaryotic cells (Bugg and Brandish, 1994; Higgins, 1994; Sprong et al., 2001). Following transfer to the luminal side of the ER membrane, the oligosaccharide moiety is extended by the progressive addition of four mannose (Man) and three glucose (Glc) residues by several luminal glycosyltransferases to form $GlcMan_9GlcNAc_2$-PP-Dol (Gomord et al., 2010). Genes encoding a variety of plant glycosyltransferases have been identified (Ross et al., 2001) and several enzymes involved in *N*-glycan biosynthesis have been characterized in recombinant forms (Leonard et al., 2004). However, the functions of many plant glycosyltransferase homologs remain to be elucidated (**Table 1**). The basic step of the biosynthesis of the *N*-glycan precursor is conserved among eukaryotic cells but the regulation mechanism and the number of intermediates vary among species, and even between tissues (Pattison and Amtmann, 2009).

The third phase of *N*-linked protein glycosylation (**Figure 1**) is the transfer of a mature $Glc_3Man_9GlcNAc_2$ oligosaccharide to select Asn residues that are components of the N-!P-[S/T] consensus sequence (Pless and Lennarz, 1977). This step is catalyzed by the oligomeric oligosaccharyltransferase (OST, **Table 1**) complex (Yan and Lennarz, 1999), which in mammals consists of seven or eight non-identical subunits, but nine protein subunits in yeast (Knauer and Lehle, 1999). The active site subunits of the eukaryotic OST are termed STT3 proteins (Nilsson and von Heijne, 1993; Yan and Lennarz, 2002) and in mammalian systems it has been shown that two STT3 isoforms (STT3A and STT3B) are responsible for co- and post-translational *N*-polypeptide glycosylation (Ruiz-Canada et al., 2009). Sequence homology searches of the *Arabidopsis* genome identified five genes encoding putative OST subunits and two STT3 isoforms (Gallois et al., 1997), but only three of these have been functionally characterized (Koiwa et al., 2003; Lerouxel et al., 2005b).

MODIFICATION IN THE ENDOPLASMIC RETICULUM

After transfer from dolichol to the nascent glycoprotein, the *N*-glycan is trimmed (**Figure 1**; **Table 1**), involving hydrolytic removal of the distal α-1,2-linked Glc by glucosidase I (Grinna and Robbins, 1979; Hubbard and Ivatt, 1981), two adjacent α-1,3-linked Glc residues by glucosidase II (Michael and Kornfeld, 1980; Kilker et al., 1981) and a single specific Man residue. Glucosidases I and II were first purified from mung bean (Szumilo et al., 1986a; Kaushal et al., 1990a, 1993; Zeng and Elbein, 1998) and the characterization of the *Arabidopsis* mutants *gsc1-1* and *gsc1-2* resulted in the cloning of a glucosidase I gene. The encoded protein has homology to animal and yeast α-glucosidase I, which is involved in the first step of *N*-glycan trimming (Boisson et al., 2001). The first plant glucosidase II gene was identified from potato (Taylor et al., 2000) and the gene encoding the catalytic subunit was discovered through a study of an *Arabidopsis* temperature sensitive mutant (*rsw3*) with perturbed cellulose synthesis and a swollen root phenotype (Burn et al., 2002a).

With regard to subsequent trimming and modification of the Man component by mannosidases, studies in mammalians systems have resulted in the definition of three subgroups of the class 1 α-mannosidase family, based on sequence similarity and proposed function: ER-α1, 2, mannosidases I (ER-MNSIs), Golgi-α-mannosidases I (Golgi-MNSIs), and ER degradation-α-enhancing mannosidase (EDEM) like proteins (Mast and Moremen, 2006). In humans, only one ER-MNS1 cleaves one terminal Man from

Table 1 | Homologous yeast, human and plants genes associated with *N*-glycosylation and phenotypes of related plant mutants.

Reaction	Yeast	Human	Plants	Plant mutant feature
CYTOPLASMIC FACE OF THE ER				
1 (Add GlcNAc-1-P)	*ALG7*	*DPAGT1*	*GPT* (Koizumi et al., 1999)	Overexpression induce BiP and tunicamycin resistance
2 (Add GlcNAc)	*ALG13/14*	*hALG13/14*	Partial purification (Kaushal and Elbein, 1986)	
3 (Add β-1,4-Man)	*ALG1*	*MAT-1*		
4 (Add α-1,3-Man)	*ALG2*	*hALG2*		
5 (Add α-1,6-Man)	*ALG2?*	*hALG2?*		
6 and 7 (Add α-1,2-Man)	*ALG11*		*LEW3* (Zhang et al., 2009)	Cell wall defect
LUMINAL SIDE OF THE ER				
8 (Add α-1,3-Man)	*ALG3*	*NOT56*	*AtALG3* (Henquet et al., 2008)	Abnormal glycosylation
9 (Add α-1,2-Man)	*ALG9*	*hALG9/DIBD1*		
10 (Add α-1,6-Man)	*ALG12*	*hALG12*	*EBS4* (Hong et al., 2009)	Abnormal glycosylation
11 (Add α-1,2-Man)	*ALG9*	*hALG9/DIBD1*		
GLUCOSYLTRANSFERASES				
12 (Add α-1,3-Glu)	*ALG6*	*hALG6*		
13 (Add α-1,3-Glu)	*ALG8*	*hALG8*		
14 (Add α-1,2-Glu)	*ALG10*	*hALG10*	*AtALG10* (Farid et al., 2011)	Defects in glycosylation and leaf development
OST COMPLEX				
	STT3	*STT3A*	*SST3A* (Koiwa et al., 2003)	Sensitive to salt and defects in glycosylation
		STT3B	*STT3B* (Koiwa et al., 2003)	
	OST1	*RPN1*	*RPN1** (Panstruga et al., 1998)	
	SWP1	*RPN2*	*HAP6** (Mayer et al., 1999)	
	WBP1	*OST48/DDOST*	*DGL1* (Lerouxel et al., 2005a)	Defective in cell growth, glycosylation and differentiation
	OST2	*DAD1*	*DAD1* (Gallois et al., 1997)	Suppressor of apoptosis in animals
	OST3 and *OST6*	*N33* (Isoform a)		
		N33 (Isoform b)		
		IAP		
	OST4		*AtOST4** (Kelleher and Gilmore, 2006)	
	OST5			
GLUCOSIDASES				
15 (Deletion α-1,2-Glu)	*CWH41* (Romero et al., 1997)	*GCS1*(Kalz-Fuller et al., 1995)	*KNF* (Szumilo et al., 1986a; Zeng and Elbein, 1998; Boisson et al., 2001; Gillmor et al., 2002)	Reduction of cellulose content and cell expansion defect
16 and 17 (Deletion α-1,3-Glu)	*GTB1* (Wilkinson et al., 2006)	GCS2 (Trombetta et al., 1996)	MALI/RSW3 (Kaushal et al., 1990a, 1993; Taylor et al., 2000; Burn et al., 2002a,b)	Change in cell wall structure and perturbation in cellulose synthesis

The plant sequences marked with asterisk have not been functionally characterized. Modified from Lehle et al. (2006).

$Man_9GlcNAc_2$ (referred to as b-branch in Liebminger et al., 2009) to generate $Man_8GlcNAc_2$. In mammals, some *N*-glycoproteins bearing $Glc_1Man_9GlcNAc_2$ (i.e., retaining an extra Glc due to incomplete processing in the ER) have been shown to traffic to the *cis*-Golgi. In such cases, a Golgi resident endo-α-D-mannosidase can then cleave this molecule internally between two Man residues, resulting in a $Man_8GlcNAc_2$ glycan (Lubas and Spiro, 1987). However, phylogenetic surveys and enzymatic assays suggests the absence of such Golgi endo-α-D-mannosidase in higher plants (Dairaku and Spiro, 1997). More recently, an ER-MNSI (MNS3) from *Arabidopsis* was identified and biochemically characterized (Liebminger et al., 2009). *Arabidopsis* MNS3 showed 47% of identity to human ER-MNSI and is required for the efficient trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ (Liebminger et al., 2009). The apparent absence of Golgi endo-α-D-mannosidases in higher plants (Dairaku and Spiro, 1997) and the fact that ER resident plant glycoproteins predominantly bear $Man_8GlcNAc_2$ and minimal amount of $Man_9GlcNAc_2$, might suggest that MNS3 resides in the ER. However, transient expression of MNS3-GFP in leaf epidermal cells of *Nicotiana benthamiana* showed overlapping

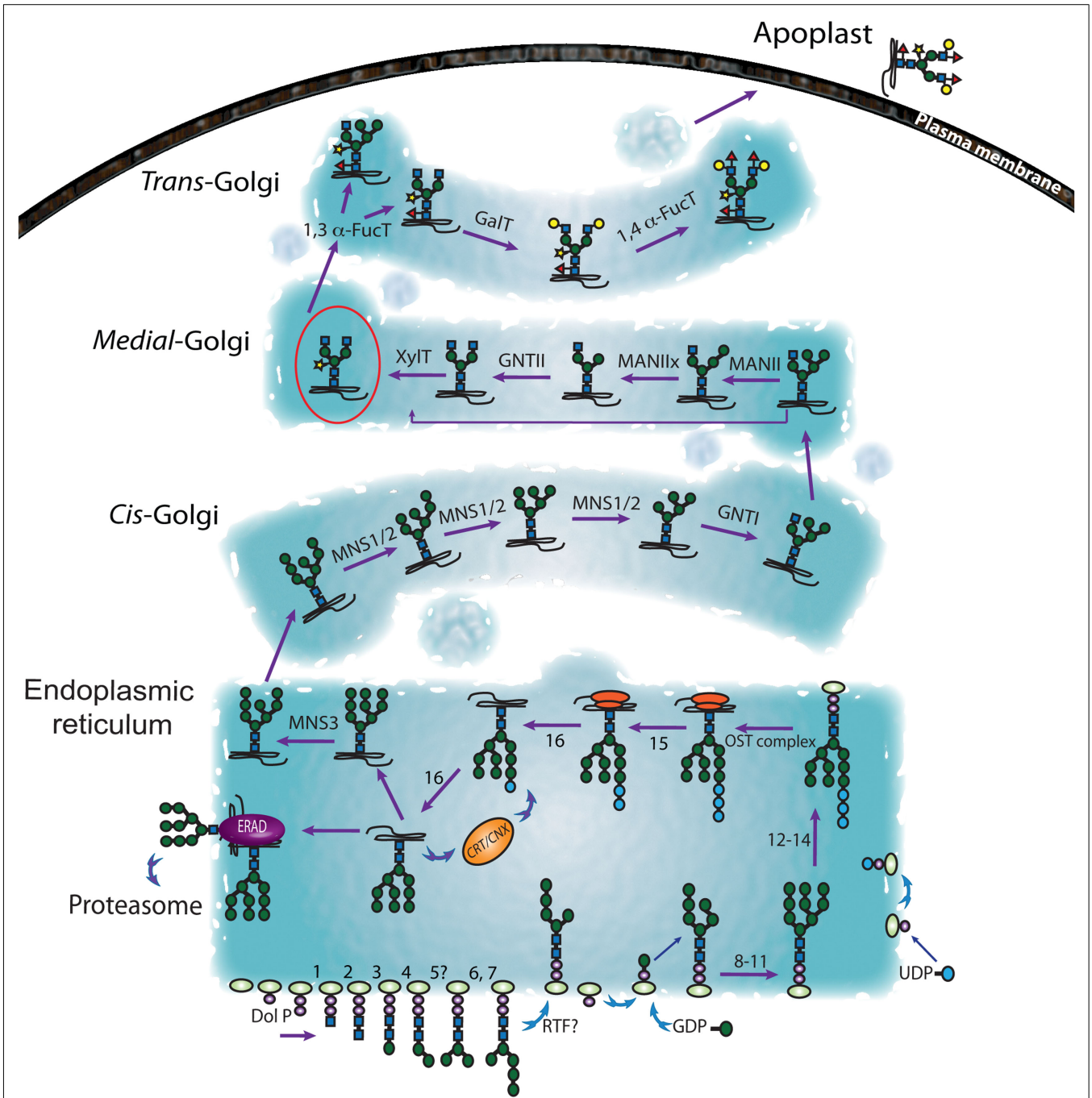


FIGURE 1 | Representation of the secretory pathway followed by the *N*-glycoproteins. The biosynthesis of the *N*-glycans is initiated on the cytosolic face of the ER. The resulting Man5GlcNAc-PP-Dol precursor is then flipped onto the luminal side ER lumen where further maturation of the sugar precursor occurs (Glc3Man9GlcNAc-PP-Dol). At this point the *N*-glycan structure is transferred to the nascent polypeptide. After removal of three Glc residues the *N*-glycoproteins enter the calnexin-calreticulin cycle (CNX/CRT; Hebert et al., 1995). The alternate action of glucosidase II and UDP-glucose:glycoprotein glucosyltransferase drives the glycoprotein through this cycle until it is correctly folded and exported from the ER to the GA. Misfolded proteins are directed from the ER to the cytosol by the ER-associated degradation (ERAD) machinery for proteasomal hydrolysis (Hebert et al., 1995; Crofts et al., 1998; Helenius and Aebi, 2004; Jin et al., 2007; Lederkremer, 2009). Glucosyl transferase and glucosidases implicated the in the sugar trimming in the ER and identified in plants so far, are enlisted in **Table 1**. Subsequent modifications of the *N*-glycans occur in the GA and potentially in other cellular compartments, such as the chloroplast and vacuole (based on a model presented in Gomord et al. (2010). The first indirect insights into the location and orientation of plant glycosyltransferases were provided by immunolocalization of the enzyme products (Laine et al., 1991; Fitchette et al., 1994, 1999). Such studies indicated that β-1,2-xylose is added to plant *N*-glycans mainly in the medial Golgi while the α-1,3-fucosylation occurs predominantly in the trans-Golgi (Fitchette et al., 1994).

expression with the Golgi marker GnTI-CTS-mRFP (Liebminger et al., 2009). In mammalian cells the ER-MNS1 has observed to be located in the ER-derived quality control compartment (Avezov et al., 2008), which is adjacent to, but not overlapping with the Golgi and the ER-to-Golgi intermediate compartment (Kamhi-Nesher et al., 2001). One hypothesis then is that MNS3 is localized in a similar, but as yet unconfirmed subcellular compartment.

QUALITY CONTROL CNX/CRT CYCLE

During translation and glycosylation in the ER, *N*-glycoproteins undergo a quality control process that prevents the arrival of incorrectly folded proteins at their final destinations and involves their redirection to the protein degradation machinery (**Figure 1**, and for more information see Liu and Howell, 2010). The *N*-glycan structure assembled in the ER serve as a recognition tags for the ER quality control and reflect the folding status of proteins (Helenius and Aebi, 2004). The membrane bound calnexin (CNX) and its soluble luminal homolog calreticulin (CRT) are chaperones that facilitate maturation and detection of defective folding (Lederkremer, 2009). The cleavage of the terminal glucose residues of the *N*-glycans can be crucial to protein folding (Lupattelli et al., 1997). Indeed, the mono-glycosylated core glycan (**Figure 1**, GlcMan9GlcNAc2) generates the interaction with the CNX/CRT complex (Ruddock and Molinari, 2006). The dissociation of the CNX/CRT complex from the glycoproteins is made possible by the removal of the third glucose linked mannose residue by glucosidase II. However, the enzyme UDP-glucose: glycoprotein glucosyltransferase (GT) can then re-glucosylate the *N*-glycan chain and recreate a ligand for CNX/CRT (Hebert et al., 1995). An *Arabidopsis* GT has been identified that plays such a role (Jin et al., 2007). It is not clear how this cycle of glycoprotein binding and glycan modification promotes protein folding or oligomerization, but one suggestion is that CNX/CRT facilitates ER retention once the GT has recognized and signaled the unfolded, or partially folded, state of a protein (Crofts et al., 1998). This cycle continues until proper folding is achieved, which prevents further recognition by the GT folding sensor (Jin et al., 2007). Another contributor to this process is the luminal binding protein (BiP). It is thought that BiP binds to translocation intermediates, misfolded proteins and peptides with exposed hydrophobic regions (Blond-Elguindi et al., 1993; Gething, 1999), preventing aggregation that could lead to permanent misfolding (Gaut and Hendershot, 1993; Hendershot et al., 1996). However, the nature and extent of any interaction between CNX/CRT and BiP that allows the proper folding of the glycoprotein intermediates through the ER is unclear at present. Similarly, it is not known whether other ER resident proteins or some other interacting molecules are also involved. Misfolded proteins released from the CNX/CRT cycle are redirected from the ER to the cytosol for proteasomal degradation; a poorly understood process in plants, referred to as ER-associated protein degradation (ERAD; Di Cola et al., 2001, 2005; Lederkremer, 2009; Liebminger et al., 2010; Liu and Howell, 2010).

ER EXPORT OF GLYCOSYLATED PROTEINS

After the initial glycosylation event involving the addition of Man and Glc residues with transfer of the *N*-glycan donor on the protein and final deletion of three glucose and one MAN residues, *N*-glycoproteins carrying $Man_8GlcNAc_2$ are delivered from the ER to the *cis*-Golgi generally via the COPII machinery (**Figure 2**) using cargo receptors or bulk flow transport (Kuehn et al., 1998; Phillipson et al., 2001). Several soluble cargo receptors have been characterized in mammals and yeasts by mutant analysis. For example, ERGIC-53 and Emp46p/47p are soluble ER resident receptors that interact with the glycosylation motif of soluble cargos, while Erv29p is a cargo receptor that interacts with the ILV motif of glycosylated proteins (Appenzeller et al., 1999; Belden and Barlowe, 2001; Otte and Barlowe, 2004). Both *in vitro* and *in vivo* interaction experiments have demonstrated that the absence of these functional cargo receptors leads to defective secretion, suggesting that they are essential for packing soluble cargo into COPII vesicles prior to transport from the ER to the Golgi. Considering their binding specificity for the glycosylation motif, ERGIC-53, and Emp46p/47p could be regarded as a glycosylation checkpoint (Appenzeller et al., 1999; Otte and Barlowe, 2004). Although receptor mediated cargo recruitment by the COPII machinery has not been characterized in plants, delivery of soluble glycoproteins by bulk flow via COPII machinery has been shown in tobacco, using calreticulin without the ER retention signal HDEL (calreticulin ΔHDEL) and α-amylase fused with HDEL (Phillipson et al., 2001). Calreticulin binds to glycosylated proteins for quality control and has the ER retention signal (HDEL) that mediates retrieval from the Golgi to ER. It has been reported that over-expressed calreticulin ΔHDEL is secreted by the default secretory pathway; however, secretion of calreticulin ΔHDEL decreases when COPII machinery is partially inhibited (Phillipson et al., 2001). These results demonstrate the existence of COPII-mediated bulk flow of glycosylated proteins in tobacco. Interestingly, no close homologs of soluble cargo receptors have been identified in plants, although given the conservation of the COPII machinery among eukaryotes, it is reasonable to hypothesize that plants may also have such receptors.

Peptide sequences in the cytosolic domain of glycoproteins have been reported to be important for their transport from the ER to the *cis*-Golgi. For example, a GDP-mannose transporter (GONST1) and *Arabidopsis* AtCASP have diacidic DXE motifs in their cytosolic domains that are required for efficient ER export (Hanton et al., 2005). Similarly, the diacidic motifs are also important for efficient ER export in yeast, where the interaction between the diacidic motif and a Sec23p/24p complex has been studied (Nishimura and Balch, 1997; Votsmeier and Gallwitz, 2001). Physical association between the diacidic motif of a K^+-channel (KAT1) and the COPII coat component Sec24 has been demonstrated *in vivo* in guard cells using a fluorescence resonance energy transfer (FRET) approach (Sieben et al., 2008). Sec24 isoforms from yeast and mammals have been reported to have their own specific binding domains for cargo selection (Miller et al., 2002; Mancias and Goldberg, 2008) and studies of the three *Arabidopsis* Sec24 isoforms (Faso et al., 2009) suggest that they may also have a specific binding motif (Wendeler et al., 2007). Further characterization of sorting signals other than a diacidic motif for the interaction with Sec24 isoforms in plants is an important future goal.

Considering the proposed specific membrane-anchored cargo selection by Sec24 isoforms, it is possible that membrane-anchored cargos are sequestered at different ER domains, dependent upon

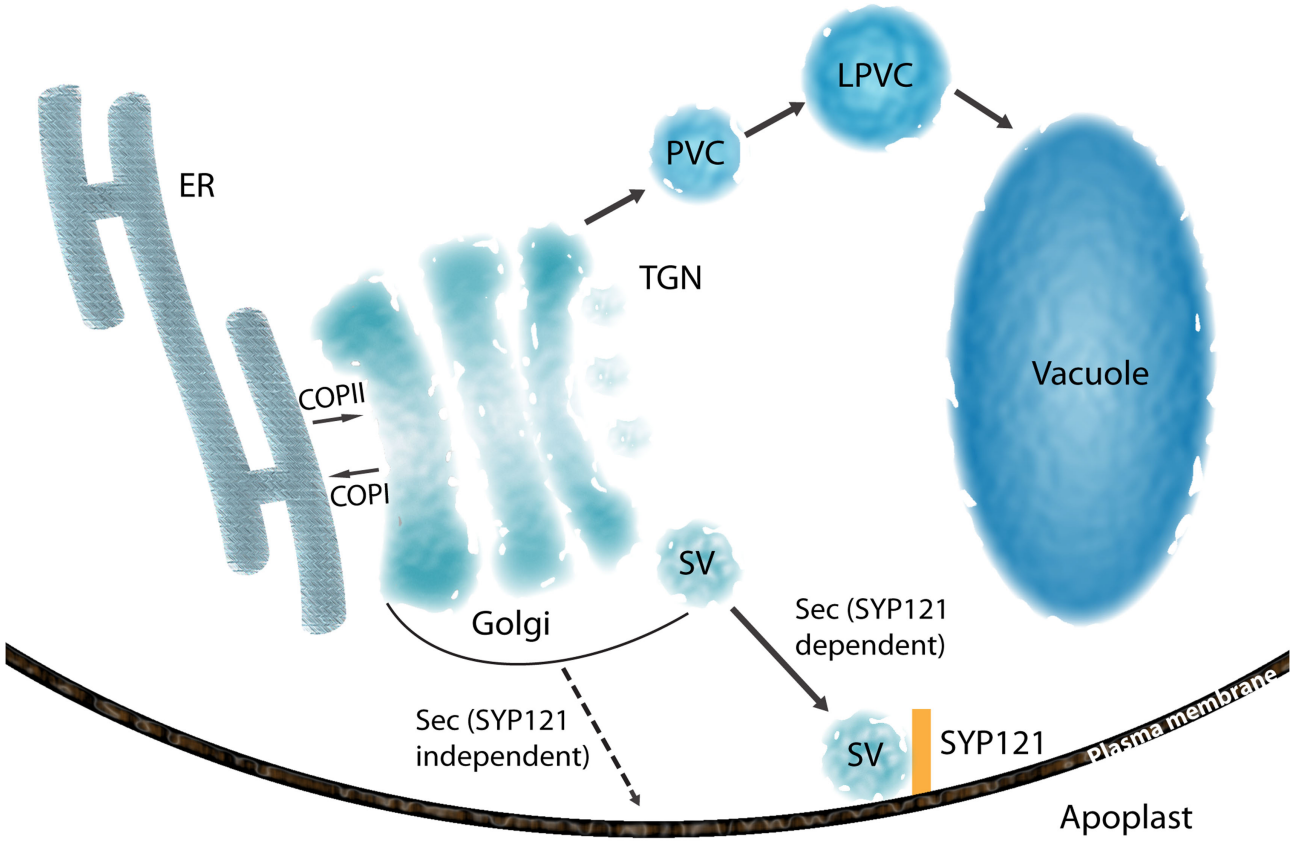


FIGURE 2 | Overview of the secretory pathway of glycosylated proteins. Glycosylated proteins are transported from ER to *cis*-Golgi by either bulk flow transport or receptor mediated transport. COPII and COPI proteins are involved in anterograde and retrograde trafficking between ER and Golgi, respectively. Secretory and vacuolar proteins are sorted at TGN. Vacuolar proteins in TGN are transported to vacuole via prevacuolar compartment (PVC) formed from maturation of TGN as well as late PVC (LPVC). Secretory proteins are accumulated in the secretory vesicles (SV) and delivered to the cell surface. A transport route indicated by dashed arrows represents a hypothetical pathway for GPI-anchored proteins (PMEI1 and PGIP2).

the isoform. This hypothesis finds further support in light of the recent result that a partial loss of function of *Arabidopsis* Sec24A led to the deformation of the ER into skein-like structures at the perinuclear area, suggesting that Sec24A may control ER export of cargo that is important for ER morphology in specific regions of the cell (Faso et al., 2009). This hypothesis has yet to be tested since it is still not know whether the COPII coat at the sites of ER protein export, the so-called ER export/exit sites (ERES), may contain different Sec24 isoforms, or only one isoform. A diversification of ERES within the cell might facilitate ER export of cargo to specific regions of the cell and allow efficient communication with the surrounding environment. A detailed analysis using fluorescent protein tagged Sec24 isoforms under the control of endogenous promoters could provide important insights into the composition of ER export sites and test the potential importance of ERES diversification.

MATURATION OF PLANT *N*-GLYCOPROTEIN GLYCANS IN THE GOLGI APPARATUS

Once *N*-glycosylated proteins reach the *cis*-Golgi, a number of enzymes then contribute to further the *N*-glycan processing and maturation (**Figure 1**; Lerouge et al., 1998). After the generation of $Man_5GlcNAc_2$ by the MNS1/2 2 (Liebminger et al., 2009; Schoberer and Strasser, 2011), the *N*-acetylglucosaminyltransferase I (GNT I) catalyze the addition of GlcNAc yielding $GlcNAcMan_5GlcNAc_2$ (Johnson and Chrispeels, 1987; Tezuka et al., 1992). The cDNA encoding GNT I was isolated from tobacco and its identity confirmed by heterologous expression and activity assays (Strasser et al., 1999) and several other cDNA clones encoding GNT I from potato and *Arabidopsis* have been characterized (Wenderoth and von Schaewen, 2000). Subsequently, α-mannosidase II (MAN II) acts to sequentially remove two Man residues (Kaushal et al., 1990b; Strasser et al., 2006) and *N*-acetylglucosaminyltransferase II (GNT) adds a single GlcNAc (Johnson and Chrispeels, 1987; Tezuka et al., 1992; Strasser et al., 1999) to generate $GlcNAc_2Man_3GlcNAc_2$. MAN II has been purified from mung bean (Szumilo et al., 1986b; Kaushal et al., 1990b) and the corresponding gene was identified from *Arabidopsis* based on homology with human and *Drosophila* sequences (Strasser et al., 2006). At this point additional structural features are introduced that are apparently unique to plants (Fitchette et al., 1994), insects (Kubelka et al., 1993; Altmann et al., 1999), certain invertebrates (van Kuik et al., 1985), and parasitic nematodes (Haslam et al., 2001; Faveeuw et al., 2003). In plants, these comprise

the addition of an β-1,2-xylose (Xyl) by β-1, 2-xylosyltransferases (Zeng et al., 1997; Strasser et al., 2000; Pagny et al., 2003) and α-1,3-fucose (Fuc) to the 3-position of the innermost GlcNAc residue by an α-1,3-fucosyltransferase (Leiter et al., 1999; Wilson et al., 2001a; **Figure 1**). The resulting *N*-glycans can be further decorated by the consecutive addition of β-1,3-galactose and α-1,4-fucose residues to the outermost GlcNAc residue to yield the so-called Lewis (Le^a) epitope (Fitchette-Laine et al., 1997; Melo et al., 1997). The first reaction is catalyzed by a β-1,3-galactosyltransferase (1,3 β-GalT), which has been identified and characterized in *Arabidopsis* (Strasser et al., 2007). The second reaction is catalyzed by an α-1,4-fucosyltransferase (1,4 α-FucT). In humans, α-1,4-fucosylation is primarily mediated by the Lewis fucosyltransferase, Fuc-TIII, a member of the α-1,3-fucosyltransferase family, which can catalyze the formation of both α-1,3- and α-1,4-fucose linkages (Palma et al., 2001). Genes encoding 1,4 α-FucT have been cloned from several plant species and the corresponding enzymatic activity shown to have strict acceptor substrate specificity for type 1 chain-based glycan structures (Galβ1-3GlcNAc), but not type 2 chains (Galβ1-4GlcNAc) that are typical of mammalian *N*-linked glycans (Fitchette-Laine et al., 1997; Oriol et al., 1999; Bakker et al., 2001; Palma et al., 2001; Wilson, 2001; Wilson et al., 2001b; Leonard et al., 2002, 2005).

These plant-associated differences in *N*-glycan structures have important implications for human health as plant glycoproteins are often extremely immunogenic (Garcia-Casado et al., 1996) and there are a number of reports that ascribe IgE cross reaction with pollen and dietary components to the carbohydrate component of glycoproteins (Wilson et al., 2001b). Indeed, it has been claimed that the major food allergens are typically water soluble glycoproteins (van Ree et al., 2000). Plant *N*-glycan structural differences are also significant in terms of experimental procedures that can be used for their analysis. For example, GlcNAc substituted with α-1, 3-fucose is not recognized by the enzyme PNGase-F, which is commonly used to deglycosylate mammalian, fungal and yeast proteins. PNGase-A can be used as an alternative, but this enzyme is only active on shorter peptides (Ytterberg and Jensen, 2010), which is an obstacle to the systematic study of plant glycoproteins.

GOLGI DISTRIBUTION OF GLYCAN MODIFYING ENZYMES

It has been proposed that the *N*-glycan modifying enzymes described above are spatially separated in the different Golgi stacks consistent with the sequence of glycan processing and maturation (Lerouge et al., 1998). Thus, their localization must be highly regulated in the Golgi. Previous efforts to explain this regulation have shown several Golgi localization or retention determinants in type-II membrane enzymes for *N*-glycosylation and type-I membrane proteins. The transmembrane domain (TMD) itself has been found to be important for the ER exit and Golgi localization (Brandizzi et al., 2002; Saint-Jore-Dupas et al., 2006). The membrane thickness is different among endomembrane systems: the ER and the Golgi membranes are 4–5 nm thick, while the post-Golgi membranes are thicker than those of the ER, or the Golgi membranes of fungi and mammals (Grove et al., 1968), suggesting that the length of the TMD domain is important for insertion into the correct membrane. Studies of the human lysosomal protein LAMP1 demonstrated that TMD lengths of 17, 20, and 23 amino acids are required for the insertion into the membranes of the ER, Golgi or the plasma membrane, respectively. A similar distribution dependency on TMD length has been shown for the TMD of pea vacuolar sorting receptor BP-80, a type-I membrane protein (Brandizzi et al., 2002), and the TMD of soybean α-1,2 mannosidase I (ManI) appears to be sufficient for the Golgi targeting (Saint-Jore-Dupas et al., 2006). While TMD length is important for membrane insertion, it has also been proposed that the composition of amino acids in the TMD, together with membrane lipid composition, is important for the Golgi localization in mammalian cells. *Cis*-, *medial*-, and *trans*-Golgi membranes have been found to have different sphingolipid:glycerophospholipid ratios and the nature of the amino acids that comprise the TMD may influence the affinities or partitioning with the membrane structural variants, and thus localization in different Golgi compartments (Patterson et al., 2008; Jackson, 2009). It is not yet known whether this model also applies to plants.

In addition to the TMD, the cytosolic tail (CT) domain of ER or Golgi membrane bound enzymes contains sorting signals. The CT domain has been investigated for ER retention in plant and mammalian cells, as well as intra-Golgi trafficking in yeast and mammalian cells. It has been suggested that there are several types of CT domain, which have a role in determining the localization of the proteins. The di-lysine motif in the CT of type-I membrane proteins and di-arginine motif in the CT of type-II membrane proteins are necessary for the COPI-mediated ER retrieval and are conserved in mammals, yeasts, and plants (Benghezal et al., 2000; McCartney et al., 2004; Kabuss et al., 2005; Okamoto et al., 2008; Boulaflous et al., 2009; Uemura et al., 2009). A number of yeast proteins have been found to have Vps74p binding motifs in their CT domain (Tu and Banfield, 2010). Vps74p forms a tetramer complex and functions as a bridge between COPI coat protein and CT domain (Schmitz et al., 2008). The localization of Vps74p is dependent on phosphatidylinositol 4-phosphate (PtdIns4P), synthesized by the enzyme phosphatidylinositol 4-kinase, and lack of PtdIns4P causes mislocalization of a Vps74p interacting Golgi resident protein, α-1,2-mannosyltransferase, to the vacuole (Tu et al., 2008; Wood et al., 2009). In addition, knock-down of a gene encoding the lipid phosphatase Suppressor of actin mutations 1 (SAC1) disrupts the restricted distribution of PtdIns4P in the *trans*-Golgi network (TGN), resulting abnormal Golgi morphology and mislocalization of Golgi enzymes in mammals. This result suggests that enrichment of PtdIns4P in the TGN is required for maintaining Golgi structure and proper distribution of Golgi proteins (Cheong et al., 2010).

In addition to Vps74p, COG complexes have been shown to be involved in the distribution of Golgi proteins in yeast and *Arabidopsis* (Bruinsma et al., 2004; Ishikawa et al., 2008). The COG complex is considered to be a tethering factor and is required for the typical SNARE- and COPI-mediated vesicle transport (Brown and Pfeffer, 2010). Mutation in one subunit in the *Arabidopsis* COG complex causes mislocalization of Golgi proteins and abnormal cell growth and organization, presumably by affecting localization of glycosylation enzymes (Ishikawa et al., 2008). These results suggest that the COPI machinery contributes to steady-state Golgi distribution of enzymes with specific CT domains, which may be required for the interaction with trafficking components

in COPI vesicles. Considering the suggested role of the COPI vesicles in retrograde trafficking from the *cis*-Golgi to the ER, or from the *trans*-Golgi to *cis-/medial*-Golgi (Donohoe et al., 2007), and the conserved role of the COG complex in Golgi distribution (Ishikawa et al., 2008), the COPI machinery could contribute to steady-state Golgi distribution of enzymes with specific CT domain, which is necessary for the interaction with trafficking components in COPI vesicles in plants. Although little is known about PtdIns4P in plants, it is known to be enriched in the *trans*-Golgi and plasma membrane in BY-2 cells, cowpea protoplasts and *Arabidopsis* (Vermeer et al., 2009). Receptors such as Vps74p and cognate interacting motif in Golgi proteins are not present in plants (Schoberer and Strasser, 2011), but a conserved role for the COG complex and high accumulation of PtdIns4P in the plant Golgi apparatus suggests that plants may utilize distinct cargo receptors and interacting motifs that utilize COPI machinery.

N-GLYCOPROTEIN SORTING AND TRAFFICKING TO THE CELL SURFACE AND APOPLAST

Following maturation in the ER and the GA (**Figure 1**), plant *N*-glycans can be further modified during the transit of glycoproteins to their final destinations, which can include the chloroplast, vacuole, and apoplast (Rose and Lee, 2010). Although cargo sorting can occur in earlier secretory compartments and also continue beyond the TGN, it may reach a particularly high level of complexity and sophistication in the TGN, where the sorting machinery controls multiple divergent pathways directed to spatially segregated acceptor compartments (De Matteis and Luini, 2008). No specific sorting determinants have been reported in secreted proteins, which are normally sorted at the TGN and transported to the plasma membrane/apoplast by the default pathway. This has been demonstrated by the secretion of non-plant soluble proteins fused with the signal peptide for ER translocation (Denecke et al., 1990; Batoko et al., 2000). At the TGN, vacuolar proteins are transported to the vacuole via the prevacuolar compartment (PVC), which originates from the TGN, and late PVC (**Figure 2**; Scheuring et al., 2011; Bottanelli et al., 2012). However, secretory vesicles have been reported to have no coat proteins and form secretory vesicle clusters at the TGN (Toyooka et al., 2007). The secretory carrier membrane protein 2 (SCAMP2) has been established as a marker of secretory vesicles containing pectin and secretory soluble GFP fusion marker (secGFP), and mammalian SCAMP2 has been reported to be involved in vesicle fusion at the PM (Liu et al., 2002, 2005a; Toyooka et al., 2007). Considering the localization and high degree of sequence conservation of plant SCAMP2 with its mammalian homolog, it could also be involved in membrane fusion at PM.

Another characterized protein in the secretory pathway is SYP121 (**Figure 2**), a soluble NSF (*N*-ethylmaleimide-sensitive factor) Attachment Protein Receptors (SNARE) at the plasma membrane. It has been reported that SYP121 is involved in many aspects of the response of plants to their environment, such as non-host resistance phenomena and cellular processes including the regulation of ion channels and membrane fusion at the plasma membrane (Geelen et al., 2002; Sutter et al., 2006; Zhang et al., 2007). The Sp2 cytosolic domain of SYP121 was reported to inhibit secretion of the fluorescent secreted marker protein secGFP, presumably by preventing SNARE-mediated vesicle fusion at the plasma membrane (Tyrrell et al., 2007). This dominant-negative feature of the Sp2 domain suggests that SYP121 is a SNARE that controls bulk flow secretory pathways. Recently, new secretory pathways for cell wall proteins, a pectin methylesterase inhibitor protein (PMEI1) and a polygalacturonase inhibitor protein (PGIP2) have been proposed (De Caroli et al., 2011). The secretion of these two proteins was not inhibited by the Sp2 domain, and a glycosylphosphatidylinositol (GPI) anchoring of PMEI1 was found to be essential for the secretion of PMEI1. The new proposed secretory pathway for PMEI1 and PGIP2 suggests that the SYP121 independent vesicles are responsible for the transport of the GPI-anchored proteins. For the characterization of the new type of vesicles, the characterization of SNAREs for membrane fusion and the visualization of vesicles containing PMEI1 or PGIP2 using electron microscopy will provide new insights into the future of the alternative secretory pathway for GPI-anchored proteins.

In addition to the above examples of features of cell wall protein associated trafficking pathways, the microtubule-dependent secretion pathway of the cellulose synthase complex (CSC) has also been established. The CSC, which is responsible for cellulose biosynthesis, is formed in the *trans*-Golgi, and microtubules and kinesins are involved in the distribution of CSC and cellulose deposition (Haigler and Brown, 1986; Zhong et al., 2002; Lloyd, 2006; Wightman and Turner, 2008). Recently, it was reported that CSC is transported from the Golgi to the plasma membrane along cortical microtubules (Crowell et al., 2009; Gutierrez et al., 2009), consistent with previous results. Additionally, CSC was found to be internalized under abiotic stress or cellulose synthesis inhibitor treatment, indicating the putative regulation of the CSC by endocytosis (Crowell et al., 2009; Gutierrez et al., 2009). Endocytosis in plants is important for maintaining cellular functions and transmitting signals resulting from developmental or environmental cues. Other examples include the polar distribution of auxin efflux carriers and the shuttling of the receptor-like kinase, BRI1 from the plasma membrane to endosomes for signaling (Geldner et al., 2003, 2007; Abas et al., 2006). Endocytosis of the CSC would presumably allow cellulose synthesis to be directed to new sites by redistribution to the PM, or rapidly inhibit cellulose synthesis by compartmentalizing CSC in endosomes, although a detailed mechanism has not yet been determined. Thus, investigation of the endocytic regulation of the CSC, as well as related trafficking components and signaling pathways by endocytosis is likely to provide important information on the regulation of cellulose synthesis during development or under various environmental conditions.

FUNCTIONAL SIGNIFICANCE OF CELL WALL PROTEIN *N*-GLYCOSYLATION

There are numerous reports, spanning several decades, describing the secretion of plant glycoproteins both *in vitro* and *in vivo* (Jamet et al., 2008; Agrawal et al., 2010; Rose and Lee, 2010; Ruiz-May et al., 2010). *In vitro* culture systems, such as suspension cell cultures (SCCs), have provided a useful platform to study secreted glycoproteins through characterization of protein populations in the cell culture medium (Kieliszewski and Lamport,

1994; Fitchette-Laine et al., 1997; Fitchette et al., 1999; Kieliszewski, 2001; Misaki et al., 2001; Luczak et al., 2008; Agrawal et al., 2010; Ruiz-May et al., 2010). While there are several reports describing the analysis of secreted plant protein populations (Rose and Lee, 2010), there have been few systematic surveys that specifically target plant glycoproteins from complex tissues. One of the first efforts involved an analysis of *N*-glycoproteins from *Arabidopsis* stems using affinity chromatography with the carbohydrate binding lectin Concanavalin A (Con A; Minic et al., 2007). This resulted in the identification of 102 glycoproteins, 94% of which were predicted to be targeted to the secretory pathway, while 87% had putative cell wall or PM localization (Minic et al., 2007). Zhang et al. (2010) identified 127 putative *Arabidopsis* glycoproteins using a combination of multidimensional lectin chromatography and boric acid chromatography. *N*-glycopeptides for 20 proteins were also predicted using ProTerNyc software (Albenne et al., 2009). Recently, Catala et al. (2011) used affinity chromatography with Con A to identify a population of proteins that are predicted to be resident in the cell wall in ripe tomato (*Solanum lycopersicum*) fruit. However, in these studies no glycosylation sites or *N*-glycan identification and characterization were described.

While the functions of large numbers of plant cell wall proteins have been determined (Lee et al., 2004; Jamet et al., 2008; Agrawal et al., 2010; Rose and Lee, 2010), it is rare that the associated glycan structures or their functional significance are addressed. One early report described proteins that are secreted into the medium of carrot cell cultures coincident with the formation of embryogenic cells and somatic embryos (van Engelen et al., 1991). These transitions were inhibited by treatment with tunicamycin at an early preglobular stage, leading to the suggestion that *N*-glycoproteins secreted to the extracellular environment contribute to cell development: the active glycoprotein was subsequently identified as a secreted cationic peroxidase (Cordewener et al., 1991). However, it is important to note that such cell cultures are highly artificial experimental systems. In a related study, it was shown that a somatic embryogenesis defect in a temperature sensitive carrot cell mutant was effectively rescued by the addition to the culture medium of a mixture of proteins secreted by wild type embryo cultures (Lo Schiavo et al., 1990). The active component was purified and shown to be an acidic endochitinase bearing complex type *N*-glycans (De Jong et al., 1992).

In addition to the glycans that are attached to proteins, a variety of free *N*-glycans have been found in plant tissues as precursors of glycosylation or resulting from glycoprotein proteolysis (Priem and Gross, 1992; Nakamura et al., 2008; Meli et al., 2010). Particular attention has been paid to *N*-glycans that are present in the pericarp tissue of tomato fruit and that both accumulate during ripening (Priem et al., 1993) and that have been reported to promote ripening when infiltrated into fruit tissues. Blocking *N*-glycosylation with tunicamycin slows the rate of ripening (Handa et al., 1985), but it is not clear whether this is a consequence of altering levels of free *N*-glycans, or modifying the properties of the many ripening related *N*-glycoproteins. Recently, Meli et al. (2010) reported that suppression of α-mannosidase or β-*N*-acetyl hexosaminidase genes reduced rates of softening in transgenic tomato fruits. α-mannosidase cleaves the terminal α-mannosidic linkages from both the high mannose type and plant complex type *N*-glycans (Kimura et al., 2002), while β-*N*-acetyl hexosaminidase removes terminal *N*-acetyl-D-hexosamine residues and generates the paucimannosidic *N*-glycans present in most plant glycoproteins (Takahashi and Nishibe, 1978; Nakamura et al., 2009). Both enzymes are present at high levels in ripening fruit from a range of species, including tomato (Suvarnalatha and Prabha, 1999; Jagadeesh et al., 2004). The authors also reported that the α-mannosidase and β-*N*-acetyl hexosaminidase suppressed fruits showed reduced levels of the transcripts expression encoding a range of glycoproteins that are associated with cell wall degradation in (Meli et al., 2010). In this study, impaired glycosylation in the transgenic fruit would clearly have affected many glycoproteins and no new insights were provided into the specific proteins that contribute to fruit softening, but the results do suggest the existence of some currently unknown mechanism of feedback regulation.

CONCLUSION AND FUTURE PERSPECTIVES

Glycoproteins have long been of interest to biochemists and biologists from a wide range of fields. It is now clear that that the glycans on glycoproteins play numerous important roles, such as influencing protein folding, regulation of protein function by differential glycan processing (Moloney et al., 2000; Lee et al., 2005; Lauc, 2006; Lauc et al., 2010), providing protection from proteases, acting as recognition motifs for specific lectins and mediating cell-cell interactions (Lee and Lee, 1995), as well as enabling intracellular protein transport (Lauc and Heffer-Lauc, 2006) and many other functions, some of which are still poorly understood (Varki, 1993).

Most studies of glycoproteins focus on their protein components (Lauc, 2006). However, in order to have a comprehensive knowledge of their functions and properties there can be great value in adopting an integrative approach to study both the sugar and protein moieties as a functional unit. For example, Con A, one of the most studied plant lectins, is synthesized as an inactive glycoprotein precursor and various post-translational events, such as endoproteolytic cleavages and deglycosylation, are required to generate the mature lectin. Importantly, deglycosylation appears to be a key step and *N*-glycanase action is sufficient for activation of the lectin precursor (Sheldon and Bowles, 1992; Ramis et al., 2001), suggesting that de-*N*-glycosylation is a control key for glycoprotein activation. Another example is a prion protein that has two variably occupied glycosylation sites and generally a GPI anchor (Stahl et al., 1987). The implications of glycosylation for the structure and function of this glycoprotein are far reaching and the regulation of glycan modifications and their effects on the subsequent structure and function continue to be an extremely important and active area of current research into prion diseases (Rudd et al., 2002). In such cases critical aspects of protein function would be obscure if the research focus was solely directed to the polypeptide.

The same generic conclusions should likely be applied to the glycans that decorate plant proteins, but studies of the plant glycoproteome are still in their infancy and are lagging

behind equivalent analyses of their microbial, yeast and animal counterparts. Indeed, as far as we are aware, there exist no reports of systematic screening of plant glycoproteins: a remarkable deficiency in the "omics" compendium that will doubtless change in the near future as plant scientists exploit the current explosion of gene sequence information and new mass spectrometry technologies.

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REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J. C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat. Cell Biol.* 8, 249–256.

Agrawal, G. K., Jwa, N. S., Lebrun, M. H., Job, D., and Rakwal, R. (2010). Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* 10, 799–827.

Albenne, C., Canut, H., Boudart, G., Zhang, Y., San Clemente, H., Pont-Lezica, R., and Jamet, E. (2009). Plant cell wall proteomics: mass spectrometry data, a trove for research on protein structure/function relationships. *Mol. Plant* 2, 977–989.

Altmann, F., Staudacher, E., Wilson, I. B., and Marz, L. (1999). Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J.* 16, 109–123.

An, H. J., Froehlich, J. W., and Lebrilla, C. B. (2009). Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. *Curr. Opin. Chem. Biol.* 13, 421–426.

Appenzeller, C., Andersson, H., Kappeler, F., and Hauri, H. P. (1999). The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat. Cell Biol.* 1, 330–334.

Avezov, E., Frenkel, Z., Ehrlich, M., Herscovics, A., and Lederkremer, G. Z. (2008). Endoplasmic reticulum (ER) mannosidase I is compartmentalized and required for N-glycan trimming to Man5-6GlcNAc2 in glycoprotein ER-associated degradation. *Mol. Biol. Cell* 19, 216–225.

Bakker, H., Schijlen, E., De Vries, T., Schiphorst, W. E., Jordi, W., Lommen, A., Bosch, D., and van Die, I. (2001). Plant members of the alpha1→3/4-fucosyltransferase gene family encode an alpha1→4-fucosyltransferase, potentially involved in Lewis(a) biosynthesis, and two core alpha1→3-fucosyltransferases. *FEBS Lett.* 507, 307–312.

Batoko, H., Zheng, H. Q., Hawes, C., and Moore, I. (2000). A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal Golgi movement in plants. *Plant Cell* 12, 2201–2218.

Belden, W. J., and Barlowe, C. (2001). Role of Erv29p in collecting soluble secretory proteins into ER-derived transport vesicles. *Science* 294, 1528–1531.

Benghezal, M., Wasteneys, G. O., and Jones, D. A. (2000). The C-terminal dilysine motif confers endoplasmic reticulum localization to type I membrane proteins in plants. *Plant Cell* 12, 1179–1201.

Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M. J. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* 75, 717–728.

Boisson, M., Gomord, V., Audran, C., Berger, N., Dubreucq, B., Granier, F., Lerouge, P., Faye, L., Caboche, M., and Lepiniec, L. (2001). *Arabidopsis* glucosidase I mutants reveal a critical role of N-glycan trimming in seed development. *EMBO J.* 20, 1010–1019.

Bottanelli, F., Gershlick, D. C., and Denecke, J. (2012). Evidence for sequential action of Rab5 and Rab7 GTPases in prevacuolar organelle partitioning. *Traffic* 13, 338–354.

Boulaflous, A., Saint-Jore-Dupas, C., Herranz-Gordo, M. C., Pagny-Salehabadi, S., Plasson, C., Garidou, F., Kiefer-Meyer, M. C., Ritzenthaler, C., Faye, L., and Gomord, V. (2009). Cytosolic N-terminal arginine-based signals together with a luminal signal target a type II membrane protein to the plant ER. *BMC Plant Biol.* 9, 144. doi:10.1186/1471-2229-9-144

Brandizzi, F., Frangne, N., Marc-Martin, S., Hawes, C., Neuhaus, J. M., and Paris, N. (2002). The destination for single-pass membrane proteins is influenced markedly by the length of the hydrophobic domain. *Plant Cell* 14, 1077–1092.

Brown, F. C., and Pfeffer, S. R. (2010). An update on transport vesicle tethering. *Mol. Membr. Biol.* 27, 457–461.

Bruinsma, P., Spelbrink, R. G., and Nothwehr, S. F. (2004). Retrograde transport of the mannosyltransferase Och1p to the early Golgi requires a component of the COG transport complex. *J. Biol. Chem.* 279, 39814–39823.

Bugg, T. D., and Brandish, P. E. (1994). From peptidoglycan to glycoproteins: common features of lipid-linked oligosaccharide biosynthesis. *FEMS Microbiol. Lett.* 119, 255–262.

Bunkenborg, J., Pilch, B. J., Podtelejnikov, A. V., and Wisniewski, J. R. (2004). Screening for N-glycosylated proteins by liquid chromatography mass spectrometry. *Proteomics* 4, 454–465.

Burda, P., and Aebi, M. (1999). The dolichol pathway of N-linked glycosylation. *Biochim. Biophys. Acta* 1426, 239–257.

Burn, J. E., Hocart, C. H., Birch, R. J., Cork, A. C., and Williamson, R. E. (2002a). Functional analysis of the cellulose synthase genes CesA1, CesA2, and CesA3 in *Arabidopsis*. *Plant Physiol.* 129, 797–807.

Burn, J. E., Hurley, U. A., Birch, R. J., Arioli, T., Cork, A., and Williamson, R. E. (2002b). The cellulose-deficient *Arabidopsis* mutant rsw3 is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. *Plant J.* 32, 949–960.

Catala, C., Howe, K. J., Hucko, S., Rose, J. K., and Thannhauser, T. W. (2011). Towards characterization of the glycoproteome of tomato (*Solanum lycopersicum*) fruit using Concanavalin A lectin affinity chromatography and LC-MALDI-MS/MS analysis. *Proteomics* 11, 1530–1544.

Cheong, F. Y., Sharma, V., Blagoveshchenskaya, A., Oorschot, V. M., Brankatschk, B., Klumperman, J., Freeze, H. H., and Mayinger, P. (2010). Spatial regulation of Golgi phosphatidylinositol-4-phosphate is required for enzyme localization and glycosylation fidelity. *Traffic* 11, 1180–1190.

Chrispeels, M. J., and Faye, L. (1996). "Production of recombinant glycoproteins with define no-immunogenic glycans," in *Transgenic Plants: A Production of Recombinant System for Industrial and Pharmaceutical Proteins*, eds M. R. L. Owen and J. Pen (Chinchester: John Wiley), 99–113.

Cordewener, J., Booij, H., Vanderzandt, H., Vanengelen, F., Vankammen, A., and Devries, S. (1991). Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. *Planta* 184, 478–486.

Crofts, A. J., Leborgne-Castel, N., Pesca, M., Vitale, A., and Denecke, J. (1998). BiP and calreticulin form an abundant complex that is independent of endoplasmic reticulum stress. *Plant Cell* 10, 813–824.

Crowell, E. F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y. D., Schumacher, K., Gonneau, M., Hofte, H., and Vernhettes, S. (2009). Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell* 21, 1141–1154.

Dairaku, K., and Spiro, R. G. (1997). Phylogenetic survey of endomannosidase indicates late evolutionary appearance of this N-linked oligosaccharide processing enzyme. *Glycobiology* 7, 579–586.

De Caroli, M., Lenucci, M. S., Di Sansebastiano, G. P., Dalessandro, G., De Lorenzo, G., and Piro, G. (2011). Protein trafficking to the cell wall occurs through mechanisms distinguishable from default sorting in tobacco. *Plant J.* 65, 295–308.

De Jong, A. J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A., and De Vries, S. C. (1992). A carrot somatic embryo mutant is rescued by chitinase. *Plant Cell* 4, 425–433.

De Matteis, M. A., and Luini, A. (2008). Exiting the Golgi complex. *Nat. Rev. Mol. Cell Biol.* 9, 273–284.

Denecke, J., Botterman, J., and Deblaere, R. (1990). Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2, 51–59.

Di Cola, A., Frigerio, L., Lord, J. M., Ceriotti, A., and Roberts, L. M. (2001). Ricin A chain without its partner B chain is degraded after retrotranslocation from the endoplasmic reticulum to the cytosol in plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14726–14731.

Di Cola, A., Frigerio, L., Lord, J. M., Roberts, L. M., and Ceriotti, A. (2005). Endoplasmic reticulum-associated degradation of ricin A chain has unique and plant-specific features. *Plant Physiol.* 137, 287–296.

Donohoe, B. S., Kang, B. H., and Staehelin, L. A. (2007). Identification and characterization of COPIa- and COPIb-type vesicle classes associated with plant and algal Golgi. *Proc. Natl. Acad. Sci. U.S.A.* 104, 163–168.

Farid, A., Pabst, M., Schoberer, J., Altmann, F., Glossl, J., and Strasser, R. (2011). *Arabidopsis thaliana* alpha1,2-glucosyltransferase (ALG10) is required for efficient N-glycosylation and leaf growth. *Plant J.* 68, 314–325.

Faso, C., Chen, Y. N., Tamura, K., Held, M., Zemelis, S., Marti, L., Saravanan, R., Hummel, E., Kung, L., Miller, E., Hawes, C., and Brandizzi, F. (2009). A missense mutation in the *Arabidopsis* COPII coat protein Sec24A induces the formation of clusters of the endoplasmic reticulum and Golgi apparatus. *Plant Cell* 21, 3655–3671.

Faveeuw, C., Mallevaey, T., Paschinger, K., Wilson, I. B., Fontaine, J., Mollicone, R., Oriol, R., Altmann, F., Lerouge, P., Capron, M., and Trottein, F. (2003). Schistosome N-glycans containing core alpha 3-fucose and core beta 2-xylose epitopes are strong inducers of Th2 responses in mice. *Eur. J. Immunol.* 33, 1271–1281.

Fitchette, A. C., Cabanes-Macheteau, M., Marvin, L., Martin, B., Satiat-Jeunemaitre, B., Gomord, V., Crooks, K., Lerouge, P., Faye, L., and Hawes, C. (1999). Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. *Plant Physiol.* 121, 333–344.

Fitchette, A. C., Gomord, V., Chekkafi, A., and Faye, L. (1994). Distribution of xylosylation and fucosylation in the plant Golgi apparatus. *Plant J.* 5, 673–682.

Fitchette-Laine, A. C., Gomord, V., Cabanes, M., Michalski, J. C., Saint Macary, M., Foucher, B., Cavelier, B., Hawes, C., Lerouge, P., and Faye, L. (1997). N-glycans harboring the Lewis a epitope are expressed at the surface of plant cells. *Plant J.* 12, 1411–1417.

Gallois, P., Makishima, T., Hecht, V., Despres, B., Laudie, M., Nishimoto, T., and Cooke, R. (1997). An *Arabidopsis thaliana* cDNA complementing a hamster apoptosis suppressor mutant. *Plant J.* 11, 1325–1331.

Garcia-Casado, G., Sanchez-Monge, R., Chrispeels, M. J., Armentia, A., Salcedo, G., and Gomez, L. (1996). Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* 6, 471–477.

Gaut, J. R., and Hendershot, L. M. (1993). The modification and assembly of proteins in the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 5, 589–595.

Geelen, D., Leyman, B., Batoko, H., Di Sansabastiano, G. P., Moore, I., and Blatt, M. R. (2002). The abscisic acid-related SNARE homolog NtSyr1 contributes to secretion and growth: evidence from competition with its cytosolic domain. *Plant Cell* 14, 387–406.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* 112, 219–230.

Geldner, N., Hyman, D. L., Wang, X. L., Schumacher, K., and Chory, J. (2007). Endosomal signaling of plant steroid receptor kinase BRI1. *Genes Dev.* 21, 1598–1602.

Gething, M. J. (1999). Role and regulation of the ER chaperone BiP. *Semin. Cell Dev. Biol.* 10, 465–472.

Gillmor, C. S., Poindexter, P., Lorieau, J., Palcic, M. M., and Somerville, C. (2002). Alpha-glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis*. *J. Cell Biol.* 156, 1003–1013.

Gomord, V., Fitchette, A. C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D., and Faye, L. (2010). Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol. J.* 8, 564–587.

Grinna, L. S., and Robbins, P. W. (1979). Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides. *J. Biol. Chem.* 254, 8814–8818.

Grove, S. N., Bracker, C. E., and Morre, D. J. (1968). Cytomembrane differentiation in endoplasmic reticulum-Golgi apparatus-vesicle complex. *Science* 161, 171–173.

Gundry, R. L., Raginski, K., Tarasova, Y., Tchernyshyov, I., Bausch-Fluck, D., Elliott, S. T., Boheler, K. R., van Eyk, J. E., and Wollscheid, B. (2009). The mouse C2C12 myoblast cell surface N-linked glycoproteome: identification, glycosite occupancy, and membrane orientation. *Mol. Cell. Proteomics* 8, 2555–2569.

Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M., and Ehrhardt, D. W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nat. Cell Biol.* 11, 797–806.

Haigler, C. H., and Brown, R. M. (1986). Transport of rosettes from the golgi-apparatus to the plasma-membrane in isolated mesophyll-cells of zinnia-elegans during differentiation to tracheary elements in suspension-culture. *Protoplasma* 134, 111–120.

Handa, A. K., Singh, N. K., and Biggs, M. S. (1985). Effect of tunicamycin on invitro ripening of tomato pericarp tissue. *Physiol. Plant* 63, 417–424.

Hanton, S. L., Renna, L., Bortolotti, L. E., Chatre, L., Stefano, G., and Brandizzi, F. (2005). Diacidic motifs influence the export of transmembrane proteins from the endoplasmic reticulum in plant cells. *Plant Cell* 17, 3081–3093.

Haslam, S. M., Morris, H. R., and Dell, A. (2001). Mass spectrometric strategies: providing structural clues for helminth glycoproteins. *Trends Parasitol.* 17, 231–235.

Hebert, D. N., Foellmer, B., and Helenius, A. (1995). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* 81, 425–433.

Helenius, A., and Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* 73, 1019–1049.

Helenius, J., and Aebi, M. (2002). Transmembrane movement of dolichol linked carbohydrates during N-glycoprotein biosynthesis in the endoplasmic reticulum. *Semin. Cell Dev. Biol.* 13, 171–178.

Hendershot, L., Wei, J., Gaut, J., Melnick, J., Aviel, S., and Argon, Y. (1996). Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5269–5274.

Henquet, M., Lehle, L., Schreuder, M., Rouwendal, G., Molthoff, J., Helsper, J., van der Krol, S., and Bosch, D. (2008). Identification of the gene encoding the alpha1,3-mannosyltransferase (ALG3) in *Arabidopsis* and characterization of downstream n-glycan processing. *Plant Cell* 20, 1652–1664.

Higgins, C. F. (1994). Flip-flop: the transmembrane translocation of lipids. *Cell* 79, 393–395.

Hong, Z., Jin, H., Fitchette, A. C., Xia, Y., Monk, A. M., Faye, L., and Li, J. (2009). Mutations of an alpha1,6 mannosyltransferase inhibit endoplasmic reticulum-associated degradation of defective brassinosteroid receptors in *Arabidopsis*. *Plant Cell* 21, 3792–3802.

Hubbard, S. C., and Ivatt, R. J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50, 555–583.

Ishikawa, T., Machida, C., Yoshioka, Y., Ueda, T., Nakano, A., and Machida, Y. (2008). EMBRYO YELLOW gene, encoding a subunit of the conserved oligomeric Golgi complex, is required for appropriate cell expansion and meristem organization in *Arabidopsis thaliana*. *Genes Cells* 13, 521–535.

Jackson, C. L. (2009). Mechanisms of transport through the Golgi complex. *J. Cell Sci.* 122, 443–452.

Jagadeesh, B. H., Prabha, T. N., and Srinivasan, K. (2004). Activities of glycosidases during fruit development and ripening of tomato (*Lycopersicum esculantum* L.): implication in fruit ripening. *Plant Sci.* 166, 1451–1459.

Jamet, E., Albenne, C., Boudart, G., Irshad, M., Canut, H., and Pont-Lezica, R. (2008). Recent advances in plant cell wall proteomics. *Proteomics* 8, 893–908.

Jin, H., Yan, Z., Nam, K. H., and Li, J. (2007). Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. *Mol. Cell* 26, 821–830.

Johnson, K. D., and Chrispeels, M. J. (1987). Substrate Specificities of N-acetylglucosaminyl-, fucosyl-, and xylosyltransferases that modify glycoproteins in the Golgi apparatus of bean cotyledons. *Plant Physiol.* 84, 1301–1308.

Jones, J., Viswanathan, K., Krag, S. S., and Betenbaugh, M. J. (2005). Polyprenyl lipid synthesis in mammalian cells expressing human cis-prenyl transferase. *Biochem. Biophys. Res. Commun.* 331, 379–383.

Kabuss, R., Ashikov, A., Oelmann, S., Gerardy-Schahn, R., and Bakker, H. (2005). Endoplasmic reticulum retention of the large splice variant of the UDP-galactose transporter is caused by a dilysine motif. *Glycobiology* 15, 905–911.

Kaji, H., Kamiie, J., Kawakami, H., Kido, K., Yamauchi, Y., Shinkawa, T., Taoka, M., Takahashi, N., and Isobe, T. (2007). Proteomics reveals N-linked glycoprotein diversity in *Caenorhabditis elegans* and suggests an atypical translocation mechanism for integral membrane

proteins. *Mol. Cell Proteomics* 6, 2100–2109.

Kalz-Fuller, B., Bieberich, E., and Bause, E. (1995). Cloning and expression of glucosidase I from human hippocampus. *Eur. J. Biochem.* 231, 344–351.

Kamhi-Nesher, S., Shenkman, M., Tolchinsky, S., Fromm, S. V., Ehrlich, R., and Lederkremer, G. Z. (2001). A novel quality control compartment derived from the endoplasmic reticulum. *Mol. Biol. Cell* 12, 1711–1723.

Kaushal, G. P., and Elbein, A. D. (1986). Purification and Properties of UDP-GlcNAc:dolichyl-pyrophosphoryl-GlcNAc GlcNAc transferase from mung bean seedling. *Plant Physiol.* 81, 1086–1091.

Kaushal, G. P., Pastuszak, I., Hatanaka, K., and Elbein, A. D. (1990a). Purification to homogeneity and properties of glucosidase II from mung bean seedlings and suspension-cultured soybean cells. *J. Biol. Chem.* 265, 16271–16279.

Kaushal, G. P., Szumilo, T., Pastuszak, I., and Elbein, A. D. (1990b). Purification to homogeneity and properties of mannosidase II from mung bean seedlings. *Biochemistry* 29, 2168–2176.

Kaushal, G. P., Zeng, Y., and Elbein, A. D. (1993). Biosynthesis of glucosidase II in suspension-cultured soybean cells. *J. Biol. Chem.* 268, 14536–14542.

Kelleher, D. J., and Gilmore, R. (2006). An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* 16, 47R–62R.

Kieliszewski, M. J. (2001). The latest hype on Hyp-O-glycosylation codes. *Phytochemistry* 57, 319–323.

Kieliszewski, M. J., and Lamport, D. T. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant J.* 5, 157–172.

Kilker, R. D. Jr., Saunier, B., Tkacz, J. S., and Herscovics, A. (1981). Partial purification from *Saccharomyces cerevisiae* of a soluble glucosidase which removes the terminal glucose from the oligosaccharide Glc3Man9GlcNAc2. *J. Biol. Chem.* 256, 5299–5603.

Kimura, Y., Matsuo, S., Tsurusaki, S., Kimura, M., Hara-Nishimura, I., and Nishimura, M. (2002). Subcellular localization of endo-beta-N-acetylglucosaminidase and high-mannose type free N-glycans in plant cell. *Biochim. Biophys. Acta* 1570, 38–46.

Knauer, R., and Lehle, L. (1999). The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*. Isolation of the OST6 gene, its synthetic interaction with OST3, and analysis of the native complex. *J. Biol. Chem.* 274, 17249–17256.

Koiwa, H., Li, F., Mccully, M. G., Mendoza, I., Koizumi, N., Manabe, Y., Nakagawa, Y., Zhu, J., Rus, A., Pardo, J. M., Bressan, R. A., and Hasegawa, P. M. (2003). The STT3a subunit isoform of the *Arabidopsis* oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. *Plant Cell* 15, 2273–2284.

Koizumi, N., Ujino, T., Sano, H., and Chrispeels, M. J. (1999). Overexpression of a gene that encodes the first enzyme in the biosynthesis of asparagine-linked glycans makes plants resistant to tunicamycin and obviates the tunicamycin-induced unfolded protein response. *Plant Physiol.* 121, 353–361.

Kubelka, V., Altmann, F., Staudacher, E., Tretter, V., Marz, L., Hard, K., Kamerling, J. P., and Vliegenthart, J. F. (1993). Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A2. *Eur. J. Biochem.* 213, 1193–1204.

Kuehn, M. J., Herrmann, J. M., and Schekman, R. (1998). COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature* 391, 187–190.

Laine, A. C., Gomord, V., and Faye, L. (1991). Xylose-specific antibodies as markers of subcompartmentation of terminal glycosylation in the Golgi apparatus of sycamore cells. *FEBS Lett.* 295, 179–184.

Lauc, G. (2006). Sweet secret of the multicellular life. *Biochim. Biophys. Acta* 1760, 525–526.

Lauc, G., and Heffer-Lauc, M. (2006). Shedding and uptake of gangliosides and glycosylphosphatidylinositol-anchored proteins. *Biochim. Biophys. Acta* 1760, 584–602.

Lauc, G., Rudan, I., Campbell, H., and Rudd, P. M. (2010). Complex genetic regulation of protein glycosylation. *Mol. Biosyst.* 6, 329–335.

Lechner, J., and Wieland, F. (1989). Structure and biosynthesis of prokaryotic glycoproteins. *Annu. Rev. Biochem.* 58, 173–194.

Lederkremer, G. Z. (2009). Glycoprotein folding, quality control and ER-associated degradation. *Curr. Opin. Struct. Biol.* 19, 515–523.

Lee, A., Kolarich, D., Haynes, P. A., Jensen, P. H., Baker, M. S., and Packer, N. H. (2009). Rat liver membrane glycoproteome: enrichment by phase partitioning and glycoprotein capture. *J. Proteome Res.* 8, 770–781.

Lee, R. T., Lauc, G., and Lee, Y. C. (2005). Glycoproteomics: protein modifications for versatile functions. Meeting on glycoproteomics. *EMBO Rep.* 6, 1018–1022.

Lee, S. J., Saravanan, R. S., Damasceno, C. M., Yamane, H., Kim, B. D., and Rose, J. K. (2004). Digging deeper into the plant cell wall proteome. *Plant Physiol. Biochem.* 42, 979–988.

Lee, Y. C., and Lee, R. T. (1995). Carbohydrate-Protein Interactions – basis of glycobiology. *Acc. Chem. Res.* 28, 321–327.

Lehle, L., Strahl, S., and Tanner, W. (2006). Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases. *Angew. Chem. Int. Ed. Engl.* 45, 6802–6818.

Leiter, H., Mucha, J., Staudacher, E., Grimm, R., Glossl, J., and Altmann, F. (1999). Purification, cDNA cloning, and expression of GDP-L-Fuc:Asn-linked GlcNAc alpha1,3-fucosyltransferase from mung beans. *J. Biol. Chem.* 274, 21830–21839.

Leonard, R., Costa, G., Darrambide, E., Lhernould, S., Fleurat-Lessard, P., Carlue, M., Gomord, V., Faye, L., and Maftah, A. (2002). The presence of Lewis a epitopes in *Arabidopsis thaliana* glycoconjugates depends on an active alpha4-fucosyltransferase gene. *Glycobiology* 12, 299–306.

Leonard, R., Kolarich, D., Paschinger, K., Altmann, F., and Wilson, I. B. (2004). A genetic and structural analysis of the N-glycosylation capabilities. *Plant Mol. Biol.* 55, 631–644.

Leonard, R., Lhernould, S., Carlue, M., Fleurat, P., Maftah, A., and Costa, G. (2005). Biochemical characterization of Silene alba alpha4-fucosyltransferase and Lewis a products. *Glycoconj. J.* 22, 71–78.

Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fischette-Laine, A. C., Gomord, V., and Faye, L. (1998). N-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol. Biol.* 38, 31–48.

Lerouxel, O., Mouille, G., Andeme-Onzighi, C., Bruyant, M. P., Seveno, M., Loutelier-Bourhis, C., Driouich, A., Hofte, H., and Lerouge, P. (2005a). Mutants in DEFECTIVE GLYCOSYLATION, an *Arabidopsis* homolog of an oligosaccharyltransferase complex subunit, show protein underglycosylation and defects in cell differentiation and growth. *Plant J.* 42, 455–468.

Lerouxel, O., Mouille, G., Andeme-Onzighi, C., Bruyant, M. P., Seveno, M., Loutelier-Bourhis, C., Driouich, A., Hofte, H., and Lerouge, P. (2005b). Mutants in DEFECTIVE GLYCOSYLATION, an *Arabidopsis* homolog of an oligosaccharyltransferase complex subunit, show protein underglycosylation and defects in cell differentiation and growth. *Plant J.* 42, 455–468.

Liebminger, E., Huttner, S., Vavra, U., Fischl, R., Schoberer, J., Grass, J., Blaukopf, C., Seifert, G. J., Altmann, F., Mach, L., and Strasser, R. (2009). Class I alpha-mannosidases are required for N-glycan processing and root development in *Arabidopsis thaliana*. *Plant Cell* 21, 3850–3867.

Liebminger, E., Veit, C., Mach, L., and Strasser, R. (2010). Mannose trimming reactions in the early stages of the N-glycan processing pathway. *Plant Signal. Behav.* 5, 476–478.

Liu, J. X., and Howell, S. H. (2010). Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* 22, 2930–2942.

Liu, L. X., Guo, Z. H., Tieu, Q. Y., Castle, A., and Castle, D. (2002). Role of secretory carrier membrane protein SCAMP2 in granule exocytosis. *Mol. Biol. Cell* 13, 4266–4278.

Liu, L. X., Liao, H. N., Castle, A., Zhang, J., Casanova, J., Szabo, G., and Castle, D. (2005a). SCAMP2 interacts with Arf6 and phospholipase D1 and links their function to exocytotic fusion pore formation in PC12 cells. *Mol. Biol. Cell* 16, 4463–4472.

Liu, T., Qian, W. J., Gritsenko, M. A., Camp, D. G. II, Monroe, M. E., Moore, R. J., and Smith, R. D. (2005b). Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. *J. Proteome Res.* 4, 2070–2080.

Lloyd, C. (2006). Plant science – microtubules make tracks for cellulose. *Science* 312, 1482–1483.

Lo Schiavo, F., Giuliano, G., De Vries, S. C., Genga, A., Bollini, R., Pitto, L., Cozzani, F., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. *Mol. Gen. Genet.* 223, 385–393.

Lubas, W. A., and Spiro, R. G. (1987). Golgi endo-alpha-D-mannosidase from rat liver, a novel N-linked carbohydrate unit processing enzyme. *J. Biol. Chem.* 262, 3775–3781.

Luczak, M., Bugajewska, A., and Wojtaszek, P. (2008). Inhibitors of protein glycosylation or secretion change the pattern of extracellular proteins in suspension-cultured

cells of *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 46, 962–969.

Lupattelli, F., Pedrazzini, E., Bollini, R., Vitale, A., and Ceriotti, A. (1997). The rate of phaseolin assembly is controlled by the glucosylation state of its N-linked oligosaccharide chains. *Plant Cell* 9, 597–609.

Mancias, J. D., and Goldberg, J. (2008). Structural basis of cargo membrane protein discrimination by the human COPII coat machinery. *EMBO J.* 27, 2918–2928.

Marino, K., Bones, J., Kattla, J. J., and Rudd, P. M. (2010). A systematic approach to protein glycosylation analysis: a path through the maze. *Nat. Chem. Biol.* 6, 713–723.

Mast, S. W., and Moremen, K. W. (2006). Family 47 alpha-mannosidases in N-glycan processing. *Meth. Enzymol.* 415, 31–46.

Mayer, K., Schuller, C., Wambutt, R., Murphy, G., Volckaert, G., Pohl, T., Dusterhoft, A., Stiekema, W., Entian, K. D., Terryn, N., Harris, B., Ansorge, W., Brandt, P., Grivell, L., Rieger, M., Weichselgartner, M., De Simone, V., Obermaier, B., Mache, R., Muller, M., Kreis, M., Delseny, M., Puigdomenech, P., Watson, M., Schmidtheini, T., Reichert, B., Portatelle, D., Perez-Alonso, M., Boutry, M., Bancroft, I., Vos, P., Hoheisel, J., Zimmermann, W., Wedler, H., Ridley, P., Langham, S. A., Mccullagh, B., Bilham, L., Robben, J., van der Schueren, J., Grymonprez, B., Chuang, Y. J., Vandenbussche, F., Braeken, M., Weltjens, I., Voet, M., Bastiaens, I., Aert, R., Defoor, E., Weitzenegger, T., Bothe, G., Ramsperger, U., Hilbert, H., Braun, M., Holzer, E., Brandt, A., Peters, S., van Staveren, M., Dirkse, W., Mooijman, P., Lankhorst, R. K., Rose, M., Hauf, J., Kotter, P., Berneiser, S., Hempel, S., Feldpausch, M., Lamberth, S., van den Daele, H., De Keyser, A., Buysshaert, C., Gielen, J., Villarroel, R., De Clercq, R., van Montagu, M., Rogers, J., Cronin, A., Quail, M., Bray-Allen, S., Clark, L., Doggett, J., Hall, S., Kay, M., Lennard, N., Mclay, K., Mayes, R., Pettett, A., Rajandream, M. A., Lyne, M., Benes, V., Rechmann, S., Borkova, D., Blocker, H., Scharfe, M., Grimm, M., Lohnert, T. H., Dose, S., De Haan, M., Maarse, A., Schafer, M., Müller-Auer, S., Gabel, C., Fuchs, M., Fartmann, B., Granderath, K., Dauner, D., Herzl, A., Neumann, S., Argiriou, A., Vitale, D., Liguori, R., Piravandi, E., Massenet, O., Quigley, F., Clabauld, G., Mündlein, A., Felber, R., Schnabl, S., Hiller, R., Schmidt, W., Lecharny, A., Aubourg, S., Chefdor, F., Cooke, R., Berger, C., Montfort, A., Casacuberta, E., Gibbons, T., Weber, N., Vandenbol, M., Bargues, M., Terol, J., Torres, A., Perez-Perez, A., Purnelle, B., Bent, E., Johnson, S., Tacon, D., Jesse, T., Heijnen, L., Schwarz, S., Scholler, P., Heber, S., Francs, P., Bielke, C., Frishman, D., Haase, D., Lemcke, K., Mewes, H. W., Stocker, S., Zaccaria, P., Bevan, M., Wilson, R. K., de la Bastide, M., Habermann, K., Parnell, L., Dedhia, N., Gnoj, L., Schutz, K., Huang, E., Spiegel, L., Sehkon, M., Murray, J., Sheet, P., Cordes, M., Abu-Threideh, J., Stoneking, T., Kalicki, J., Graves, T., Harmon, G., Edwards, J., Latreille, P., Courtney, L., Cloud, J., Abbott, A., Scott, K., Johnson, D., Minx, P., Bentley, D., Fulton, B., Miller, N., Greco, T., Kemp, K., Kramer, J., Fulton, L., Mardis, E., Dante, M., Pepin, K., Hillier, L., Nelson, J., Spieth, J., Ryan, E., Andrews, S., Geisel, C., Layman, D., Du, H., Ali, J., Berghoff, A., Jones, K., Drone, K., Cotton, M., Joshu, C., Antonoiu, B., Zidanic, M., Strong, C., Sun, H., Lamar, B., Yordan, C., Ma, P., Zhong, J., Preston, R., Vil, D., Shekher, M., Matero, A., Shah, R., Swaby, I K., O'Shaughnessy, A., Rodriguez, M., Hoffmann, J., Till, S., Granat, S., Shohdy, N., Hasegawa, A., Hameed, A., Lodhi, M., Johnson, A., Chen, E., Marra, M., Martienssen, R., and McCombie, W. R. (1999). Sequence and analysis of chromosome 4 of the plant *Arabidopsis thaliana*. *Nature* 402, 769–777.

McCartney, A. W., Dyer, J. M., Dhanoa, P. K., Kim, P. K., Andrews, D. W., Mcnew, J. A., and Mullen, R. T. (2004). Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. *Plant J.* 37, 156–173.

Meli, V. S., Ghosh, S., Prabha, T. N., Chakraborty, N., Chakraborty, S., and Datta, A. (2010). Enhancement of fruit shelf life by suppressing N-glycan processing enzymes. *Proc. Natl. Acad. Sci. U.S. A.* 107, 2413–2418.

Melo, N. S., Nimtz, M., Conradt, H. S., Fevereiro, P. S., and Costa, J. (1997). Identification of the human Lewis(a) carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus* L.). *FEBS Lett.* 415, 186–191.

Messner, P. (1997). Bacterial glycoproteins. *Glycoconj. J.* 14, 3–11.

Michael, J. M., and Kornfeld, S. (1980). Partial purification and characterization of the glucosidases involved in the processing of asparagine-linked oligosaccharides. *Arch. Biochem. Biophys.* 199, 249–258.

Miller, E., Antonny, B., Hamamoto, S., and Schekman, R. (2002). Cargo selection into COPII vesicles is driven by the Sec24p subunit. *EMBO J.* 21, 6105–6113.

Minic, Z., Jamet, E., Negroni, L., Arsene Der Garabedian, P., Zivy, M., and Jouanin, L. (2007). A sub-proteome of *Arabidopsis thaliana* mature stems trapped on Concanavalin A is enriched in cell wall glycoside hydrolases. *J. Exp. Bot.* 58, 2503–2512.

Misaki, R., Kimura, Y., Fujiyama, K., and Seki, T. (2001). Glycoproteins secreted from suspension-cultured tobacco BY2 cells have distinct glycan structures from intracellular glycoproteins. *Biosci. Biotechnol. Biochem.* 65, 2482–2488.

Mohnen, D., and Tierney, M. L. (2011). Plant science. Plants get Hyp to O-glycosylation. *Science* 332, 1393–1394.

Moloney, D. J., Shair, L. H., Lu, F. M., Xia, J., Locke, R., Matta, K. L., and Haltiwanger, R. S. (2000). Mammalian Notch1 is modified with two unusual forms of O-linked glycosylation found on epidermal growth factor-like modules. *J. Biol. Chem.* 275, 9604–9611.

Nakamura, K., Inoue, M., Maeda, M., Nakano, R., Hosoi, K., Fujiyama, K., and Kimura, Y. (2009). Molecular cloning and gene expression analysis of tomato endo-beta-N-acetylglucosaminidase, an endoglycosidase involved in the production of high-mannose type free N-glycans during tomato fruit ripening. *Biosci. Biotechnol. Biochem.* 73, 461–464.

Nakamura, K., Inoue, M., Yoshiie, T., Hosoi, K., and Kimura, Y. (2008). Changes in structural features of free N-glycan and endoglycosidase activity during tomato fruit ripening. *Biosci. Biotechnol. Biochem.* 72, 2936–2945.

Nilsson, I. M., and von Heijne, G. (1993). Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J. Biol. Chem.* 268, 5798–5801.

Nishimura, N., and Balch, W. E. (1997). A di-acidic signal required for selective export from the endoplasmic reticulum. *Science* 277, 556–558.

Nothaft, H., and Szymanski, C. M. (2010). Protein glycosylation in bacteria: sweeter than ever. *Nat. Rev. Microbiol.* 11, 765–778.

Okamoto, M., Yoko-O, T., Miyakawa, T., and Jigami, Y. (2008). The cytoplasmic region of alpha-1,6-mannosyltransferase Mnn9p is crucial for retrograde transport from the Golgi apparatus to the endoplasmic reticulum in *Saccharomyces cerevisiae*. *Eukaryotic Cell* 7, 310–318.

O'Reilly, M. K., Zhang, G., and Imperiali, B. (2006). In vitro evidence for the dual function of Alg2 and Alg11: essential mannosyltransferases in N-linked glycoprotein biosynthesis. *Biochemistry* 45, 9593–9603.

Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., and Breton, C. (1999). Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology* 9, 323–334.

Otte, S., and Barlowe, C. (2004). Sorting signals can direct receptor-mediated export of soluble proteins into COPII vesicles. *Nat. Cell Biol.* 6, 1189–1194.

Pagny, S., Bouissonnie, F., Sarkar, M., Follet-Gueye, M. L., Driouich, A., Schachter, H., Faye, L., and Gomord, V. (2003). Structural requirements for *Arabidopsis* beta1,2-xylosyltransferase activity and targeting to the Golgi. *Plant J.* 33, 189–203.

Palma, A. S., Vila-Verde, C., Pires, A. S., Fevereiro, P. S., and Costa, J. (2001). A novel plant alpha4-fucosyltransferase (*Vaccinium myrtillus* L.) synthesises the Lewis(a) adhesion determinant. *FEBS Lett.* 499, 235–238.

Panstruga, R., Buschges, R., Piffanelli, P., and Schulze-Lefert, P. (1998). A contiguous 60 kb genomic stretch from barley reveals molecular evidence for gene islands in a monocot genome. *Nucleic Acids Res.* 26, 1056–1062.

Patterson, G. H., Hirschberg, K., Polishchuk, R. S., Gerlich, D., Phair, R. D., and Lippincott-Schwartz, J. (2008). Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system. *Cell* 133, 1055–1067.

Pattison, R. J., and Amtmann, A. (2009). N-glycan production in the endoplasmic reticulum of plants. *Trends Plant Sci.* 14, 92–99.

Phillipson, B. A., Pimpl, P., Dasilva, L. L. P., Crofts, A. J., Taylor, J. P., Movafeghi, A., Robinson, D. G., and Denecke, J. (2001). Secretory bulk flow of soluble proteins is efficient and COPII dependent. *Plant Cell* 13, 2005–2020.

Pless, D. D., and Lennarz, W. J. (1977). Enzymatic conversion of proteins to glycoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 74, 134–138.

Priem, B., Gitti, R., Bush, C. A., and Gross, K. C. (1993). Structure of 10 free N-glycans in ripening tomato fruit – arabinose is a constituent of a plant N-glycan. *Plant Physiol.* 102, 445–458.

Priem, B., and Gross, K. C. (1992). Mannosyl-containing and xylosyl-containing glycans promote tomato (*Lycopersicon-esculentum* Mill) fruit ripening. *Plant Physiol.* 98, 399–401.

Ramis, C., Gomord, V., Lerouge, P., and Faye, L. (2001). Deglycosylation is necessary but not sufficient for activation of proconcanavalin A. *J. Exp. Bot.* 52, 911–917.

Romero, P. A., Dijkgraaf, G. J., Shahinian, S., Herscovics, A., and Bussey, H. (1997). The yeast CWH41 gene encodes glucosidase I. *Glycobiology* 7, 997–1004.

Rose, J. K. C., and Lee, S. J. (2010). Straying off the highway: trafficking of secreted plant proteins and complexity in the plant cell wall proteome. *Plant Physiol.* 153, 433–436.

Ross, J., Li, Y., Lim, E., and Bowles, D. J. (2001). Higher plant glycosyltransferases. *Genome Biol.* 2, reviews3004.

Rudd, P. M., Merry, A. H., Wormald, M. R., and Dwek, R. A. (2002). Glycosylation and prion protein. *Curr. Opin. Struct. Biol.* 12, 578–586.

Ruddock, L. W., and Molinari, M. (2006). N-glycan processing in ER quality control. *J. Cell. Sci.* 119, 4373–4380.

Ruiz-Canada, C., Kelleher, D. J., and Gilmore, R. (2009). Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. *Cell* 136, 272–283.

Ruiz-May, E., De La Peña, C., Ayil-Gutiérrez, B. A., Nic-Can, G. I., Mukul-López, H. G., Galaz-Ávalos, R. M., and Loyola-Vargas, V. M. (2010). Protein secretion by cell cultures: an essential biological issue. *Acta Hortic.* 849, 213–222.

Saint-Jore-Dupas, C., Nebenfuhr, A., Boulaflous, A., Follet-Gueye, M. L., Plasson, C., Hawes, C., Driouich, A., Faye, L., and Gomord, V. (2006). Plant N-glycan processing enzymes employ different targeting mechanisms for their spatial arrangement along the secretory pathway. *Plant Cell* 18, 3182–3200.

Scheuring, D., Viotti, C., Kruger, F., Kunzl, F., Sturm, S., Bubeck, J., Hillmer, S., Frigerio, L., Robinson, D. G., Pimpl, P., and Schumacher, K. (2011). Multivesicular bodies mature from the trans-golgi network/early endosome in *Arabidopsis*. *Plant Cell* 23, 3463–3481.

Schmitz, K. R., Liu, J., Li, S., Setty, T. G., Wood, C. S., Burd, C. G., and Ferguson, K. M. (2008). Golgi localization of glycosyltransferases requires a Vps74p oligomer. *Dev. Cell* 14, 523–534.

Schoberer, J., and Strasser, R. (2011). Sub-compartmental organization of Golgi-resident N-glycan processing enzymes in plants. *Mol. Plant* 4, 220–228.

Sheldon, P. S., and Bowles, D. J. (1992). The Glycoprotein precursor of concanavalin-a is converted to an active lectin by deglycosylation. *EMBO J.* 11, 1297–1301.

Showalter, A. M. (2001). Arabinogalactan-proteins: structure, expression and function. *Cell. Mol. Life Sci.* 58, 1399–1417.

Sieben, C., Mikosch, M., Brandizzi, F., and Homann, U. (2008). Interaction of the K(+)-channel KAT1 with the coat protein complex II coat component Sec24 depends on a di-acidic endoplasmic reticulum export motif. *Plant J.* 56, 997–1006.

Spiro, R. G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12, 43R–56R.

Sprong, H., van der Sluijs, P., and van Meer, G. (2001). How proteins move lipids and lipids move proteins. *Nat. Rev. Mol. Cell Biol.* 2, 504–513.

Stahl, N., Borchelt, D. R., Hsiao, K., and Prusiner, S. B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* 51, 229–240.

Strasser, R., Bondili, J. S., Vavra, U., Schoberer, J., Svoboda, B., Glossl, J., Leonard, R., Stadlmann, J., Altmann, F., Steinkellner, H., and Mach, L. (2007). A unique beta1,3-galactosyltransferase is indispensable for the biosynthesis of N-glycans containing Lewis a structures in *Arabidopsis thaliana*. *Plant Cell* 19, 2278–2292.

Strasser, R., Mucha, J., Mach, L., Altmann, F., Wilson, I. B., Glossl, J., and Steinkellner, H. (2000). Molecular cloning and functional expression of beta1, 2-xylosyltransferase cDNA from *Arabidopsis thaliana*. *FEBS Lett.* 472, 105–108.

Strasser, R., Mucha, J., Schwihla, H., Altmann, F., Glossl, J., and Steinkellner, H. (1999). Molecular cloning and characterization of cDNA coding for beta1, 2N-acetylglucosaminyltransferase I (GlcNAc-TI) from *Nicotiana tabacum*. *Glycobiology* 9, 779–785.

Strasser, R., Schoberer, J., Jin, C., Glossl, J., Mach, L., and Steinkellner, H. (2006). Molecular cloning and characterization of *Arabidopsis thaliana* Golgi alpha-mannosidase II, a key enzyme in the formation of complex N-glycans in plants. *Plant J.* 45, 789–803.

Sutter, J. U., Campanoni, P., Tyrrell, M., and Blatt, M. R. (2006). Selective mobility and sensitivity to SNAREs is exhibited by the *Arabidopsis* KAT1 K+ channel at the plasma membrane. *Plant Cell* 18, 935–954.

Suvarnalatha, G., and Prabha, T. N. (1999). Alpha-D-mannosidase from *Lycopersicon esculentum* – II. *Phytochemistry* 50, 1111–1115.

Szumilo, T., Kaushal, G. P., and Elbein, A. D. (1986a). Purification and properties of glucosidase I from mung bean seedlings. *Arch. Biochem. Biophys.* 247, 261–271.

Szumilo, T., Kaushal, G. P., Hori, H., and Elbein, A. D. (1986b). Purification and properties of a glycoprotein processing alpha-mannosidase from mung bean seedlings. *Plant Physiol.* 81, 383–389.

Takahashi, N., and Nishibe, H. (1978). Some characteristics of a new glycopeptidase acting on aspartylglycosylamine linkages. *J. Biochem.* 84, 1467–1473.

Taylor, C. M., Karunaratne, C. V., and Xie, N. (2011). Glycosides of hydroxyproline: some recent, unusual discoveries. *Glycobiology* 22, 757–767.

Taylor, M. A., Ross, H. A., Mcrae, D., Stewart, D., Roberts, I., Duncan, G., Wright, F., Millam, S., and Davies, H. V. (2000). A potato alpha-glucosidase gene encodes a glycoprotein-processing alpha-glucosidase II-like activity. Demonstration of enzyme activity and effects of down-regulation in transgenic plants. *Plant J.* 24, 305–316.

Tezuka, K., Hayashi, M., Ishihara, H., Akazawa, T., and Takahashi, N. (1992). Studies on synthetic pathway of xylose-containing N-linked oligosaccharides deduced from substrate specificities of the processing enzymes in sycamore cells (*Acer pseudoplatanus* L.). *Eur. J. Biochem.* 203, 401–413.

Toyooka, K., Goto, Y., Asatsuma, S., and Matsuoka, K. (2007). SCAMP-containing vesicle cluster (SVC), a post-Golgi exocytotic organelle. *Plant Cell Physiol.* 48, S122–S122.

Trombetta, E. S., Simons, J. F., and Helenius, A. (1996). Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. *J. Biol. Chem.* 271, 27509–27516.

Tu, L., Tai, W. C. S., Chen, L., and Banfield, D. K. (2008). Signal-mediated dynamic retention of glycosyltransferases in the Golgi. *Science* 321, 404–407.

Tu, L. N., and Banfield, D. K. (2010). Localization of Golgi-resident glycosyltransferases. *Cell. Mol. Life Sci.* 67, 29–41.

Tyrrell, M., Campanoni, P., Sutter, J. U., Pratelli, R., Paneque, M., Sokolovski, S., and Blatt, M. R. (2007). Selective targeting of plasma membrane and tonoplast traffic by inhibitory (dominant-negative) SNARE fragments. *Plant J.* 51, 1099–1115.

Uemura, S., Yoshida, S., Shishido, F., and Inokuchi, J. (2009). The cytoplasmic tail of Gm3 synthase defines its subcellular localization, stability, and in vivo activity. *Mol. Biol. Cell* 20, 3088–3100.

van Engelen, F. A., Sterk, P., Booij, H., Cordewener, J. H., Rook, W., van Kammen, A., and De Vries, S. C. (1991). Heterogeneity and cell type-specific localization of a cell wall glycoprotein from carrot suspension cells. *Plant Physiol.* 96, 705–712.

van Kuik, J. A., van Halbeek, H., Kamerling, J. P., and Vliegenthart, J. F. (1985). Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* alpha-hemocyanin. Xylose as a constituent of N-linked oligosaccharides in an animal glycoprotein. *J. Biol. Chem.* 260, 13984–13988.

van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J. P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L., and Lerouge, P. (2000). Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.* 275, 11451–11458.

Varki, A. (1993). Biological roles of oligosaccharides – all of the theories are correct. *Glycobiology* 3, 97–130.

Velasquez, S. M., Ricardi, M. M., Dorosz, J. G., Fernandez, P. V., Nadra, A. D., Pol-Fachin, L., Egelund, J., Gille, S., Harholt, J., Ciancia, M., Verli, H., Pauly, M., Bacic, A., Olsen, C. E., Ulvskov, P., Petersen, B. L., Somerville, C., Iusem, N. D., and Estevez, J. M. (2011). O-glycosylated cell wall proteins are essential in root hair growth. *Science* 332, 1401–1403.

Vermeer, J. E., Thole, J. M., Goedhart, J., Nielsen, E., Munnik, T., and Gadella, T. W. Jr. (2009). Imaging phosphatidylinositol 4-phosphate

dynamics in living plant cells. *Plant J.* 57, 356–372.

Votsmeier, C., and Gallwitz, D. (2001). An acidic sequence of a putative yeast Golgi membrane protein binds COPII and facilitates ER export. *EMBO J.* 20, 6742–6750.

Wendeler, M. W., Paccaud, J. P., and Hauri, H. P. (2007). Role of Sec24 isoforms in selective export of membrane proteins from the endoplasmic reticulum. *EMBO Rep.* 8, 258–264.

Wenderoth, I., and von Schaewen, A. (2000). Isolation and characterization of plant N-acetyl glucosaminyltransferase I (GntI) cDNA sequences. Functional analyses in the *Arabidopsis* cgl mutant and in antisense plants. *Plant Physiol.* 123, 1097–1108.

Wightman, R., and Turner, S. R. (2008). The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. *Plant J.* 54, 794–805.

Wilkinson, B. M., Purswani, J., and Stirling, C. J. (2006). Yeast GTB1 encodes a subunit of glucosidase II required for glycoprotein processing in the endoplasmic reticulum. *J. Biol. Chem.* 281, 6325–6333.

Wilson, I. B. (2001). Identification of a cDNA encoding a plant Lewis-type alpha1,4-fucosyltransferase. *Glycoconj. J.* 18, 439–447.

Wilson, I. B., Rendic, D., Freilinger, A., Dumic, J., Altmann, F., Mucha, J., Muller, S., and Hauser, M. T. (2001a). Cloning and expression of cDNAs encoding alpha1,3-fucosyltransferase homologues from *Arabidopsis thaliana*. *Biochim. Biophys. Acta* 1527, 88–96.

Wilson, I. B., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C. J., Kamerling, J. P., and Altmann, F. (2001b). Analysis of Asn-linked glycans from vegetable foodstuffs: widespread occurrence of Lewis a, core alpha1,3-linked fucose and xylose substitutions. *Glycobiology* 11, 261–274.

Wollscheid, B., Bausch-Fluck, D., Henderson, C., O'Brien, R., Bibel, M., Schiess, R., Aebersold, R., and Watts, J. D. (2009). Mass-spectrometric identification and relative quantification of N-linked cell surface glycoproteins. *Nat. Biotechnol.* 27, 378–386.

Wood, C. S., Schmitz, K. R., Bessman, N. J., Setty, T. G., Ferguson, K. M., and Burd, C. G. (2009). PtdIns4P recognition by Vps74/GOLPH3 links PtdIns 4-kinase signaling to retrograde Golgi trafficking. *J. Cell Biol.* 187, 967–975.

Yan, Q., and Lennarz, W. J. (1999). Oligosaccharyltransferase: a complex multisubunit enzyme of the endoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 266, 684–689.

Yan, Q., and Lennarz, W. J. (2002). Studies on the function of oligosaccharyl transferase subunits. Stt3p is directly involved in the glycosylation process. *J. Biol. Chem.* 277, 47692.

Ytterberg, A. J., and Jensen, O. N. (2010). Modification-specific proteomics in plant biology. *J. Proteomics* 73, 2249–2266.

Zeng, Y., Bannon, G., Thomas, V. H., Rice, K., Drake, R., and Elbein, A. (1997). Purification and specificity of beta1,2-xylosyltransferase, an enzyme that contributes to the allergenicity of some plant proteins. *J. Biol. Chem.* 272, 31340–31347.

Zeng, Y. C., and Elbein, A. D. (1998). Purification to homogeneity and properties of plant glucosidase I. *Arch. Biochem. Biophys.* 355, 26–34.

Zhang, H., Ohyama, K., Boudet, J., Chen, Z., Yang, J., Zhang, M., Muranaka, T., Maurel, C., Zhu, J. K., and Gong, Z. (2008). Dolichol biosynthesis and its effects on the unfolded protein response and abiotic stress resistance in *Arabidopsis*. *Plant Cell* 20, 1879–1898.

Zhang, M., Henquet, M., Chen, Z., Zhang, H., Zhang, Y., Ren, X., van der Krol, S., Gonneau, M., Bosch, D., and Gong, Z. (2009). LEW3, encoding a putative alpha-1,2-mannosyltransferase (ALG11) in N-linked glycoprotein, plays vital roles in cell-wall biosynthesis and the abiotic stress response in *Arabidopsis thaliana*. *Plant J.* 60, 983–999.

Zhang, Y., Giboulot, A., Zivy, M., Valot, B., Jamet, E., and Albenne, C. (2010). Combining various strategies to increase the coverage of the plant cell wall glycoproteome. *Phytochemistry* 72, 1109–1123.

Zhang, Z., Feechan, A., Pedersen, C., Newman, M. A., Qiu, J. L., Olesen, K. L., and Thordal-Christensen, H. (2007). A SNARE-protein has opposing functions in penetration resistance and defence signalling pathways. *Plant J.* 49, 302–312.

Zhong, R., Burk, D. H., Morrison, W. H. III, and Ye, Z. H. (2002). A kinesin-like protein is essential for oriented deposition of cellulose microfibrils and cell wall strength. *Plant Cell* 14, 3101–3117.

Zielinska, D. F., Gnad, F., Wisniewski, J. R., and Mann, M. (2010). Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. *Cell* 141, 897–907.

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Combining polysaccharide biosynthesis and transport in a single enzyme: dual-function cell wall glycan synthases

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Extracellular polysaccharides are synthesized by a wide variety of species, from unicellular bacteria and Archaea to the largest multicellular plants and animals in the biosphere. In every case, the biosynthesis of these polymers requires transport across a membrane, from the cytosol to either the lumen of secretory pathway organelles or directly into the extracellular space. Although some polysaccharide biosynthetic substrates are moved across the membrane to sites of polysaccharide synthesis by separate transporter proteins before being incorporated into polymers by glycosyltransferase proteins, many polysaccharide biosynthetic enzymes appear to have both transporter and transferase activities. In these cases, the biosynthetic enzymes utilize substrate on one side of the membrane and deposit the polymer product on the other side. This review discusses structural characteristics of plant cell wall glycan synthases that couple synthesis with transport, drawing on what is known about such dual-function enzymes in other species.

Keywords: glycosyltransferase, membrane transport, polysaccharide synthesis, protein topology, cell wall

INTRODUCTION

Extracellular matrices (ECMs) are complex biopolymer mixtures produced by cells and deposited outside the cell membrane. Diverse eukaryotic and prokaryotic organisms throughout the biosphere produce ECMs, making the ECM a common feature of most living cells. ECMs play an integral role in many essential processes, from influencing the growth and development of cellular and organismal morphology to mediating interactions between cells and their environments.

The specific composition and structure of the ECM varies widely depending on species, cell type, developmental stage, and environmental conditions. Despite these differences, there are some aspects of ECM composition that are similar throughout most of biology. One common feature of the ECM produced by diverse species is the prevalence of polysaccharides as major structural components. Examples include pectin, hemicellulose, and cellulose polymers of the plant cell wall, chitin found in fungal, mollusk, and arthropod ECMs, chondroitin and hyaluronan synthesized by vertebrates, and peptidoglycan of bacterial cell walls.

Although there is wide variation in composition, structure, and function of ECM polysaccharides found in nature, there are certain required steps that must be accomplished during the biosynthesis of any extracellular polysaccharide. Essential processes include the synthesis of precursor molecules and polymerization of the component sugars. Additionally, because the sugar-nucleotide precursors of ECM polymers are synthesized inside the cell, transport across a membrane of glycosyltransferase (GT) substrates or polysaccharide products is also required for ECM polysaccharide synthesis. This transport might occur across the plasma membrane (PM) directly into the extracellular space, or across an endomembrane into an intracellular compartment with the polymer later secreted to the ECM.

Transport across the membrane might occur before, during, or after polysaccharide synthesis. Each of these modes of transport probably occurs in nature, depending on the polysaccharide. In some cases, different sugar moieties incorporated into a polysaccharide may be transported across the membrane by different mechanisms. ECM polysaccharides whose activated sugar precursors are transported before polymerization include the glycan components of extracellular glycoproteins (Spiro, 2002), plant pectins (Mohnen, 2008), and ECM mannans in plants and fungi (Keegstra and Raikhel, 2001; Latgé, 2007; Reyes and Orellana, 2008). These polymers are synthesized in the lumen of secretory pathway compartments from sugar-nucleotides imported from the cytosol by transport proteins. ECM polymers transported across a membrane following at least partial synthesis include bacterial peptidoglycan precursor Lipid II (undecaprenyl-pyrophosphoryl-*N*-acetylmuramyl-(pentapeptide)-*N*-acetylglucosamine) and hyaluronan synthesized by "class II" hyaluronan synthases (HSs). These molecules are transported to the extracellular space by a lipid flippase (FtsW) and an ABC (ATP-binding cassette) transporter (such as MRP5), respectively (Schulz et al., 2007; Mohammadi et al., 2011).

In contrast to transport that occurs before or after polymer synthesis, this review focuses on another mechanism of transport across the membrane: transport that occurs at the same time as polymer synthesis and directly involves the biosynthetic enzyme. Enzymes that appear to processively synthesize ECM polysaccharides while transporting the polymer across a membrane are

present in bacterial, fungal, animal, and plant systems. Such enzymes are referred to hereafter as dual-function GTs. Among the products of dual-function GTs are the some of the most economically and medically important polymers in nature. Included in this list are cellulose, the glucan backbone of the hemicellulose xyloglucan, chitin, much of the hyaluronan produced in animals, and at least two examples of polysaccharides produced by bacterial human pathogens (DeAngelis, 1999; Oglesby et al., 2008).

Because the structures have not been solved, the idea that some GTs also participate in polymer transport has not been rigorously proven. However, the topology of these proteins makes membrane transport of the polysaccharide by the glycan synthase seem likely. Dual-function GT proteins are characterized by a similar predicted topology with multiple transmembrane helices (TMHs) and an active site facing the cytosol.

In one mechanistic model for dual-function GTs, the TMHs are arranged to form a pore in the membrane. In this model, the cytosolic active site adds sugars from cytosolic substrate pools to an elongating polymer that extends across the membrane through the pore (**Figure 1**). Unfortunately, because dual-function GTs have multiple TMHs and many appear to function in protein complexes, determining their structures by traditional crystallographic methods is difficult. Nevertheless, evidence confirming the predicted topology described above continues to mount for a number of these proteins from different species (Heldermon et al., 2001; Urbanowicz et al., 2004; Zeng and Keegstra, 2008; Davis et al., 2010; Merzendorfer, 2011).

Although the group of proteins predicted to be dual-function GTs spans a large evolutionary distance, structural commonalities are likely considering their functional similarities. Because all dual-function GTs must accomplish similar processes, applying what we have learned about even distantly related proteins can be helpful in better understanding dual-function GTs in plants. Several plant ECM polysaccharide synthases have the topological requirements to be dual-function GTs. These enzymes include cellulose synthase (CESA), callose synthase (CalS), xyloglucan glucan synthase (cellulose synthase-like C4-CSLC4), and multiple related glycan synthases whose products have not been identified with certainty, including members of CSLD and CSLH clades (Verma and Hong, 2001; Zeng and Keegstra, 2008; Doblin et al., 2009; Davis et al., 2010; Carpita, 2011).

STRUCTURAL CHARACTERISTICS OF DUAL-FUNCTION GLYCOSYLTRANSFERASES

Although we are just beginning to understand the fine structure of these proteins, we can identify some factors that are likely to be necessary for any dual-function GT. One strategy to accomplish this is to attempt to predict specific structural constraints based on how these proteins function. Another useful approach is to draw upon observations about diverse dual-function GTs to identify common features. These are important initial steps to generate realistic models and to design experimental approaches that are more likely to succeed.

For a GT to transport its product across a membrane, the transmembrane regions of the protein or proteins comprising the active complex must create a pore large enough to accommodate the polysaccharide. An unsubstituted β-(1 → 4)-linked polymer of glucose (cellulose) is approximately 1 nm across in its widest axis. Because of the relatively high energy cost of partitioning glucose to a hydrophobic environment (Ha et al., 1991; Kollman, 1993; Mazzobre et al., 2005), it is likely that a glucose polymer remains hydrated as it moves across the membrane. Therefore, the absolute minimum diameter for a GT transmembrane pore would be about 1.8 nm, allowing for water molecules associated with the polysaccharide. Assuming the outer diameters of standard alpha helices range from 1 to 1.4 nm, the minimum number of TMHs required to form a pore larger than 1.8 nm is between 10 and 18 helices.

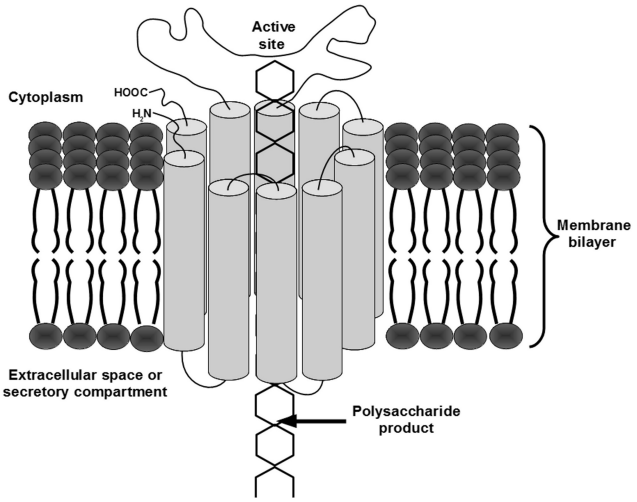


FIGURE 1 | A model for dual-function GT transport mechanism. The TMHs of the enzyme are depicted as cylinders connected by curved lines representing the extra-membrane regions of the protein. A polymer is shown being transported through a pore formed by the TMHs, from the cytosolic active site where sugars are added, across the membrane to the extracellular space or lumen of the secretory pathway.

Although useful as a starting point for modeling the number of TMHs required for a dual-function GT, the calculations above are necessarily based on assumptions about the minimum diameter of a polysaccharide transport pore and the diameter of TMHs. In addition, this estimate assumes that TMHs are oriented perpendicular to the plane of the membrane. In reality, it is likely that some pore-forming TMHs of a dual-function GT span the membrane at an angle, creating a larger pore. For example, one bacterial HS has five to six membrane domains, functions as a monomer, and transports a polysaccharide with a wider diameter than the β-(1 → 4)-linked glucan described above (Tlapak-Simmons et al., 1998). This is possible in part because one or more of the membrane domains of this bacterial HS appear to be reentrant loops, occupying more space parallel to the plane of the membrane than a TMH inserted perpendicular to the membrane plane (Heldermon et al., 2001).

Another likely structural requirement for a pore-forming membrane protein is the ability to restrict free diffusion of water and other small molecules across the membrane. If a pore large enough to accommodate a hydrated polysaccharide were to remain open in the absence of a polymer occupying the pore, the electrochemical potential of the membrane would be compromised. Therefore,

it is likely that dual-function GTs have a mechanism for pore gating. Gating could be mediated by the presence of an accessory protein, as occurs with the Sec61 translocon complex and the Hsp70 chaperone BiP (Alder et al., 2005). Alternatively, GT pore gating could be controlled by conformational changes in the GT itself. This might be accomplished through substrate binding or post-translational modification such as phosphorylation. Precedent for these mechanisms can be found in ligand-gated ion channels (Keramidasa et al., 2004) and the regulation of aquaporins by phosphorylation (Chaumont et al., 2005). In addition to the general requirement of cells to maintain membrane potential, this can be important for the function of dual-function GTs themselves, as evidenced by the ion potential requirement for the activity of CESA in plants and bacteria (Bacic and Delmer, 1981; Delmer et al., 1982).

There is a third possibility for how organisms with dual-function GTs might prevent compromise of the membrane gradient. By the time a dual-function GT protein is mature and competent to form a pore, it could be localized to a specialized vesicular compartment where free diffusion across the membrane has little consequence. Fusion of such vesicles to the appropriate membrane where polysaccharide biosynthesis occurs would be coupled with activation of the GT, followed by removal of the GT from the diffusion-sensitive membrane when synthesis is complete. If such a mechanism exists, it could provide a partial explanation for atypical vesicles observed in chitin (chitosomes) and cellulose biosynthesis (MASCs), and might relate to why CESA proteins spend such a short time at the PM (Bartnicki-Garcia, 2006; Crowell et al., 2010; Wightman and Turner, 2010).

Many dual-function GTs function in enzyme complexes, so proteins other than the glycan synthase may participate in the formation of a pore. Cocuron et al. (2007) found that *Pichia pastoris* expressing both the xyloglucan glucan synthase CSLC4 and the xyloglucan xylosyltransferase XXT1 accumulated insoluble, unsubstituted β-(1 → 4)-linked glucan. Much lower levels of this polymer accumulated in both wild-type *Pichia* and lines expressing only CSLC4, which produced shorter, soluble oligoglucans that were absent in dual-expressing and wild-type lines. In other words, CSLC4 functions as an active glucan synthase in both transgenic yeast cell lines, but produces a product with a much higher degree of polymerization when co-expressed with XXT1. Interestingly, *Pichia* does not synthesize UDP-xylose, the substrate of XXT1, and the β-(1 → 4)-linked glucan produced is unsubstituted. One explanation for this observation could be that XXT1 (a type II membrane protein) participates in the formation of a pore with CSLC4.

At least some dual-function GTs may be dependent on non-protein membrane components. A major advance in our understanding of plant CESA biochemistry has been the development of techniques to measure cellulose and callose synthesis *in vitro* (for a review, see Harris et al., 2010). This is accomplished through the isolation of detergent-resistant membranes (DRMs) and subsequent incubation of these membrane fractions with UDP-glucose. To date, there have been no successful attempts to solubilize active CESA away from DRMs.

Known as lipid "rafts" or membrane microdomains, the biological structures from which DRMs are derived have specific lipid and protein compositions that are distinct from the rest of the PM (Mongrand et al., 2010). A requirement for a specific lipid or sterol environment for active cellulose synthase complexes (CSCs) is consistent with these lipids playing a structural role in the complex, perhaps in allowing the formation of a transport pore(s). In support of this idea, a monomeric bacterial HS from *Streptococcus* requires multiple cardiolipin molecules for activity. It has been suggested that a specific number of cardiolipin molecules are associated with each active HS, playing a structural role to form a pore sufficient to accommodate the polymer product (Tlapak-Simmons et al., 1998).

One plant sterol in particular, sitosterol-β-glucoside (SG), has been implicated in cellulose biosynthesis. It has been suggested that this molecule may serve as a primer for initiation of cellulose synthesis (Peng et al., 2002). However, *Arabidopsis* plants with T-DNA insertions in two UDP-glucose:sterol glucosyltransferases are deficient in SG synthesis but not affected in cellulose accumulation (DeBolt et al., 2009). This observation is not consistent with SG functioning as a primer for cellulose, though the possibility that residual levels of SG in these plants is sufficient cannot be completely ruled out. An alternative explanation is that SG plays a structural role in the CSC, perhaps functioning in a manner similar to the putative role of cardiolipin in bacterial HS. If SG functions to promote proper folding of CESA proteins in the membrane, such a role might be more readily filled by unglycosylated sitosterol compared to the theoretical priming function. It is important to note that these two alternative functions for SG are not necessarily mutually exclusive.

Enzymes that are able to synthesize a polymer while extruding it across the membrane have a number of common structural features required for function, some of which are discussed above. Accomplishing membrane transport of a polymer product coupled with synthesis requires a certain level of complexity and imposes specific structural and compositional constraints on the enzyme and its lipid environment. It is perhaps surprising, then, that dual-function GTs are so widely distributed in diverse biological systems.

WHY DID DUAL-FUNCTION GTs EVOLVE?

With the prevalence of putative dual-function GTs in ECM polysaccharide biosynthesis from diverse species, it seems likely that the dual-function mechanism provides an evolutionary advantage. One possibility is that dual-function GTs allow greater control over enzyme function from within the cell. For example, the predicted topology of CESA proteins places over 90% of the hydrophilic regions of these proteins facing the cytosol. This presents large surfaces of CESA proteins to the cytosolic milieu, providing ample opportunity for interactions with cytosolic factors.

Multiple phosphorylation sites have been identified on CESA proteins using mass spectrometry-based approaches (Nühse et al., 2004; Taylor, 2007). Work by Taylor (2007) connected CESA7 protein phosphorylation at sites near the protein's amino terminus with proteasome-dependent degradation. This study opens many potential avenues of exploration. For instance, under what circumstances is CESA7 targeted for phosphorylation, and which proteins

control the phosphorylation status? Does phosphorylation target CESA7 for proteasome degradation directly? Alternatively, might phosphorylation regulate another process such as CSC assembly or motility, which subsequently results in proteasome degradation?

Chen et al. (2010) investigated potential physiological roles of multiple phosphorylation sites on CESA1. The researchers expressed versions of CESA1 with mutations designed to eliminate or mimic protein phosphorylation at several sites. Depending on the phosphorylation site and modification, some transgenic plants exhibited altered cell expansion anisotropy. In the plants with altered cell expansion, movement of CSCs along microtubules was also affected. Though the molecular mechanisms are not understood, this work indicates that CESA phosphorylation can influence microtubule-directed translation of actively synthesizing CSCs, potentially through interactions with proteins connecting the CSC to microtubule "tracks."

Recently, Gu et al. (2010) identified a protein that appears to be involved with physically connecting CSCs to cortical microtubules, highlighting another important interaction between the dual-function GT CESA proteins and cytosolic factors. Through a yeast two-hybrid approach, Gu et al. (2010) identified an armadillo repeat protein they termed cellulose synthase-interactive protein 1 (CSI1). Plants with disrupted *CSI1* have reduced CSC motility and less uniformity of cellulose microfibril orientation (Gu et al., 2010). The *CSI1* gene was later shown to be allelic with a previously identified cell expansion mutant *POM-POM2* (Hauser et al., 1995) and further characterized by Bringmann et al. (2012). This work convincingly demonstrates that POM2/CSI1 protein is important for CESA protein association with microtubules (Bringmann et al., 2012). Considering the results of Chen et al. (2010) and the recent work on CSI1, it will be interesting to see whether CESA phosphorylation is related to CSI1 function.

In addition to controlling motility and degradation of CESA, another potential advantage of a cytosolic active site involves substrate availability. As mentioned above, the activated sugars that are substrates for polysaccharide synthesis are made primarily in the cytosol. So cytosolic active sites allow GTs to access substrate without requiring an independent transport protein. This might be of particular advantage for PM-localized dual-function GTs, where relatively labile activated sugars might degrade or diffuse away from sites of synthesis if exported before polymer synthesis. Taking this idea a step further, a cytosolic active site might allow the development of protein interactions for metabolite channeling. Indeed, sucrose synthase (SUS), which converts sucrose into fructose and UDP-glucose, the substrate for cellulose biosynthesis, has been observed at the PM (Amor et al., 1995) directly associated with sites of cellulose synthesis and structures isolated from membranes that appear to be CSCs (Salnikov et al., 2000; Fujii et al., 2010).

Another potentially advantageous structural property of dual-function GTs involves the TMHs. If the model shown in **Figure 1** is representative of the true structures of these proteins, the acceptor substrate (elongating polysaccharide) is situated among the TMHs during elongation. These regions may have other functions in addition to forming a pore for polymer transport across the membrane. It is possible that the TMHs could affect enzyme processivity by holding the product/acceptor substrate in place during catalysis. Recent work by Harris et al. (2012) showed that mutations within a TMH of a CESA protein can reduce cellulose microfibril crystallinity and alter the speed of CSC movement on the PM. The authors present evidence that the TMHs of dual-function GTs may have a role in promoting appropriate interactions between the polymer product and other molecules in the extracellular space. For example, the TMHs might participate in orienting the polysaccharide as it is extruded across the membrane, which could in turn affect cellulose crystallinity.

CONCLUSION

Dual-function GTs are fascinating biochemical machines that synthesize ECM polysaccharides in a remarkable variety of biological systems. We are just beginning to understand how these proteins function, and ongoing research is likely to produce exciting advances in the coming years. A better understanding of these proteins will provide insight into ECM synthesis, one of the most fundamental biological processes in nature. Knowing how these enzymes function can also lead to the ability to modulate their activities, which could have tremendous impact on plant biotechnology and human health.

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REFERENCES

Alder, N. N., Shen, Y., Brodsky, J. L., Hendershot, L. M., and Johnson, A. E. (2005). The molecular mechanisms underlying BiP-mediated gating of the Sec61 translocon of the endoplasmic reticulum. *J. Cell Biol.* 168, 389–399.

Amor, Y., Haigler, C. H., Johnson, S., Wainscott, M., and Delmer, D. P. (1995). A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9353–9357.

Bacic, A., and Delmer, D. P. (1981). Stimulation of membrane-associated polysaccharide synthetases by a membrane potential in developing cotton fibers. *Planta* 152, 346–351.

Bartnicki-Garcia, S. (2006). Chitosomes: past, present and future. *FEMS Yeast Res.* 6, 957–965.

Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M.-T., and Persson, S. (2012). POM-POM2/CELLULOSE SYNTHASE INTERACTING1 is essential for the functional association of cellulose synthase and microtubules in *Arabidopsis*. *Plant Cell* 24, 163–177.

Carpita, N. (2011). Update on mechanisms of plant cell wall biosynthesis: how plants make cellulose and other (1 → 4)-β-D-glycans. *Plant Physiol.* 155, 171–184.

Chaumont, F., Moshelion, M., and Daniels, M. J. (2005). Regulation of plant aquaporin activity. *Biol. Cell* 97, 749–764.

Chen, S., Ehrhardt, D. W., and Somerville, C. R. (2010). Mutations of cellulose synthase (CESA1) phosphorylation sites modulate anisotropic cell expansion and bidirectional mobility of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17188–17193.

Cocuron, J.-C., Lerouxel, O., Drakakaki, G., Alonso, A.-P., Liepman, A., Keegstra, K., Raikhel, N., and Wilkerson, C. (2007). A gene from the cellulose synthase-like C family encodes a xyloglucan synthase.

Proc. Natl. Acad. Sci. U.S.A. 104, 8550–8555.

Crowell, E. F., Gonneau, M., Stierhof, Y.-D., Höfte, H., and Vernhettes, S. (2010). Regulated trafficking of cellulose synthases. *Curr. Opin. Plant Biol.* 13, 700–705.

Davis, J., Brandizzi, F., Liepman, A., and Keegstra, K. (2010). *Arabidopsis* mannan synthase CSLA9 and glucan synthase CSLC4 have opposite orientations in the Golgi membrane. *Plant J.* 64, 1028–1037.

DeAngelis, P. L. (1999). Hyaluronan synthases: fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. *Cell. Mol. Life Sci.* 56, 670–682.

DeBolt, S., Scheible, W.-R., Schrick, K., Auer, M., Beisson, F., Bischoff, V., Bouvier-Navé, P., Carroll, A., Hematy, K., Li, Y., Milne, J., Nair, M., Schaller, H., Zemla, M., and Somerville, C. R. (2009). Mutations in UDP-glucose:sterol glucosyltransferase in *Arabidopsis* cause transparent testa phenotype and suberization defect in seeds. *Plant Physiol.* 151, 78–87.

Delmer, D. P., Benziman, M., and Padan, E. (1982). Requirement for a membrane potential for cellulose synthesis in intact cells of *Acetobacter xylinum*. *Proc. Natl. Acad. Sci. U.S.A.* 79, 5282–5286.

Doblin, M. S., Pettolino, F. A., Wilson, S. M., Campbell, R., Burton, R. A., Fincher, G. B., Newbigin, E., and Bacic, A. (2009). A barley cellulose synthase-like CSLH gene mediates (1,3;1,4)-β-D-glucan synthesis in transgenic *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5996–6001.

Fujii, S., Hayashi, T., and Mizuno, K. (2010). Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant Cell Physiol.* 51, 294–301.

Gu, Y., Kaplinsky, N., Bringmannc, M., Cobb, A., Carrolla, A., Sampathkumar, A., Baskin, T. I., Persson, S., and Somerville, C. R. (2010). Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12866–12871.

Ha, S., Gao, J., Tidor, B., Brady, J. W., and Karplus, M. (1991). Solvent effect on the anomeric equilibrium in D-glucose: a free energy simulation analysis. *J. Am. Chem. Soc.* 113, 1553–1557.

Harris, D. M., Bulone, V., Ding, S.-Y., and DeBolt, S. (2010). Tools for cellulose analysis in plant cell walls. *Plant Physiol.* 153, 420–426.

Harris, D. M., Corbin, K., Wang, T., Gutierrez, R., Bertolo, A. L., Petti, C., Smilgiese, D.-M., Estevez, J. M., Bonetta, D., Urbanowicz, B. R., Ehrhardt, D. W., Somerville, C. R., Rose, J. K. C., Hong, M., and DeBolt, S. (2012). Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CESA1^{A903V} and CESA3^{T942I} of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4098–4103.

Hauser, M. T., Morikami, A., and Benfey, P. N. (1995). Conditional root expansion mutants of *Arabidopsis*. *Development* 121, 1237–1252.

Heldermon, C., DeAngelis, P. L., and Weigel, P. (2001). Topological organization of the hyaluronan synthase from *Streptococcus pyogenes*. *J. Biol. Chem.* 276, 2037–2046.

Keegstra, K., and Raikhel, N. (2001). Plant glycosyltransferases. *Curr. Opin. Plant Biol.* 4, 219–224.

Keramidasa, A., Moorhousea, A. J., Schofieldb, P. R., and Barrya, P. H. (2004). Ligand-gated ion channels: mechanisms underlying ion selectivity. *Prog. Biophys. Mol. Biol.* 86, 161–204.

Kollman, P. (1993). Free energy calculations: applications to chemical and biochemical phenomena. *Chem. Rev.* 93, 2395–2417.

Latgé, J. (2007). The cell wall: a carbohydrate armour for the fungal cell. *Mol. Microbiol.* 66, 279–290.

Mazzobre, M. F., Román, M. V., Mourelle, A. F., and Corti, H. R. (2005). Octanol–water partition coefficient of glucose, sucrose, and trehalose. *Carbohydr. Res.* 340, 1207–1211.

Merzendorfer, H. (2011). The cellular basis of chitin synthesis in fungi and insects: common principles and differences. *Eur. J. Cell Biol.* 90, 759–769.

Mohammadi, T., van Dam, V., Sijbrandi, R., Vernet, T., Zapun, A., Bouhss, A., Diepeveen-de Bruin, M., Nguyen-Distèche, M., de Kruijff, B., and Breukink, E. (2011). Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *EMBO J.* 30, 1425–1432.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant Biol.* 11, 266–277.

Mongrand, S., Stanislas, T., Baye, E. M. F., Lherminier, J., and Simon-Plas, F. (2010). Membrane rafts in plant cells. *Trends Plant Sci.* 15, 656–663.

Nühse, T. S., Stensballe, A., Jensen, O. N., and Peck, S. C. (2004). Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell* 16, 2394–2405.

Oglesby, L. L., Jain, S., and Ohman, D. E. (2008). Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. *Microbiology* 154, 1605–1615.

Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002). Sitosterol-β-glucoside as primer for cellulose synthesis in plants. *Science* 295, 147–150.

Salnikov, V. V., Grimson, M. J., Delmer, D. P., and Haigler, C. H. (2000). Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. *Phytochemistry* 57, 823–833.

Schulz, T., Schumacher, U., and Prehm, P. (2007). Hyaluronan export by the ABC transporter MRP5 and its modulation by intracellular cGMP. *J. Biol. Chem.* 282, 20999–21004.

Spiro, R. G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12, 43R–56R

Reyes, F., and Orellana, A. (2008). Golgi transporters: opening the gate to cell wall polysaccharide biosynthesis. *Curr. Opin. Plant Biol.* 11, 244–251.

Taylor, N. G. (2007). Identification of cellulose synthase AtCesA7 (IRX3) *in vivo* phosphorylation sites – a potential role in regulating protein degradation. *Plant Mol. Biol.* 64, 161–714.

Tlapak-Simmons, V. L., Kempner, E. S., Baggenstoss, B. A., and Weigel, P. H. (1998). The active streptococcal hyaluronan synthases (HASs) contain a single HAS monomer and multiple cardiolipin molecules. *J. Biol. Chem.* 273, 26100–26109.

Urbanowicz, B. R., Rayon, C., and Carpita, N. C. (2004). Topology of the maize mixed linkage (1 → 3),(1 → 4)-β-D-glucan synthase at the Golgi membrane. *Plant Physiol.* 134, 758–768.

Verma, D. P. S., and Hong, Z. (2001). Plant callose synthase complexes. *Plant Mol. Biol.* 47, 693–701.

Wightman, R., and Turner, S. (2010). Trafficking of the plant cellulose synthase complex. *Plant Physiol.* 153, 427–432.

Zeng, W., and Keegstra, K. (2008). AtCSLD2 is an integral Golgi membrane protein with its N-terminus facing the cytosol. *Planta* 228, 823–838.

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Xyloglucan and its biosynthesis

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The hemicellulosic polysaccharide xyloglucan (XyG), found in the primary cell walls of most plant tissues, is important for structural organization of the cell wall and regulation of growth and development. Significant recent progress in structural characterization of XyGs from different plant species has shed light on the diversification of XyG during plant evolution. Also, identification of XyG biosynthetic enzymes and examination of their interactions suggests the involvement of a multiprotein complex in XyG biosynthesis. This mini-review presents an updated overview of the diversity of XyG structures in plant taxa and recent findings on XyG biosynthesis.

Keywords: xyloglucan structure, biosynthesis, glycosyltransferases, multiprotein complex

STRUCTURAL DIVERSITY OF THE XYLOGLUCANS

Xyloglucan (XyG) is a hemicellulosic polysaccharide found in all land plants (Popper and Fry, 2003, 2004; Popper, 2008; Sarkar et al., 2009) in varying amounts; for example, XyG is a major hemicellulosic component of the primary cell wall of flowering plants (up to 25%), but a minor, sometimes barely detectable, constituent of grasses (less than 2%; Hsieh and Harris, 2009). XyGs have a β-1,4-glucan backbone that is highly branched, with characteristic repetitive patterns of α-Xyl residues linked to glucose at the *O*-6 position. The side chain xylosyl residues can be further substituted with different mono-, di-, or trisaccharides; the pattern of these substitutions produces the broad diversity of XyG structural motifs that are present in different plant species (Hoffman et al., 2005; Pena et al., 2008; Hsieh and Harris, 2009). A single-letter nomenclature was introduced by Fry et al. (1993), to describe the XyG backbone substitution pattern. For example, the letter G indicates an unbranched Glc residue, and X denotes the α-D-Xyl-(1 → 6)-β-D-Glc motif in the xylosylated glucan backbone. Xyl residues can carry a β-D-Gal (L motif), an α-L-Ara (S motif), or a β-D-Xyl (U motif; Hoffman et al., 2005). In turn, Gal residues in the L side chain can be linked to an α-L-Fuc residue (F motif; Hoffman et al., 2005), or an α-L-Gal (J motif; Hantus et al., 1997); also, the Ara residue in the S side chain can carry a β-L-Ara (T motif; York et al., 1996). In addition, Pena et al. (2008) found that avascular plants, such as mosses and liverworts, can form XyG with a 2,4-linked Xyl residue, where xylosyl can be substituted with a β-D-GalA and a β-D-Gal (P motif), with an α-L-Ara and a β-D-Gal (M motif), an α-L-Ara and the disaccharide β-D-Gal-(1 → 6)-β-D-GalA (N motif), or the disaccharide β-D-Gal-(1 → 6)-β-D-GalA and a β-D-Gal at the *O*-2 and *O*-4 positions, respectively.

Different patterns and types of XyG subunits can be found in different species and different tissues. Most vascular seed-bearing plants synthesize XXXG-type XyG (O'Neill and York, 2003; Hoffman et al., 2005), but grasses and some lamiids produce XXGG- and XXGGG-type XyG with fewer Xyls on the glucan backbone (Gibeaut et al., 2004; Hoffman et al., 2005). Typically, XXXG-type XyG comprises XXXG, XXFG, XXLG, and XLFG subunits, which are present in different proportions depending on the plant tissue and developmental stage (Pauly et al., 2001; Obel et al., 2009). In many flowering and non-flowering plants, the Fuc residue is linked to β-D-Gal at the *O*-2 position, while the XyG in Equisetum and Selaginella has the Fuc linked to an α-L-Ara residue at the *O*-2 position (E motif; Pena et al., 2008). It was proposed that fucosylated XyG was first synthesized in a common ancestor of hornworts and vascular plants (Pena et al., 2008). Gal residues in XXLG, XXFG, and XLFG can carry acetyl groups as was found in *Arabidopsis* and sycamore XyG (Kiefer et al., 1989; Gille et al., 2011).

The commelinid monocotyledons have predominantly non-fucosylated XXGn-type XyG. Thus, the Zingiberales and Commelinales have both XXGn and XXXG core motifs with few XXFG units, and no XLFG (Hsieh and Harris, 2009). In the Poales, the Poaceae have exclusively XXGn-type XyG without Fuc, but the other families contain either the mixed type XyG with XXXG and XXGn core motifs or only XXXG and XXFG motifs, but no XLFG (Hsieh and Harris, 2009). Frequently, in XXGG- or XXGGG-type XyG, one or two unbranched Glc residues have acetyl groups instead of the α-Xyl (Hoffman et al., 2005).

del Bem and Vincentz (2010) constructed an evolutionary model of the emergence of XyG-related genes in Viridiplantae, proposing a stepwise increase in XyG branching complexity starting from XyG-like molecules that contained only Glc and Xyl, which are found in streptophyte algae, to galactosylated motifs, which emerged in embryophytes, and finally to fucosylated XyGs, which emerged in the last common ancestors of spermatophytes. Although XyG has never been directly released from algae cell walls, some indirect evidence indicates that certain green algae contain XyG-like polysaccharide (VanSandt et al., 2007; Fry et al., 2008), additionally, XyG epitopes were immunologically detected in some members of evolutionarily advanced charophycean green algae orders (Sorensen et al., 2010, 2011). Since liverworts, which

are believed to be the oldest extant land plant family (Qiu et al., 2006), have XXGG- and XXGGG-type XyG, it was proposed that XXXG-type motifs evolved later in hornworts and many vascular plants (Pena et al., 2008). Demonstration in hornworts of the presence of fucosylated XyG with similarities to seed-bearing plant XyG motifs allowed Pena et al. (2008) to propose that in some Lamiids and grasses, the genes encoding XyG-specific fucosyltransferases may have been eliminated completely, or are expressed only in specific cells or conditions.

XYLOGLUCAN BIOSYNTHESIS

Xyloglucan biosynthesis requires glucan synthase to form the glucan backbone and requires multiple different types of glycosyl transferases to decorate the glucan chain to produce the broad diversity of XyG side chains found in various plants. Considering the high specificity of glycosyl transferases (Keegstra and Raikhel, 2001), the formation of each linkage is believed to require a distinct transferase; therefore, a combination of at least one (1,4)-β-glucan synthase, three (1,6)-α-xylosyltransferases, two (1,2)-β-galactosyltransferases, and one (1,2)-α-fucosyltransferase is needed to assemble a complete XLFG subunit.

The presence of other XyG motifs discovered in different taxa, and briefly described in the first section, implicates involvement of other types of glycosyltransferases in various plant species; these transferases may include XyG specific arabinosyltransferases, galacturonyltransferases, additional galactosyltransferases, and xylosyltransferases that would elongate diverse XyG side chains. For example, in *Arabidopsis*, XyG biosynthesis requires at least five types of enzymatic activities: UDP-Glc-dependent glucan synthase to synthesize the glucan chain, UDP-Xyl-dependent xylosyltransferases to transfer Xyl onto a specific Glc in the glucan chain, UDP-Gal-dependent galactosyltransferases to transfer Gal onto Xyl and elongate side chains attached to the second and third Glc in the XyG subunit, GDP-Fuc-dependent fucosyltransferase to transfer Fuc onto Gal in the side chain attached to the third Glc in the XyG subunit, and finally XyG specific acetyltransferase to attach the acetyl group to Gal.

Candidate genes encoding all these enzymes have been identified and partially characterized using a combination of biochemical and genetic approaches (**Figure 1**). For example, the amino acid sequence of fucosyltransferase (FUT1) purified from pea was used to identify *Arabidopsis FUT1* from the GT37 gene family (Perrin et al., 1999), and heterologous expression demonstrated that *Arabidopsis* and pea *FUT1* genes encode proteins with XyG fucosyltransferase activity (Perrin et al., 1999; Faik et al., 2000). Complete elimination of fucosylated XyG subunits in the *Arabidopsis FUT1* T-DNA knock-out mutant suggests that XyG specific fucosyltransferase activity in *Arabidopsis* is encoded by a single gene (Keegstra and Cavalier, 2011).

XyG galactosyltransferases have also been identified. Discovered in a screen for mutants with aberrant cell wall formation, *Arabidopsis thaliana MUR3* (Reiter et al., 1997) encodes a XyG galactosyltransferase that adds galactose specifically to the third xylosyl residue, forming an XXLG subunit (Madson et al., 2003). More recently, a second XyG galactosyltransferase was discovered by RNA-Seq analysis of developing nasturtium seeds and then confirmed by mutation of the *Arabidopsis* ortholog (At5g62220).

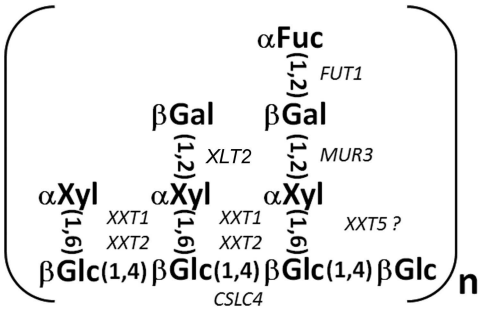


FIGURE 1 | The structure of the XLFG XyG subunit. Glycosyl transferases known to form particular linkages are shown in corresponding positions. The catalytic activity of XXT5 and XLT2 has not been confirmed.

The *Arabidopsis* gene, named XyG *L-side chain galactosyltransferase* (*XLT2*) showed no redundancy with *MUR3*, and is required for galactosylation of the second xylose in the XyG subunit (Jensen et al., 2012).

A gene family (*CAZy* GT34Family), containing seven genes spread among three clades in *Arabidopsis*, was identified and predicted to encode XyG xylosyltransferases (Faik et al., 2002). Heterologous expression of two of those genes, *XXT1* and *XXT2*, demonstrated that the encoded proteins have xylosyltransferase activity (Cavalier and Keegstra, 2006). The studies also demonstrated that XXT1 and XXT2 have the same substrate-acceptor specificity, catalyzing the substitution of two glycosidic residues in adjacent positions, and thereby generating a GGXXGG structure. Also, *xxt1 xxt2* double mutant plants lack detectable XyG in their cell walls (Cavalier et al., 2008), confirming that XXT1 and XXT2 are XyG xylosyltransferases that are essential for XyG formation. Application of reverse genetics demonstrated that another member of the GT34 gene family, *XXT5*, is also involved in XyG biosynthesis, although its activity has not been demonstrated *in vitro* (Zabotina et al., 2008). The lack of detectable XyG in the *xxt1 xxt2* double mutant plants challenges conventional models for the functional organization of the primary cell wall and also existing assumptions about linkage-specific enzyme relationships in polysaccharide biosynthesis. The *xxt1* and *xxt2* single mutant plants each have only a slight decrease in XyG content, but the *xxt5* single mutant has a 50% reduction in XyG content and the XyG that is made in the *xxt5* mutant plant shows an altered subunit composition (Zabotina et al., 2008). However, the knock-out of *XXT5* does not eliminate xylosylation of any particular glucose in the XyG backbone, which suggests that the absence of XXT5 protein is compensated for, at least in part, by the presence of the other two xylosyltransferases. Thus, the ability of XXT1 and XXT2 to partially compensate for the lack of XXT5 in synthesizing fully xylosylated XyG subunits raises questions about their specificity with respect to which glucose in the glucan backbone is the target of their activity. The studies of two additional double mutants, *xxt1 xxt5* and *xxt2 xxt5*, as well as a triple mutant line, *xxt1 xxt2 xxt5*, revealed further complexity in the functional relationship of XXT proteins. A combination of biochemical and immunological analyses (Zabotina et al., 2012) demonstrated that either XXT1

or XXT2 alone is sufficient to synthesize the complete set of XyG subunits, although in significantly lower amounts compared with plants that have XXT5 in addition to XXT1 and XXT2. Thus, either XXT1 or XXT2 is capable of adding all three of the xylosyl residues present in XyG; this confirms earlier in *in vitro* experiments (Cavalier and Keegstra, 2006). However *in vivo*, the efficiency of XXT1 or XXT2 when functioning alone is significantly lower than when the three proteins are present together. Also, although the XXT5 protein itself does not seem to be capable of adding xylosyl residues to the XyG backbone *in vivo*, the lack of this protein causes the most dramatic impact on XyG biosynthesis.

The *XXT1*, *XXT2*, and *XXT5* double and single mutant phenotypes indicate that these loci encode the major XyG xylosyltransferases. Two members of the *Arabidopsis GT34* gene family (At4g37690 and At2g22900) are closely related to the galactomannan galactosyltransferases identified by Edwards et al. (1999), which suggests that they encode galactosyltransferases (Keegstra and Cavalier, 2011). Two other members of this family (At1g18690 and At1g74380) are expressed at very low levels in all tissues that were examined (http://www.weigelworld.org/resources/microarray/AtGenExpress/; Schmid et al., 2005) and are therefore unlikely to contribute significantly to XyG biosynthesis. This supports the hypothesis that XXT1, XXT2, and XXT5 are the main XyG xylosyltransferases that synthesize XyG, at least in the major plant tissues. Analysis of microarray data showed that these three *XXT* genes are expressed in all tissues that were analyzed, but have different levels of expression. In the majority of tissues, *XXT2* has approximately twofold higher expression than *XXT1* and *XXT5*, but *XXT2* and *XXT5* have comparable expression levels except in a few specialized tissues such as stamen and root pericycle, where *XXT1* has the highest expression level of the three genes.

The members of the GT2 gene family, *Arabidopsis cellulose synthase-like C* genes (*CSLC4*, *CSLC5*, and *CSLC6*) are believed to be involved in XyG biosynthesis, being implicated in glucan backbone synthesis. Initially, *CSLC4* was identified as a candidate gene for the (1,4)-β-glucan synthase in nasturtium and *Arabidopsis* (Cocuron et al., 2007). Heterologously expressed in *Pichia pastoris* cells, CSLC4 produces cellodextrins. Moreover, when XXT1 was co-expressed in the same cells, longer chains were detected, indicating that XXT1 can assist CSLC4 in glucan synthesis. Since yeast cells cannot produce UDP-xylose, xylosylation of the synthesized glucan oligomers was not observed. Later, reverse-genetic studies suggested that two other genes, *CSLC5* and *CSLC6*, are also involved in XyG biosynthesis (Cavalier and Keegstra, 2010). Among these three genes, *CSLC4* has higher levels of expression in all *Arabidopsis* tissues and also is expressed in the same tissues and with the same developmental timing as the *XXT*s, but expression of *CSLC5* and *CSLC6* is limited to specific tissues (Schmid et al., 2005).

In *Arabidopsis* XyG, acetyl groups are exclusively linked to galactosyl residues. Recently, the putative XyG specific acetyltransferases, AXY4 and AXY4L, were discovered in *Arabidopsis* using a forward genetic screen (Gille et al., 2011), although their catalytic activity and substrate specificity have yet to be confirmed. It is also unclear whether AXY4 attaches acetyl groups to all galactosyl residues or only to some of them, and whether this happens during XyG biosynthesis or after XyG is completely synthesized. When AXY4 was overexpressed in wild type *Arabidopsis*, cell wall XyG still contained unacetylated galactoses (Gille et al., 2011), suggesting the possible involvement of other AXY proteins. Whether AXY4 can interact with any of the proteins involved in XyG biosynthesis remains to be investigated as part of an exploration of the biological role of XyG acetylation.

DOES A XyG SYNTHASE COMPLEX EXIST?

Identification of glycosyl transferases that can potentially fully decorate the XyG glucan backbone and synthesize the complete XyG structure (**Figure 1**) stimulated further investigations to understand their functional organization in the Golgi. The few available examples suggest that glycosyl transferases localized in the Golgi might function in multiprotein complexes (Atmodjo et al., 2011; Harholt et al., 2012). Indeed, recent data suggest that proteins involved in XyG biosynthesis are co-localized in multiprotein homo- and hetero-complexes and most likely interact through their catalytic domains. For example, BiFC assays using *Arabidopsis* protoplasts transiently expressing transferases fused with the C and N parts of YFP demonstrated approximate co-localization of XyG xylosyltransferases in two hetero-complexes, XXT2-XXT5 and XXT1-XXT2; XXT2 was also shown to form an XXT2-XXT2 homo-complex (Chou et al., 2012). Using the same approach, XXT5 and CSLC4 were shown to form a hetero-complex, XXT5-CSLC4. Results from *in vitro* pull-down experiments using recombinant proteins expressed without transmembrane domains confirmed the interactions between XXT2 and XXT5, and XXT1 and XXT2, suggesting that these proteins can physically interact through their catalytic domains (Chou et al., 2012).

A recent study (Davis et al., 2010) suggested that CSLC4 is positioned in the Golgi membrane with its active loop and both C- and N-termini protruding to the cytosolic side of the Golgi. BiFC assays showed that CSLC4 forms homo-complexes and switching the C and N YFP fragments fused to either the C or N-terminus of CSLC4 did not affect the intensity of reconstituted YFP fluorescence (Chou et al., 2012). This suggests that both termini are localized close to each other, while the CSLC4 active loops are positioned on the outer sides of the homo-complex, farther from each other.

Similarly, BiFC assays demonstrated that MUR3 and FUT1 also co-localize and interact with each other and with either XXT2 or CSLC4. These results have yet to be confirmed by an independent approach, but it is plausible to believe that during XyG formation, these proteins likely come into close proximity to XXTs to finalize the complete XyG structure. Whether all these proteins form a single multiprotein complex or a more dynamic structure that differs in different situations has yet to be investigated. For example, it has been suggested that the fucosyltransferase FUC1 functions independently from glucan elongation and xylosylation (Faik et al., 1997a,b). After using pea microsomal fractions to study XyG formation *in vitro*, the authors proposed that fucosylation of galactosylated heptasaccharide occurs after complete formation of the glucan backbone. Additionally, Chevalier et al. (2010), using *Arabidopsis* XXT1, MUR3, and FUT1 fused with GFP and expressed in *N. tabacum* BY-2 cells, showed that XXT1 and MUR3 localized predominantly in Golgi cis and medial cisternae, respectively, but FUT1 was detected in trans cisternae. This

localization supports the hypothesis that the fucosyltransferase may be spatially independent. However, it is worth noting that tobacco XyG does not contain terminal fucose in its subunits; therefore *Arabidopsis* FUT1 may not have been able to establish the proper interactions to be correctly localized in *N. tabacum* Golgi. Further elucidation of the structure of the XyG synthase complex is needed to understand its functions, and the regulation of XyG biosynthesis. It is hypothesized (Gunl et al., 2011) that XyG synthesized in Golgi has, most likely, an XXXGXXFG repeating pattern and the diversity of XyG structure is created after its deposition into the cell wall. This regular structure would derive most efficiently from cooperative action of multiple XyG biosynthetic enzymes organized in a multiprotein complex in the Golgi, rather than from spatially and functionally independent enzymes.

CONCLUDING REMARKS

During the last decade, significant progress has been made in revealing the proteins involved in XyG biosynthesis, which, together with detailed structural studies, makes this important cell wall polysaccharide a good model for examining the mystery of polysaccharide evolution, formation, and modification. Accumulating evidence demonstrates that the evolution of polysaccharide structural complexity during land colonization produced key adaptations, including cell wall mechanical strengthening needed to support plants in the absence of water buoyancy and to protect them against biotic and abiotic environmental stresses. To provide this complexity, plants evolved complex and dynamic polysaccharide synthesizing machinery localized in the Golgi. Enzymes involved in polysaccharide biosynthesis are most likely organized in multiprotein complexes, the structure of which is probably dynamic, varying in different plant tissues and throughout plant development. The structural organization and regulation of XyG and other polysaccharide synthase complexes remain to be uncovered in the future.

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REFERENCES

Atmodjo, M. A., Sakuragi, Y., Zhu, X., Burrell, A. J., Mohanty, S. S., Atwood, J. A. III, Orlando, R., Scheller, H. V., and Mohnen, D. (2011). Galacturonosyltransferase (GAUT)1 and GAUT7 are the core of a plant cell wall pectin biosynthetic homogalacturonan:galacturonosyltransferase complex. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20225–20230.

Cavalier, D., and Keegstra, K. (2010). "Members of Arabidopsis cellulose synthase-like C (CSLC) family are involved in xyloglucan biosynthesis," in *Book of Abstracts of XII Cell Wall Meeting*, Porto.

Cavalier, D. M., and Keegstra, K. (2006). Two xyloglucan xylosyltransferases catalyze the addition of multiple xylosyl residues to cellohexaose. *J. Biol. Chem.* 281, 34197–34207.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Chevalier, L., Bernard, S., Ramdani, Y., Lamour, R., Bardor, M., Lerouge, P., Follet-Gueye, M.-L., and Driouich, A. (2010). Subcompartment localization of the side chain xyloglucan-synthesizing enzymes within Golgi stacks of tobacco suspension-cultured cells. *Plant J.* 64, 977–989.

Chou, Y.-H., Pogorelko, G., and Zabotina, O. A. (2012). Xyloglucan xylosyltransferases XXT1, XXT2, XXT5, and the glucan synthase CSLC4 form Golgi-localized multiprotein complexes. *Plant Physiol.* doi:10.1104/pp.112.199356

Cocuron, J. C., Lerouxel, O., Drakakaki, G., Alonso, A. P., Liepman, A. H., Keegstra, K., Raikhel, N., and Wilkerson, C. G. (2007). A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8550–8555.

Davis, J., Brandizzi, F., Liepman, A. H., and Keegstra, K. (2010). Arabidopsis mannan synthase CSLA9 and glucan synthase CSLC4 have opposite orientations in the Golgi membrane. *Plant J.* 64, 1028–1037.

del Bem, L., and Vincentz, M. (2010). Evolution of xyloglucan-related genes in green plants. *BMC Evol. Biol.* 10, 341. doi:10.1186/1471-2148-10-341

Edwards, M. E., Dickson, C. A., Chengappa, S., Sidebottom, C., Gidley, M. J., and Reid, J. S. G. (1999). Molecular characterisation of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. *Plant J.* 19, 691–697.

Faik, A., Bar-Peled, M., Derocher, A. E., Zeng, W., Perrin, R. M., Wilkerson, C., Raikhel, N. V., and Keegstra, K. (2000). Biochemical characterization and molecular cloning of an alpha-1,2-fucosyltransferase that catalyzes the last step of cell wall xyloglucan biosynthesis in pea. *J. Biol. Chem.* 275, 15082–15089.

Faik, A., Chileshe, C., Sterling, J., and Maclachlan, G. (1997a). Xyloglucan galactosyl- and fucosyltransferase activities from pea epicotyl microsomes. *Plant Physiol.* 114, 245–254.

Faik, A., Desveaux, D., and Maclachlan, G. (1997b). Xyloglucan galactosyl- and fucosyl-transferase activities in the cotyledons of developing nasturtium seeds. *Plant Physiol.* 114, 716–716.

Faik, A., Price, N. J., Raikhel, N. V., and Keegstra, K. (2002). An Arabidopsis gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7797–7802.

Fry, S. C., Mohler, K. E., Nesselrode, B. H., and Frankova, L. (2008). Mixed-linlage bet-glucan: xyloglucan endotransglucosylase, a novel wall-remodelling enzyme from Equisetum (horsetails) and charophytic algae. *Plant J.* 55, 240–252.

Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J. P., Kato, Y., Lorences, E. P., Maclachlan, G. A., McNeil, M., Mort, A. J., Reid, J. S. G., Seitz, H. U., Selvendran, R. R., Voragen, A. G. J., and White, A. R. (1993). An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiol. Plant.* 89, 1–3.

Gibeaut, D. M., Pauly, M., Bacic, T., and Fincher, G. B. (2004). Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. *Planta* 221, 729–738.

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Schultink, A., Reca, I. B., and Pauly, M. (2011). O-acetylation of arabidopsis hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. *Plant Cell* 23, 4041–4053.

Gunl, M., Neumetzler, L., Kraemer, F., de Souza, A., Schultink, A., Pena, M., York, W. S., and Pauly, M. (2011). AXY8 encodes an alpha-fucosidase, underscoring the importance of apoplastic metabolism on the fine structure of Arabidopsis cell wall polysaccharides. *Plant Cell* 23, 4025–4040.

Hantus, J., Pauly, M., Darvill, A. G., Albersheim, P., and York, W. S. (1997). Structural characterization of novel L-galactose-containing oligosaccharide subunits of jojoba seed xyloglucans. *Carbohydr. Res.* 304, 11–20.

Harholt, J., Jensen, J. K., Verhertbruggen, Y., Sogaard, C., Bernard, S., Nafisi, M., Poulsen, C. P., Geshi, N., Sakuragi, Y., Driouich, A., Knox, J. P., and Scheller, H. V. (2012). ARAD proteins associated with pectic Arabinan biosynthesis form complexes when transiently overexpressed in planta. *Planta.* doi:10.1007/s00425-012-1592–1593

Hoffman, M., Jia, Z. H., Pena, M. J., Cash, M., Harper, A., Blackburn, A. R., Darvill, A., and York, W. S. (2005). Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. *Carbohydr. Res.* 340, 1826–1840.

Hsieh, Y. S. Y., and Harris, P. J. (2009). Xyloglucans of monocotyledons have diverse structures. *Mol. Plant* 2, 943–965.

Jensen, J. K., Schultink, A., Keegstra, K., Wilkerson, C. G., and Pauly, M. (2012). RNA-Seq analysis of developing nasturtium seeds (*Tropaeolum majus*): identification and characterization of an additional, galactosyltransferase involved in xyloglucan biosynthesis. *Mol. Plant.* doi:10.1093/mp/sss032

Keegstra, K., and Cavalier, D. (2011). "Glycosyltransferases of the GT34 and GT37 families," in *Annual Plant Reviews, Vol. 41, Plant Polysaccharides: Biosynthesis and Bioengineering*, ed. P. Ulskov (Oxford: Blackwell Publishing), 235–250.

Keegstra, K., and Raikhel, N. (2001). Plant glycosyltransferases. *Curr. Opin. Plant Biol.* 4, 219–224.

Kiefer, L. L., York, W. S., Darvill, A. G., and Albersheim, P. (1989). Structure of plant-cell walls.27. xyloglucan isolated from suspension-cultured sycamore cell-walls is o-acetylated. *Phytochemistry* 28, 2105–2107.

Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G. F., Caplan, J., Shoue, D. A., Carpita, N. C., and Reiter, W. D. (2003). The MUR3 gene of Arabidopsis encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *Plant Cell* 15, 1662–1670.

Obel, N., Erben, V., Schwarz, T., Kuhnel, S., Fodor, A., and Pauly, M. (2009). Microanalysis of plant cell wall polysaccharides. *Mol. Plant* 2, 922–932.

O'Neill, M. A., and York, W. S. (2003). "The composition and structure of plants primary cell walls," in *The Plant Cell Wall*, ed. J. K. C. Rose (Oxford: Blackwell), 1–54.

Pauly, M., Qin, Q., Greene, H., Albersheim, P., Darvill, A., and York, W. S. (2001). Changes in the structure of xyloglucan during cell elongation. *Planta* 212, 842–850.

Pena, M. J., Darvill, A. G., Eberhard, S., York, W. S., and O'Neill, M. A. (2008). Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants. *Glycobiology* 18, 891–904.

Perrin, R. M., Derocher, A. E., Bar-Peled, M., Zeng, W., Norambuena, L., Orellana, A., Raikhel, N. V., and Keegstra, K. (1999). Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* 284, 1976–1979.

Popper, Z. A. (2008). Evolution and diversity of green plant cell walls. *Curr. Opin. Plant Biol.* 11, 1–7.

Popper, Z. A., and Fry, S. C. (2003). Primary cell wall composition of bryophytes and charophytes. *Ann. Bot.* 91, 1–12.

Popper, Z. A., and Fry, S. C. (2004). Primary cell wall composition of pteridophytes and spermatophytes. *New Phytol.* 64, 165–174.

Qiu, Y.-L., Li, L., Wang, B., Chen, Z., Knoop, V., Groth-Malonek, M., Dombrovska, O., Lee, J., Kent, L., Rest, J., Estabrook, G. F., Hendry, T. A., Taylor, D. W., Testa, C. M., Ambros, M., Crandall-Stotler, B., Duff, R. J., Stech, M., Frey, W., Quandt, D., and Davis, C. C. (2006). The deepest divergences in land plants inferred from phylogenomic evidence. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15511–15516.

Reiter, W. D., Chapple, C., and Somerville, C. R. (1997). Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J.* 12, 335–345.

Sarkar, P., Bosneaga, E., and Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *J. Exp. Bot.* 60, 3615–3635.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., and Lohmann, J. U. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nat. Genet.* 37, 501–506.

Sorensen, I., Domozych, D., and Willats, W. G. T. (2010). How have plant cell walls evolved? *Plant Physiol.* 153, 366–372.

Sorensen, I., Pettolino, F. A., Bacic, A., Ralph, J., Lu, F., O'Neill, M. A., Fei, Z., Rose, J. K. C., Domozych, D. S., and Willats, W. G. T. (2011). The charophycean green algae provide insights into the early origins of plant cell walls. *Plant J.* 68, 201–211.

Van Sandt, V. S., Stieperaere, H., Guisez, Y., Verbelen, J. P., and Vissenberg, K. (2007). XET activity is found near sites of growth and cell elongation in bryphytes and some green algae: new insights into the evolution of primary cell wall elongation. *Ann. Bot.* 99, 39–51.

York, W. S., Kolli, V. S. K., Orlando, R., Albersheim, P., and Darvill, A. G. (1996). The structure of arabinoxyloglucans produced by solanaceous plants. *Carbohydr. Res.* 285, 99–128.

Zabotina, O. A., Avci, U., Cavalier, D., Pattathil, S., Chou, Y.-H., Eberhard, S., Danhof, L., Keegstra, K., and Hahn, M. G. (2012). Mutations in multiple *XXT* genes of Arabidopsis reveal the complexity of xyloglucan biosynthesis. *Plant Physiol.* doi: 10.1104/pp.112.198119

Zabotina, O. A., van de Ven, W. T. G., Freshour, G., Drakakaki, G., Cavalier, D., Mouille, G., Hahn, M. G., Keegstra, K., and Raikhel, N. V. (2008). Arabidopsis XXT5 gene encodes a putative alpha-1,6-xylosyltransferase that is involved in xyloglucan biosynthesis. *Plant J.* 56, 101–115.

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Arabinogalactan-proteins and the research challenges for these enigmatic plant cell surface proteoglycans

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Arabinogalactan-proteins (AGPs) are complex glycoconjugates that are commonly found at the cell surface and in secretions of plants. Their location and diversity of structures have made them attractive targets as modulators of plant development but definitive proof of their direct role(s) in biological processes remains elusive. Here we overview the current state of knowledge on AGPs, identify key challenges impeding progress in the field and propose approaches using modern bioinformatic, (bio)chemical, cell biological, molecular and genetic techniques that could be applied to redress these gaps in our knowledge.

Keywords: arabinogalactan-proteins, glycosylphosphatidylinositol anchor, cell surface signaling, AGP polysaccharide complexes, fasciclin-like AGP, galactosyltransferase

INTRODUCTION

Arabinogalactan-proteins (AGPs), ubiquitous cell surface proteoglycans in both terrestrial and aquatic plants (and algae), are proposed to play essential roles in a range of plant growth and development processes, including cell expansion, cell division, reproductive development, somatic embryogenesis, xylem differentiation, abiotic stress responses, and hormone signaling pathways (Seifert and Roberts, 2007; Ellis et al., 2010). These roles emerge from largely indirect evidence and from the "recognition" potential arising from the incredible diversity of their glycan and protein backbone moieties as well as their location; attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor and in some instances cross-linked into the wall. Despite intense research to unravel AGP function, their molecular mechanism(s) of action remain elusive. AGPs exhibit complexity at many levels: First, AGP protein backbone genes are part of large gene families, and this makes the study of AGP function through characterization of single AGP mutants a major challenge due to gene redundancy (Ma and Zhao, 2010; Showalter et al., 2010). Second, AGP protein backbones are highly glycosylated, hindering production of antibodies specifically directed to the protein moiety that would allow for identification and isolation of single AGPs. Third, the variety of monosaccharides present in AGP carbohydrate moieties, the variety of linkages between these monosaccharides and the special arrangement of these linkages provides high heterogeneity and complexity to the AGP carbohydrates. These properties make purification of individual AGPs difficult and expression of properly glycosylated AGPs in heterologous systems problematic. Consequently, functional evaluation of specific AGPs is not trivial. Incredible advances in cell and molecular biology, 'omics and computational sciences, however, offer the potential to unlock the mysteries of AGP structure and function if harnessed in a synergistic manner. In this overview we briefly review the AGP field and identify and explain the key research challenges.

STRUCTURES

Arabinogalactan-proteins belong to the hydroxyproline-rich superfamily of glycoproteins (Schultz et al., 2002; Johnson et al., 2003) being composed largely of carbohydrate (90–98% w/w) with some protein typically rich in the amino acids, Hyp/Pro, Ala, Ser/Thr, that is usually covalently modified with a GPI anchor at the C-terminus (see **Figure 1**). Historically, AGPs were defined if they met three criteria: the presence of arabinogalactan chains, a Hyp-rich protein backbone, and the ability to bind to a class of synthetic phenylazo dyes, the β-glucosyl Yariv reagent (see Du et al., 1996). The significant advances in our knowledge of their carbohydrate structures, protein backbone sequences, and variability in Yariv binding has considerably complicated how an AGP is defined. For instance, the diversity of protein backbones has led to a classification of the AGPs into different sub-classes based on the presence/absence of particular motifs/domains (Johnson et al., 2003). The carbohydrate moiety is typically in the form of type II arabinogalactans (AGs) although some AGPs also contain short arabino-oligosaccharide chains (**Figure 1**; Fincher et al., 1983; Tan et al., 2004, 2010; Ellis et al., 2010). Type II AGs have also

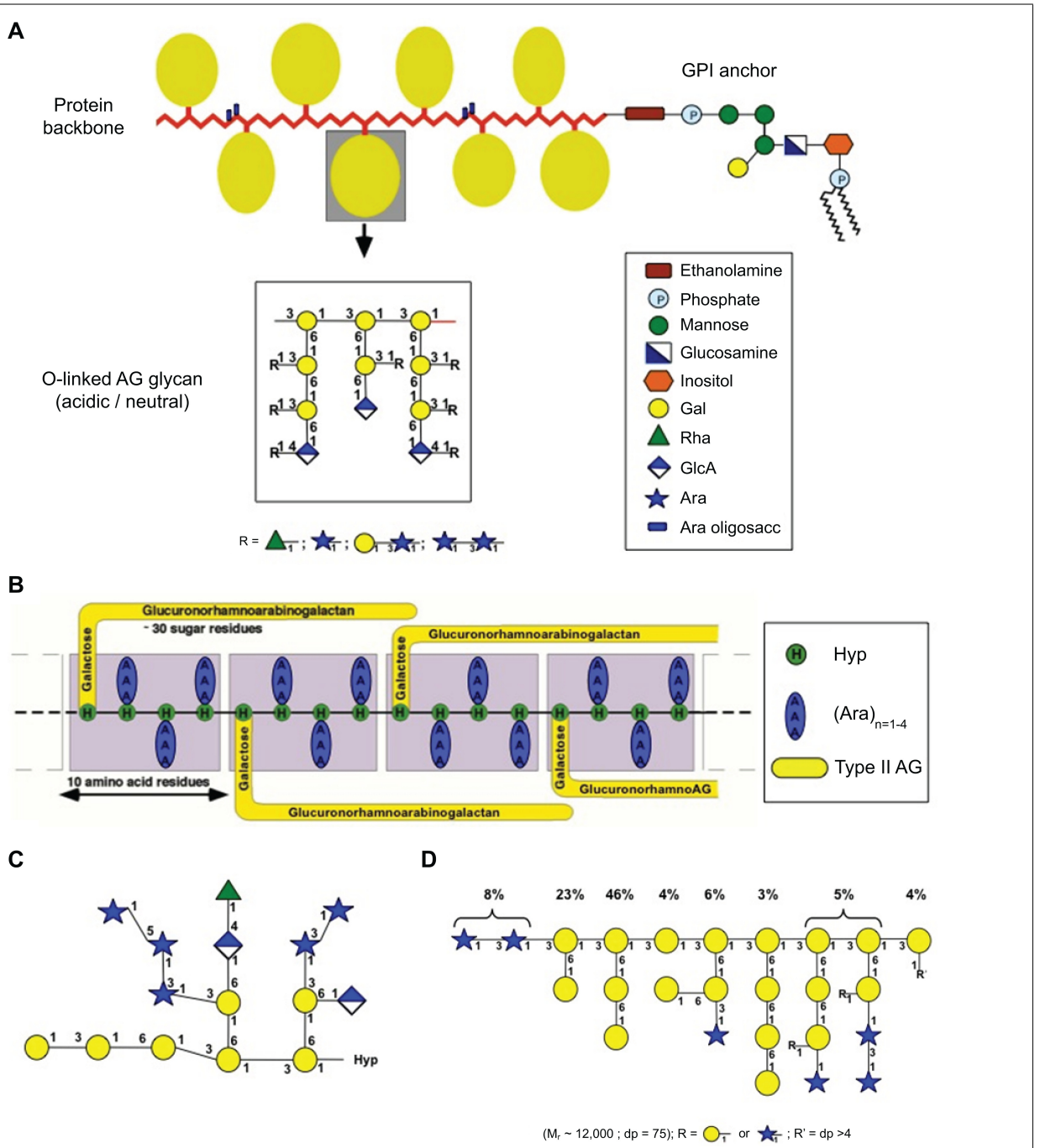


FIGURE 1 | Schematic representation of the diversity of AGP glycan structures (taken from Ellis et al., 2010). (A) The "wattle blossom" model of the structure of AGPs with a GPI-membrane anchor attached (modified from Fincher et al., 1983). **(B)** The "twisted-hairy rope" model of the structure of the Gum Arabic glycoprotein (GAGP; from Qi et al., 1991). **(C)** Primary structure of a representative Hyp-AG polysaccharide (AHP-1) released by base hydrolysis from a synthetic AGP $(Ala\text{-}Hyp)_{51}$ from tobacco BY2 cells (modified from Tan et al., 2004). **(D)** Larch AG structure (modified from Ponder and Richards, 1997).

been reported either as free polysaccharides (Ponder and Richards, 1997) or as side chains of rhamnogalacturonan I (RG-I; Caffall and Mohnen, 2009). The existence of different forms of type II AGs raises a few questions. Are free type II AGs generated from AGPs by hydrolases in the wall or synthesized *de novo*? Are AG side chains of RG-I derived from either AGPs by transglycosylases or from covalently linked RG-I–AGP complexes? To understand how this diversity impacts biological function, we face the challenge of isolating "individual" AGPs and sequencing their glycans (and protein backbones).

Another aspect of AGP research is the intriguing possibility that they are one form of covalent cross linker for wall matrix phase polysaccharides. In the early 1970s, Keegstra et al. (1973) hypothesized that Rha residues on AG side chains of AGPs might be attachment sites for RG-I. Since then, AGPs/AGs have been reported to form complexes with pectins (Yamada et al., 1987; Saulnier et al., 1988; Kwan and Morvan, 1991; Pellerin et al., 1995; Yamada, 2000; Duan et al., 2003, 2004) and xylans (Kwan and Morvan, 1995). However, residues involved in the covalent cross-link between AG(P)s and wall polysaccharides have not been defined. Several major challenges must be addressed to determine if AGP polysaccharide complexes (APCs) exist and to determine the structure and function of any such complexes.

CHALLENGE 1: ISOLATION AND PURIFICATION OF AGPs

The incredible heterogeneity of AGP structures has hampered purification of individual AGPs. As a consequence, most studies on AGPs have been on a family of molecules and often in the presence of contaminating polymers. There are a few examples of AGPs purified by a combination of traditional chromatographic methods (for example, anion exchange/lectin affinity/gel permeation using chaotropic reagents) and/or Yariv precipitation that are "pure" AGPs; for example, the AGPs from tobacco floral tissues (Gane et al., 1995) and larch AG exudates (Ponder and Richards, 1997). The application of molecular biology techniques to both isolate heterologously expressed AGP protein backbones or synthetic peptides as green fluorescent protein tagged (GFP)-fusion proteins by the Kieliszewski/Showalter, Matsuoka, and Somerville laboratories (Shpak et al., 1999; Zhao et al., 2002; Tan et al., 2004; Shimizu et al., 2005; Estévez et al., 2006) was an ingenious innovation that allowed the purification of AGPs with a single protein backbone and therefore the study of inherent glycan heterogeneity. However, the low DP of these glycans raises some questions of the fidelity of glycosylation in heterologous/high expression systems.

Thus, a combination of purification techniques is necessary to purify relatively homogenous AGPs (and APC complexes extracted as described below). These techniques take advantage of the heterogeneous structural features of AGPs and wall polysaccharides including size, charge, hydrophobicity (Serpe and Nothnagel, 1996; Lamport et al., 2011), the ability to co-precipitate with Yariv reagents, the availability of anti-AG antibodies (Pattathil et al., 2010), and the use of tagged heterologously expressed protein backbones.

CHALLENGE 2: EXTRACTION AND PURIFICATION OF PUTATIVE APCs FROM WALLS

Because pectins and non-cellulosic polysaccharides are embedded within the highly cross-linked wall, the first obstacle to studying putative APCs is to extract intact macromolecules from the wall, especially from secondary walls. Traditional methods to release polysaccharides from the wall include either the use of wall-specific degrading enzymes (York et al., 1986) or the extraction of walls with increasingly harsh solvents (Fry, 1988). Since the enzymatic and strong base treatments could also potentially break covalent linkages between AGPs and wall polysaccharides, the released polymers may only contain partial structural information of potential APCs and may still contain contaminating wall polysaccharides.

To avoid these extraction complications it may be possible to source APCs from potentially rich sources such as suspension culture media, especially of xylogenic calli, polysaccharide-rich seed mucilages, and exudates, such as gums (Defaye and Wong, 1986) and root mucilages (Moody et al., 1988) since these are released in a "solubilized" form.

CHALLENGE 3: SEQUENCING OF AGs AND APCs

Our current knowledge of AG carbohydrate sequences are based on experiments using tools that include monosaccharide composition, linkage analysis, chemical or enzymatic degradation of glycans, mass spectrometry (MS), and NMR analysis. Partial acid hydrolysis (Defaye and Wong, 1986), acetolysis, alkaline degradation, and Smith degradation (Churms et al., 1981; Bacic et al., 1987) have supported the basic structures summarized in **Figure 1** and led to the suggestion that the AG glycans contain a backbone of β-(1,3)-galacto-oligosaccharides interrupted at regular intervals with a periodate-sensitive residue. However, few of the large AG chains have been *de novo*-sequenced due to the inherent biosynthetic heterogeneity and the current limitations of sequencing technologies.

The availability of linkage-specific enzymes has greatly assisted the sequencing of glycans although their lack of commercial accessibility has hampered progress. Thus a breakthrough in AGP analysis was the identification of an AGP-specific exo-β-(1,3)-galactanase that can bypass the β-(1,6)-galactosyl side chains (Tsumuraya et al., 1990; Kotake et al., 2005; Ichinose et al., 2006). This enzyme, together with the recently characterized β-glucuronidase (Haque et al., 2005), α-arabinofuranosidase (Hata et al., 1992), and endo-β-(1,6)-galactanase form a enzyme tool kit specific for AG side-chain analysis which enabled Tryfona et al. (2010) to characterize some long β-(1,6)-galacto-oligosaccharide AG side chains with the aid of MS/MS fragmentation (see Oxley et al., 2004). A recent study of *Arabidopsis* AGP31 (Hijazi et al., 2012), a chimeric AGP, illustrates the power of a multipronged approach to purification and characterization of AGPs.

Therefore, the best solution is to sequence small structural units of AGs, generated using a combination of chemical and enzymic techniques, and then to re-construct models of the intact AGs. Discovery of new chemicals and enzymes that can selectively cleave AGs would facilitate future progress in the sequencing of the AG glycan chains.

BIOINFORMATICS

Genomics and its related technologies have revolutionized the study of biology, facilitated the development of other 'omics platforms, and created a need for bioinformatics to handle the acquisition, storage, and analysis of the vast amount of data generated from 'omics and 'omics-related projects. The AGP field has greatly benefited from genomics and bioinformatics. Given that AGP protein backbone sequences often have low sequence similarity, BLAST-type searches typically identify only a few closely related AGP family members and, therefore, are not a particularly effective means to comprehensively identify members of the AGP family. In contrast, bioinformatics approaches have provided a

broader and more complete picture of AGP gene/protein families. Schultz et al. (2002) conducted the first comprehensive bioinformatics analysis to identify and characterize AGP genes/proteins from the *Arabidopsis* genome/proteome with respect to their protein backbones. This study was refined by Showalter et al. (2010), who found 85 AGPs, including 22 classical AGPs, 3 lysine-rich AGPs, 16 AG-peptides, 21 fasciclin-like AGPs (FLAs), 17 plastocyanin AGPs, and 6 other chimeric AGPs. Ma and Zhao (2010) have conducted the only other comprehensive bioinformatics analysis for AGP protein backbones in rice. They identified 69 rice AGP protein backbones from the rice genome/proteome, including 13 classical AGPs, 15 AG-peptides, 3 non-classical AGPs, 3 early nodulin-like AGPs (eNod-like AGPs), 8 non-specific lipid transfer protein-like AGPs (nsLTP-like AGPs), and 27 FLAs. A few other bioinformatic studies are reported for AGP protein backbones, but these studies were not focused exclusively on AGPs and/or concentrated only on one particular sub-class (e.g., GPI-anchored AGPs or FLAs). For example, Borner et al. (2002, 2003) used bioinformatics to identify GPI-anchored proteins in *Arabidopsis* from genomic and proteomic data. In addition, Irshad et al. (2008) applied bioinformatic analysis to their cell wall proteomic data in *Arabidopsis* to identify several AGPs and Faik et al. (2006) used bioinformatic analyses to identify 34 wheat and 24 rice FLAs. Bioinformatic tools have also been used to provide insight to the glycosyltransferases (GTs) involved in the assembly of AGP glycans (see Biosynthesis of Glycan Moieties) and in this way Bacic and colleagues have proposed that the CAZy GT 31 family comprise putative β-(1,3)-GalTs (Qu et al., 2008; Ellis et al., 2010; Egelund et al., 2011).

The comprehensive bioinformatic studies on AGPs also took advantage of other related genomic technologies, including microarray data to reveal organ-specific expression patterns, abiotic- and biotic-regulated expression profiles, and genes which are co-expressed. Co-expression analysis has the potential to reveal networks of genes that are related to particular aspects of AGP biology, including their biosynthesis, interacting partners, and physiological functions. These kinds of downstream bioinformatic analyses are just in their infancy and many bioinformatics challenge lie ahead relating to AGPs, as outlined below.

CHALLENGE 4: IDENTIFYING AND CLASSIFYING AGPs FROM OTHER SEQUENCED PLANT GENOMES

Over 30 plant and algal genomes/proteomes are now known (see http://en.wikipedia.org/wiki/List_of_sequenced_eukaryotic_genomes#Algae). It would be useful to apply either the current or improved bioinformatics programs to these various datasets. Suggested enhancements to the programs would include making the bioinformatic analysis more automated and integrating the programs for predicting signal peptides, GPI anchor addition sites, gene expression, co-expression analysis, etc. into a single program. In addition, based on existing protein sequence and carbohydrate data on AGPs, a bioinformatics program predicting sites of prolyl hydroxylation and corresponding sites and type of glycosylation (i.e., AGs and arabino-oligosaccharides) could be developed and used. This relies on our knowledge that the types of *O*-glycosylation on the AGP protein backbone can be predicted from the Hyp-contiguity hypothesis that defines Hyp (arabino)galactosylation as occurring on the clustered, non-contiguous Hyp residues separated by Ala or Ser residues in a protein backbone whereas blocks of contiguous Hyp residues, such as occur in extensins, are arabinosylated with short oligosaccharides (Kieliszewski and Lamport, 1994; Shpak et al., 1999; Goodrum et al., 2000; Zhao et al., 2002); *N*-glycosylation, is predicted by the universally conserved consensus amino acid sequence Asn-X-Ser/Thr, where X can be any amino acid except for Pro. Similarly, the specificity of prolyl hydroxylation by prolyl-4-hydroxylase, although not as well defined in plants as in mammalian systems (Gorres and Raines, 2010), can be used together with the Hyp-contiguity hypothesis to inform design of bioinformatics programs.

CHALLENGE 5: APPLYING AND IMPROVING BIOINFORMATIC ANALYSES OF MICROARRAY DATA TO ELUCIDATE PATTERNS OF AGP (CO-)EXPRESSION

Unfortunately, not all of the sequenced plant genomes have extensive publically available microarray data, unlike *Arabidopsis* and rice (e.g., see PLEXdb, http://www.plexdb.org/). Thus, in addition to generating new microarray data, it would be convenient to utilize and integrate expression analysis programs like Genevestigator and co-expression analyzer tools (see **Table 1**) to mine data and provide it in a more tailored manner. Analysis of such data can provide remarkable insight into the function (and functional redundancy) of AGP protein backbone genes as well as elucidate networks of AGPs and AGP-related genes involved in various metabolic pathways.

CHALLENGE 6: IMPROVING AND DEVELOPING NEW BIOINFORMATICS PROGRAMS TO ELUCIDATE MOLECULAR PHYLOGENIES OF AGP PROTEIN BACKBONE GENES

It would be interesting from an evolutionary standpoint to understand how AGPs are related within and between species, since such analysis may explain how the AGP gene family evolved and provide insight into AGP function. From a functional perspective, it would be useful to be able to identify AGP gene orthologs and paralogs. Software developers would use the gene families identified in Challenge 4 through packages summarized in **Table 1** and the extensive web-based resources developed for studying gene ontology to focus on the AGP protein backbone genes.

CHALLENGE 7: DEVELOP BIOINFORMATICS TOOLS TO IDENTIFY AND CLASSIFY GENES/PROTEINS INVOLVED WITH AGP METABOLISM

Bioinformatic tools to identify genes involved with the biosynthesis and possible modification and degradation of AGPs would be of great benefit. In particular, bioinformatics analysis has the potential to identify GTs likely to be involved in the biosynthesis of AG chains. Currently, sequence similarities to mammalian GTs represent one approach to identifying these enzymes, for example, as recently described by Egelund et al. (2011) in which the authors adopted a bioinformatic approach to identify and systematically characterize putative GalTs from CAZy GT-family-31 responsible for synthesizing the β-(1,3)-Gal linkage. This study revealed that the *Arabidopsis* accessions grouped into four plant-specific clades (1, 7, 10, and 11; **Table 2**). Furthermore, the investigators attempted to predict the possible substrate specificity of these

Table 1 | Bioinformatic programs used to identify and characterize AGPs.

Program	Program use	Web address
PAST percentage calculator	Identification of AGP backbones	http://www.adelaide.edu.au/directory/carolyn.schultz (under Files)
BIO OHIO	Identification of AGP backbones and more	http://code.google.com/p/prot-class/
SignalP	Identification of signal peptides	http://www.cbs.dtu.dk/services/SignalP/
Plant big-PI predictor	Identification of GPI anchor addition sites	http://mendel.imp.ac.at/gpi/plant_server.html
Genevestigator	Identification of gene expression	https://www.genevestigator.ethz.ch/
Arabidopsis Co-Response Database	Identification of co-expressed genes	http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath.html

Table 2 | The 31 putative GalTs from the *Arabidopsis thaliana* CAZy GT-family-31 and their proposed function.

Sub-clade	Accession	Proposed function
1	At1g33250, At5g12460, At4g15240, At4g00300, At1g01570, At4g11350, At2g37730, At3g11420, At1g05280, At4g23490, At1g07850	β-(1,3)-GalTs; substrate unknown!
7	At1g27120, At1g74800, At5g62620, At3g06440, At1g26810, At4g21060	β-(1,3)-GalTs involved in the synthesis of N- and *O*-glycans, and β-(1,3)-GlcNAcTs; substrate unknown!
10	At4g32120, At1g11730, At1g22015, At5g53340, At2g25300, At1g77810, At2g32430, At1g05170, At1g53290, At2g26100, At3g14960, At1g33430, At4g26940, At1g32930	β-GalTs involved in the synthesis of AGPs
11	At5g57500	β-(1,3)-GalTs; substrate unknown!

The accessions clustered into four plant-specific sub-groups according to Egelund et al. (2011). Substrate specificity were predicted based on secondary structure and conserved motifs shared with known β-(1,3)-GalTs (Qu et al., 2008).

GalTs based on secondary structure and conserved motifs shared with known β-(1,3)-GalTs (Qu et al., 2008). These predictions have formed the basis for detailed biochemical and molecular studies to define the precise substrate specificities of GT-31 family members.

In a similar manner co-expression analysis of either selected AGP or groups of AGP protein backbones provides another, largely unexplored, option to identify candidate GTs responsible for AG biosynthesis (and/or degradation *in muro*). This idea is based on the premise that once the gene encoding the AGP protein backbone is expressed, other genes needed for AGP biosynthesis should also be co-expressed. In addition, co-expression analysis in conjunction with computational prediction of sub-cellular location and known protein–protein interaction data of candidate proteins involved in AGP biosynthesis could be used to identify proteins that function together in a complex (Mostafavi et al., 2008; http://www.genemania.org). Such information could be integrated into an "interactome" focusing on AGP biosynthesis.

CHALLENGE 8: DEVELOP BIOINFORMATICS TOOLS TO IDENTIFY REGULATORY SEQUENCES IN AGP PROTEIN BACKBONE GENES

Bioinformatics has the potential to reveal gene regulatory sequences involved in regulated expression of AGP genes with respect to developmental expression (e.g., tissue- and temporal-specific expression) and a variety of stresses. Bioinformatic programs that have the ability to recognize either conserved nucleotide patterns alone or in combination with chromatin immunoprecipitation (ChIP) assays followed by DNA sequencing have the potential to reveal AGP gene regulatory sequences and the corresponding trans-acting factors. Knowledge of such regulatory sequences would reveal commonly regulated networks of AGP genes as well as other co-regulated genes. As such, this information may be complementary to co-expression data and would provide another avenue to elucidating AGP function(s).

BIOSYNTHESIS OF GLYCAN MOIETIES

Many mammalian, fungal, and bacterial GTs have been identified, cloned, and biochemically characterized (Cantarel et al., 2009; Ellis et al., 2010). In contrast, only a few plant cell wall polysaccharide/proteoglycan-related GTs have been characterized biochemically (Doblin et al., 2010). From studies of *Arabidopsis* at the molecular and biochemical level (Strasser et al., 2007; Qu et al., 2008), and from assembly of mammalian proteoglycans, it is expected that AG glycan chains that decorate AGPs are synthesized by type II membrane-bound GTs located in the Golgi apparatus. This includes members of CAZy GT-family-31 with putative β-(1,3)-GalT activity, that are suggested to be involved in synthesis of the β-(1,3)-Gal backbone in AG glycans (Qu et al., 2008; Egelund et al., 2011).

Early studies showed that the Golgi apparatus plays an important role in synthesis of β-(1,6)-Gal of the AG glycan chains of AGPs (Mascara and Fincher, 1982; Schibeci et al., 1984), whereas the initial enzyme in the AG biosynthetic pathway, adding the first Gal residue to a Hyp residue on the protein backbone (the Hyp-*O*-galactosyltransferase or HGT), is predominantly located in the ER (Oka et al., 2010). Outside of the development of *in vitro* assays to monitor GalT activity (Qu et al., 2008; Liang et al., 2010;

Oka et al., 2010), no significant progress on biochemical characterization of GalTs involved in synthesis of the AG glycans on AGPs has been made since the mid-1980s, severely restricting our understanding of AGP biology and potential industrial/pharmaceutical applications. It is reasonable to assume that for assembly of AG chains, several GalTs will be required, such as HGT(s), β-(1,3)-GalTs, and β-(1,6)-GalTs, and these enzymes will work co-ordinately to regulate the density, length, and sequence of the galactan chain. In addition, several GTs responsible for decorating termini of AG chains, i.e., arabinosyltransferases (AraTs), rhamnosyltransferases (RhaTs), fucosyltransferases (FUTs), and glucuronosyltransferases (GlcATs) are also involved. Recently, AtFUT4 and AtFUT6, two members of CAZy GT-family-37, were characterized as Golgi located α(1,2)FUTs and are the first enzymes demonstrated to have a specific function in AGP glycosylation (Wu et al., 2010). To ensure continued momentum in the field, we suggest a focused co-ordinated approach on three core challenges:

CHALLENGE 9: AN ALTERNATIVE APPROACH FOR THE IDENTIFICATION OF THE GLYCOSYLATION MACHINERY INVOLVED IN AG CHAIN SYNTHESIS

An alternative approach to the one described in Challenge 7, centers on the analysis of Gum Arabic, a tree exudate from the *Acacia* species, whose main fraction is an AG (Defaye and Wong, 1986; Randall et al., 1989; Al-Assaf et al., 2005). AG chains comprise as much as 90–98% of the gum exudate (Osman et al., 1993), thus making Gum Arabic-producing cells from the *Acacia* trees an obvious choice as starting material to identify enzymes involved in AG biosynthesis.

CHALLENGE 10: BIOSYNTHESIS OF PUTATIVE APCs

The challenge is to determine in which sub-cellular compartment putative APCs are assembled and by what mechanism? One possibility is that APCs are synthesized intracellularly in the ER/Golgi apparatus by multiple GTs (as proposed for AGPs and other non-cellulosic polysaccharides) by either *en bloc* transfer of pre-assembled oligosaccharides or stepwise sugar addition, followed by delivery into the wall. Another possibility is that APCs are assembled in the extracellular matrix, possibly by transglycosylases, a mechanism that has been well studied in xyloglucan remodeling within the wall (Rose et al., 2002) and is commonly utilized by yeast to modify their wall in response to abiotic/biotic stimuli (Kollár et al., 1997).

CHALLENGE 11: HETEROLOGOUS EXPRESSION SYSTEMS

Expression of non-cellulosic/cellulosic plant GTs in functional assay systems remains a key challenge. The past lack of success of this approach has been ascribed to the mismatch between biochemical assays and native activity, failure of the expressed protein to accumulate to sufficient levels, incorrect folding or improper post-translational modifications (Petersen et al., 2009). The most obvious choice would be to develop an "*in planta*" system, however, the endogenous GT activities can make it difficult to distinguish the specific activity of the expressed protein (Petersen et al., 2009). Prokaryotes, of which some have limited capacity for post-translational processing, pose other problems. We therefore suggest developing multiple heterologous expression systems to maximize the likelihood that at least one will allow for successful expression where the biochemical activity is retained. Additionally, testing new expression systems that may prove "universal" (e.g., *Aspergillus*), which has served as one of the preferred expression systems in the biotechnology industry, as well as cell-free expression systems may prove useful for heterologous expression of plant GTs.

CHALLENGE 12: A HIGH-THROUGHPUT ENZYME ACTIVITY SCREENING SYSTEM

The assignment of substrate specificity to GTs is often hindered by difficulties related to limited availability of relevant candidate acceptor molecules for biochemical assays. To overcome this challenge the next step should be to employ carbohydrate array technology (Moller et al., 2007) with AGP/Gum Arabic-specific sugars and peptides, related acceptor substrates, i.e., natural acceptors from Gum Arabic and AGPs [e.g., β-(1,3)-galacto-oligosaccharides, generated by Smith degradation (see Challenge 3), de-arabinosylated AGPs generated by mild acid, chemically synthesized β-(1,3)-Gal oligosaccharides and isolated AGP protein backbones] together with other "AGP-enriched" fractions from wild type, AGP GT mutants, and Gum Arabic exudates.

Combining AGP-related arrays with established *in vitro* assays will facilitate a high-throughput screening system that can be used to test heterologously expressed candidate GTs in mixtures with either radio-labeled or fluorescently tagged NDP-sugar as the donor to identify AGP-specific carbohydrate acceptor molecules on the array. Development of such a comprehensive screening system would be a significant step in identifying the many GTs responsible for AG biosynthesis.

FUNCTION

Arabinogalactan-protein glycan-specific antibodies and β-Glc Yariv reagent have been broadly used to investigate AGP activity in tissue culture and *in planta* (Seifert and Roberts, 2007; Ellis et al., 2010). The current use of these two indirect tools continues to provide information on AGP activity in new biological systems, e.g., European larch, *Larix decidua* (Rafinska and Bednarska, 2011), and little studied developmental processes, in this case, ovule development in gymnosperms, confirming the relevance and the conservation of function of these molecules within the plant kingdom. Unfortunately, the broad specificity of these techniques makes it impossible to assign function to a single AGP. This limitation has been partially overcome by genetic and molecular studies, including the characterization of AGP single or double mutants, RNAi and over-expressing lines, although these approaches also have complications.

The usefulness of reverse genetics approaches to investigate AGP backbone function is well demonstrated. The function of one cotton FLA, GhAGP24, in cotton fiber initiation and elongation (Li et al., 2010a) and four *Arabidopsis* members, *FLA1*, *FLA3*, *FLA11*, and *FLA12* have recently been published (Li et al., 2010a,b; MacMillan et al., 2010; Johnson et al., 2011). Roles for *FLA1* in lateral root and shoot development in tissue culture prior to cell-type specification (Johnson et al., 2011) and *FLA3* in microspore development, possibly by participation in cellulose deposition within

the intine (Li et al., 2010b), have been described. FLAs 11 and 12 have also been implicated in the process of cellulose deposition, contributing to plant stem strength and elasticity by affecting cell wall integrity (MacMillan et al., 2010). Such a function is consistent with an earlier report by Shi et al. (2003) implicating FLA4/SOS5 in maintaining proper cell expansion under salt-stressed conditions. The apparent diversity of FLA function may be due to the ability of FLAs to mediate protein–protein interactions with cell wall or plasma membrane-associated ligands via their fasciclin-like domains, shown in other eukaryotic systems to facilitate cell adhesion.

In addition to FLA3, AGP6, and AGP11, two classical AGPs specifically expressed in pollen, have been demonstrated to be involved in the control of timing of pollen germination, as pollen of the *agp6 agp11* double mutant germinates precociously inside the anthers (Coimbra et al., 2010). How the presence of AGP6 and AGP11 avoids precocious pollen germination is unknown, but it may occur by regulating water uptake.

The Lys-rich AGP sub-family has been the focus of several studies in tomato and *Arabidopsis*. Functional characterization of *AtAGP18*, one of the three Lys-rich AGPs, by over-expression of the genomic sequence in *Arabidopsis* indicates that *AGP18* plays a role in vegetative growth and sexual reproduction (Acosta-Garcia and Vielle-Calzada, 2004; Zhang et al., 2011a). The bushy phenotype resembles that of tomato lines over-expressing LeAGP1 and is similar to tobacco plants over-producing cytokinins (Zhang et al., 2011b) leading to the suggestion that it may participate in a cytokinin signal transduction pathway as a co-receptor of cytokinins. A similar model has been proposed for FLA4/SOS5 in its interactions with two members of the leucine-rich repeat receptor-like kinase family, FEI1 and FEI2, shown by double mutant analyses to have non-additive genetic interactions with FLA4/SOS5 (Xu et al., 2008). SOS5 has been hypothesized to act as the ligand of a signal molecule that then either binds directly to FEI1/FEI2 or assists in presenting the signal molecule to FEI1/FEI2, initiating a signaling cascade that regulates the synthesis of cellulose and ultimately cell growth.

Several questions arise from this ligand model of AGP function. Given the effects on cellulose in the *fla11 fla12* double null mutant (MacMillan et al., 2010) and the abnormal cellulose deposition *fla3* RNAi lines (Li et al., 2010b), may FLA11, FLA12, and FLA3, as well as other GPI-anchored or non-anchored AGPs, also be a part of this same network of components involved in wall sensing? Does this model explain the observation of AGPs as cell fate markers in tissues undergoing cell differentiation? Considering that the appearance of AGPs during specific developmental stages has been described using antibodies that recognize AGP-carbohydrate epitopes, is the heterogeneity of AGP glycosylation also involved in providing the necessary specificity to interact with different signal molecules and generate specific responses? What is the relevance of the presence and number of fasciclin domains of FLAs? Further investigation of the possible function of AGPs in wall sensing is of fundamental importance to uncover some of the components and mechanisms involved in the regulation of wall biosynthesis and ultimately plant cell growth. To address some of these challenges, we propose the use of the following experimental approaches, techniques, and resources:

CHALLENGE 13: TARGETING FUNCTIONAL REDUNDANCY OF AGPs

The application of multiple gene knock-down technologies such as double-stranded RNAi or artificial micro-RNAs could allow the silencing of putative redundant genes within the different AGP protein backbone subfamilies and therefore overcome the problems associated with functional redundancy. The detection of specific expression patterns and changes in transcript levels of AGP protein backbone genes have also assisted in directing the application of targeted experimental approaches to reveal their function, highlighting the importance of the availability and analysis of transcriptional data.

In addition, the use of co-expression gene network analyses to identify genes possibly related with AGP function, including those implicated in environmental sensing and signal transduction, would help to deepen our knowledge of the relationship, if any, between AGPs and the regulation of wall growth and integrity. The characterization of the promoters of AGP genes specifically expressed in pollen is generating detailed information of the tissue and spatiotemporal location of AGP transcripts that will allow the implementation of more targeted experimental approaches to test the function of pollen AGPs (Anand and Tyagi, 2010; Choi et al., 2010; Yang et al., 2011). However, when using transcriptional data as a guide to study gene function, we should be aware that in some cases mRNA levels have not been in agreement with protein levels (Yang et al., 2011).

CHALLENGE 14: PRODUCTION OF SPECIFIC AGP PROTEIN BACKBONE ANTIBODIES

The recent production of antibodies specifically recognizing the Lys-rich region of AtAGP17 and AtAGP19 protein backbones demonstrates both the veracity of this approach and also provides tools to study in more detail their tissue and cellular distribution and ultimately their function (Yang et al., 2011). Either these antibodies or alternatively antibodies to tagged versions of AGPs could be used in co-location and immunoprecipitation experiments to identify possible interacting partners.

CHALLENGE 15: DETERMINING THE FUNCTIONAL SIGNIFICANCE OF AG GLYCAN CHAIN HETEROGENEITY

One approach to address the functional importance of the glycan moiety of AGPs is to characterize AGP-specific GT mutants. Mutants implicated in AGP glycan moiety biosynthesis by transcript co-expression analysis could also by analyzed as single mutants and in combination with other GTs to potentially increase plant phenotypic severity. Limiting analyses to either single cell types (e.g., pollen/pollen tubes), or simple tissues with limited cell-types, would help in these analyses and provide a more restricted list of candidate GT genes. These genes could then be heterologously co-expressed and cellular fractions used in biochemical assays for functional assessment. While the initial aim of this work is to identify the GTs involved in AGP glycan synthesis, the underlying objective is to use these and other AGP mutants as functional assay systems to dissect the mechanism and pathway of AGP synthesis in greater detail. Such mutants are only useful as a means to manipulate AGPs if a visible and/or measurable or assayable AGP phenotype is observed.

CONCLUSION

In this brief overview we have attempted to summarize what we believe to be the major challenges facing the research community in attempting to unravel the structure, function, and biosynthesis of AGPs and to provide some indicators on how we might progress. In addition, we believe there is much to be learnt from advances our colleagues in the microbial, fungal, and mammalian proteoglycan fields have made and encourage our colleagues to embrace these findings as a guide to advancing AGP research.

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REFERENCES

Acosta-Garcia, G., and Vielle-Calzada, J.-P. (2004). A classical arabinogalactan protein is essential for the initiation of female gametogenesis in *Arabidopsis*. *Plant Cell* 16, 2614–2628.

Al-Assaf, S., Phillips, G. O., and Williams, P. A. (2005). Studies on *Acacia* exudate gums: part II. Molecular weight comparison of the Vulgares and Gummiferae series of *Acacia* gums. *Food Hydrocoll.* 19, 661–667.

Anand, S., and Tyagi, A. K. (2010). Characterization of a pollen-preferential gene OSIAGP from rice (*Oryza sativa* L. subspecies *indica*) coding for an arabinogalactan protein homologue, and analysis of its promoter activity during pollen development and pollen tube growth *Transgenic Res.* 19, 385–397.

Bacic, A., Churms, S. C., Stephen, A. M., Cohen, P. B., and Fincher, G. B. (1987). Fine structure of the arabinogalactan-protein from *Lolium multiflorum*. *Carbohydr. Res.* 162, 85–93.

Borner, G. H. H., Lilley, K. S., Stevens, T. J., and Dupree, P. (2003). Identification of glycosylphosphatidylinositol-anchored proteins in *Arabidopsis*. A proteomic and genomic analysis. *Plant Physiol.* 132, 568–577.

Borner, G. H. H., Sherrier, D. J., Stevens, T. J., Arkin, I. T., and Dupree, P. (2002). Prediction of glycosylphosphatidylinositol-anchored proteins in *Arabidopsis*. A genomic analysis. *Plant Physiol.* 129, 486–499.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238.

Choi, Y.-O., Kim, S.-S., Lee, S., Kim, S., Yoon, G.-B., Kim, H., Lee, Y.-P., Yu, G.-H., Hyung, N.-I., and Sung, S.-K. (2010). Isolation and promoter analysis of anther-specific genes encoding putative arabinogalactan proteins in *Malus* × *domestica*. *Plant Cell Rep.* 29, 15–24.

Churms, S. C., Stephen, A. M., and Siddiqui, I. R. (1981). Evidence for repeating sub-units in the molecular structure of the acidic arabinogalactan from rapeseed (*Brassica compestris*). *Carbohydr. Res.* 94, 119–122.

Coimbra, S., Costa, M., Mendes, M. A., Pereira, A. M., Pinto, J., and Pereira, L. G. (2010). Early germination of *Arabidopsis* pollen in a double null mutant for the arabinogalactan protein genes AGP6 and AGP11. *Sex. Plant Reprod.* 23, 199–205.

Defaye, J., and Wong, E. (1986). Structural studies of gum arabic, the exudate polysaccharide from *Acacia senegal*. *Carbohydr. Res.* 150, 221–231.

Doblin, M. S., Pettolino, F., and Bacic, A. (2010). Plant cell walls: the skeleton of the plant world. *Funct. Plant Biol.* 37, 357–381.

Du, H., Clarke, A. E., and Bacic, A (1996). Arabinogalactan-proteins: a class of extracellular matrix proteoglycans involved in plant growth and development. *Trends Cell Biol.* 6, 411–414.

Duan, J., Wang, X., Dong, Q., Fang, J.-N., and Li, X. (2003). Structural features of a pectic arabinogalactan with immunological activity from the leaves of *Diospyros kaki*. *Carbohydr. Res.* 338, 1291–1297.

Duan, J., Zheng, Y., Dong, Q., and Fang, J. (2004). Structural analysis of a pectic polysaccharide from the leaves of *Diospyros kaki*. *Phytochemistry* 65, 609–615.

Egelund, J., Ellis, M. A., Doblin, M. S., Qu, Y., and Bacic, A. (2011). "Genes and enzymes of the GT31 family: towards unravelling the function(s) of the plant glycosyltransferase family members," in *Plant Polysaccharides: Biosynthesis and Bioengineering*, ed. P. Ulvskov (Oxford: Wiley-Blackwell), 213–234.

Ellis, M., Egelund, J., Schultz, C. J., and Bacic, A. (2010). Arabinogalactan-proteins: key regulators at the cell surface? *Plant Physiol.* 153, 403–419.

Estévez, J. M., Kieliszewski, M. J., Khitrov, N., and Somerville, C. (2006). Characterization of synthetic hydroxyproline-rich proteoglycans with arabinogalactan protein and extensin motifs in *Arabidopsis*. *Plant Physiol.* 142, 458–470.

Faik, A., Abouzouhair, J., and Sarhan, F. (2006). Putative fasciclin-like arabinogalactan-proteins (FLA) in wheat (*Triticum aestivum*) and rice (*Oryza sativa*): identification and bioinformatic analysis. *Mol. Genet. Genomics* 276, 478–494.

Fincher, G. B., Stone, B. A., and Clarke, A. E. (1983). Arabinogalactan-proteins: structure, biosynthesis, and function. *Annu. Rev. Plant Physiol.* 34, 47–70.

Fry, S. C. (1988). *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*. London: Longman Scientific & Technical.

Gane, A. M., Craik, D., Munro, S. L. A., Howlett, G. J., Clarke, A. E., and Bacic, A. (1995). Structural analysis of the carbohydrate moiety of arabinogalactan-proteins from stigmas and styles of *Nicotiana alata*. *Carbohydr. Res.* 277, 67–85.

Goodrum, L. J., Patel, A., Leykam, J. F., and Kieliszewski, M. J. (2000). Gum arabic glycoprotein contains glycomodules of both extensin and arabinogalactan-glycoproteins. *Phytochemistry* 54, 99–106.

Gorres, K. L., and Raines, R. T. (2010). Prolyl-4-hydroxylase. *Crit. Rev. Biochem. Mol. Biol.* 45, 106–124.

Haque, M. A., Kotake, T., and Tsumuraya, Y. (2005). Mode of action of β-glucuronidase from *Aspergillus niger* on the sugar chains of arabinogalactan-protein. *Biosci. Biotechnol. Biochem.* 69, 2170–2177.

Hata, K., Tanaka, M., Tsumuraya, Y., and Hashimoto, Y. (1992). α-L-arabinofuranosidase from radish (*Raphanus sativus* L.) seeds. *Plant Physiol.* 100, 388–396.

Hijazi, M., Durand, J., Pichereaux, C., Pont, F., Jamet, E., and Albenne, C (2012). Characterization of the arabinogalactan protein 31 (AGP31) of *Arabidopsis thaliana*: new advances on the Hyp-*O*-glycosylation of the pro-rich domain. *J. Biol. Chem.* 287, 9623–9632.

Ichinose, H., Kuno, A., Kotake, T., Yoshida, M., Sakka, K., Hirabayashi, J., Tsumuraya, Y., and Kaneko, S. (2006). Characterization of an exo-β-1,3-galactanase from *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 72, 3515–3523.

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

Johnson, K. L., Jones, B. J., Schultz, C. J., and Bacic, A. (2003). "Non-enzymatic cell wall (glyco)proteins," in *The Plant Cell Wall*, ed. J. K. C. Rose (Oxford: Blackwell Publishing Ltd/CRC Press), 111–154.

Johnson, K. L., Kibble, N. A. J., Bacic, A., and Schultz, C. J. (2011). A fasciclin-like arabinogalactan-protein (FLA) mutant of *Arabidopsis thaliana*, fla1, shows defects in shoot regeneration. *PLoS ONE* 6, e25154. doi: 10.1371/journal.pone.0025154

Keegstra, K., Talmadge, K. W., Bauer, W. D., and Albersheim, P. (1973). The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the

interconnections of the macromolecular components. *Plant Physiol.* 51, 188–196.

Kieliszewski, M. J., and Lamport, D. T. A. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant J.* 5, 157–172.

Kollár, R., Reinhold, B. B., Petrákova, E., Yeh, H. J. C., Ashwell, G., Drgonová, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997). Architecture of the yeast cell wall – β(1 → 6)-glucan interconnects mannoprotein, β(1 → 3)-glucan, and chitin. *J. Biol. Chem.* 272, 17762–17775.

Kotake, T., Dina, S., Konishi, T., Kaneko, S., Igarashi, K., Samejima, M., Watanabe, Y., Kimura, K., and Tsumuraya, Y. (2005). Molecular cloning of a β-galactosidase from radish that specifically hydrolyzes β-(1 → 3)- and β-(1 → 6)-galactosyl residues of arabinogalactan protein. *Plant Physiol.* 138, 1563–1576.

Kwan, J. S., and Morvan, H. (1991). Extracellular branched xylans an acidic arabinogalactans from suspension cultured cells of white campion (*Silene alba* (Miller) E.H.L. Krause). *Food Hydrocoll.* 5, 163–166.

Kwan, J. S., and Morvan, H. (1995). Characterization of extracellular β(1,4)-xylan backbone O-substituted by arabinogalactans type II in a plant cell suspension. *Carbohydr. Polym.* 26, 99–107.

Lamport, D. T., Tan, L., and Kieliszewski, M. J. (2011). "Structural proteins of the primary cell wall: extraction, purification, and analysis," in *The Plant Cell Wall: Methods and Protocols*, ed. Z. Popper (New York: Springer-Verlag New York Inc.), 209–219.

Li, Y., Liu, D., Tu, L., Zhang, X., Wang, L., Zhu, L., Tan, J., and Deng, F. (2010a). Suppression of GhAGP4 gene expression repressed the initiation and elongation of cotton fiber. *Plant Cell Rep.* 29, 193–202.

Li, J., Yu, M., Geng, L.-L., and Zhao, J. (2010b). The fasciclin-like arabinogalactan protein gene, FLA3, is involved in microspore development of *Arabidopsis*. *Plant J.* 64, 482–497.

Liang, Y., Faik, A., Kieliszewski, M., Tan, L., Xu, W. L., and Showalter, A. M. (2010). Identification and characterization of *in vitro* galactosyltransferase activities involved in arabinogalactan-protein glycosylation in tobacco and *Arabidopsis*. *Plant Physiol.* 154, 632–642.

Ma, H., and Zhao, J. (2010). Genome-wide identification, classification, and expression analysis of the arabinogalactan protein gene family in rice (*Oryza sativa* L.). *J. Exp. Bot.* 61, 2647–2668.

MacMillan, C. P., Mansfield, S. D., Stachurski, Z. H., Evans, R., and Southerton, S. G. (2010). Fasciclin-like arabinogalactan proteins: specialization for stem biomechanics and cell wall architecture in *Arabidopsis* and *Eucalyptus*. *Plant J.* 62, 689–703.

Mascara, T., and Fincher, G. B. (1982). Biosynthesis of arabinogalactan-protein in *Lolium multiflorum* (*Ryegrass*) endosperm cells. II. *In vitro* incorporation of galactosyl residues from UDPgalactose into polymeric products. *Funct. Plant Biol.* 9, 31–45.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A., and Willats, W. G. T. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Moody, S. F., Clarke, A. E., and Bacic, A. (1988). Structural analysis of secreted slime from wheat and cowpea roots. *Phytochemistry* 27, 2857–2861.

Mostafavi, S., Ray, D., Warde-Farley, D., Grouios, C., and Morris, Q. (2008). GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. *Genome Biol.* 9, S4.

Oka, T., Saito, F., Shimma, Y.-I., Yoko-O, T., Nomura, Y., Matsuoka, K., and Jigami, Y. (2010). Characterization of endoplasmic reticulum-localized UDP-D-galactose: hydroxyproline *O*-galactosyltransferase using synthetic peptide substrates in *Arabidopsis*. *Plant Physiol.* 152, 332–340.

Osman, M. E., Menzies, A. R., Williams, P. A., Phillips, G. O., and Baldwin, T. C. (1993). The molecular characterisation of the polysaccharide gum from *Acacia senegal*. *Carbohydr. Res.* 246, 303–318.

Oxley, D., Currie, G. C., and Bacic, A. (2004). "Analysis of carbohydrate from glycoproteins," in *Purifying Proteins for Proteomics: A Laboratory Manual*, ed. R. J. Simpson (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 579–636.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., Mcgill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Pellerin, P., Vidal, S., Williams, P., and Brillouet, J. M. (1995). Characterization of five type II arabinogalactan-protein fractions from red wine of increasing uronic acid content. *Carbohydr. Res.* 277, 135–143.

Petersen, B., Egelund, J., Damager, I., Faber, K., Krüger Jensen, J., Yang, Z., Bennett, E., Scheller, H., and Ulvskov, P. B. (2009). Assay and heterologous expression in *Pichia pastoris* of plant cell wall type-II membrane anchored glycosyltransferases. *Glycoconj. J.* 26, 1235–1246.

Ponder, G. R., and Richards, G. N. (1997). Arabinogalactan from Western larch, Part III: alkaline degradation revisited, with novel conclusions on molecular structure. *Carbohydr. Polym.* 34, 251–261.

Qi, W., Fong, C., and Lamport, D. T. A. (1991). Gum arabic glycoprotein is a twisted hairy rope: a new model based on *O*-galactosylhydroxyproline as the polysaccharide attachment site. *Plant Physiol.* 96, 848–855.

Qu, Y., Egelund, J., Gilson, P. R., Houghton, F., Gleeson, P. A., Schultz, C. J., and Bacic, A. (2008). Identification of a novel group of putative *Arabidopsis thaliana* β-(1,3)-galactosyltransferases. *Plant Mol. Biol.* 68, 43–59.

Rafinska, K., and Bednarska, E. (2011). Localisation pattern of homogalacturonan and arabinogalactan proteins in developing ovules of the gymnosperm plant *Larix decidua* Mill. *Sex. Plant Reprod.* 24, 75–87.

Randall, R. C., Phillips, G. O., and Williams, P. A. (1989). Fractionation and characterization of gum from *Acacia senegal*. *Food Hydrocoll.* 3, 65–75.

Rose, J. K. C., 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.

Saulnier, L., Brillouet, J.-M., and Joseleau, J.-P. (1988). Structural studies of pectic substances from the pulp of grape berries. *Carbohydr. Res.* 182, 63–78.

Schibeci, A., Pnjak, A., and Fincher, G. B. (1984). Biosynthesis of arabinogalactan-protein in *Lolium multiflorum* (Italian ryegrass) endosperm cells. *Biochem. J.* 218, 633–636.

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.

Seifert, G. J., and Roberts, K. (2007). The biology of arabinogalactan proteins. *Annu. Rev. Plant Biol.* 58, 137–161.

Serpe, M. D., and Nothnagel, E. A. (1996). Heterogeneity of arabinogalactan-proteins on the plasma membrane of rose cells. *Plant Physiol.* 112, 1261–1271.

Shi, H., Kim, Y., Guo, Y., Stevenson, B., and Zhu, J.-K. (2003). The *Arabidopsis SOS5* locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. *Plant Cell* 15, 19–32.

Shimizu, M., Igasaki, T., Yamada, M., Yuasa, K., Hasegawa, J., Kato, T., Tsukagoshi, H., Nakamura, K., Fukuda, H., and Matsuoka, K. (2005). Experimental determination of proline hydroxylation and hydroxyproline arabinogalactosylation motifs in secretory proteins. *Plant J.* 42, 877–889.

Showalter, A. M., Keppler, B., Lichtenberg, J., Gu, D., and Welch, L. R. (2010). A bioinformatics approach to the identification, classification, and analysis of hydroxyproline-rich glycoproteins. *Plant Physiol.* 153, 485–513.

Shpak, E., Leykam, J. F., and Kieliszewski, M. J. (1999). Synthetic genes for glycoprotein design and the elucidation of hydroxyproline-*O*-glycosylation codes. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14736–14741.

Strasser, R., Bondili, J. S., Vavra, U., Schoberer, J., Svoboda, B., Glossl, J., Leonard, R., Stadlmann, J., Altmann, F., Steinkellner, H., and Mach, L. (2007). A unique β1,3-galactosyltransferase is indispensable for the biosynthesis of N-glycans containing Lewis a structures in *Arabidopsis thaliana*. *Plant Cell* 19, 2278–2292.

Tan, L., Qiu, F., Lamport, D. T. A., and Kieliszewski, M. J. (2004). Structure of a hydroxyproline (Hyp)-arabinogalactan polysaccharide from repetitive Ala-Hyp expressed in transgenic *Nicotiana tabacum*. *J. Biol. Chem.* 279, 13156–13165.

Tan, L., Varnai, P., Lamport, D. T. A., Yuan, C., Xu, J., Qiu, F., and Kieliszewski, M. J. (2010). Plant *O*-hydroxyproline arabinogalactans are composed of repeating trigalactosyl subunits with short bifurcated side chains. *J. Biol. Chem.* 285, 24575–24583.

Tryfona, T., Liang, H.-C., Kotake, T., Kaneko, S., Marsh, J., Ichinose, H., Lovegrove, A., Tsumuraya, Y., Shewry,

P. R., Stephens, E., and Dupree, P. (2010). Carbohydrate structural analysis of wheat flour arabinogalactan protein. *Carbohydr. Res.* 345, 2648–2656.

Tsumuraya, Y., Mochizuki, N., Hashimoto, Y., and Kovac, P. (1990). Purification of an exo-b-(1-3)-D-galactanase of *Irpex lacteus* (*Polyporus tulipiferae*) and its action on arabinogalactan-proteins. *J. Biol. Chem.* 265, 7207–7215.

Wu, Y., Williams, M., Bernard, S., Driouich, A., Showalter, A. M., and Faik, A. (2010). Functional identification of two nonredundant *Arabidopsis* α(1,2)fucosyltransferases specific to arabinogalactan proteins. *J. Biol. Chem.* 285, 13638–13645.

Xu, S. L., Rahman, A., Baskin, T. I., and Kieber, J. J. (2008). Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. *Plant Cell* 20, 3065–3079.

Yamada, H. (2000). "Bioactive arabinogalactan-proteins and related pectic polysaccharides in Sino-Japanese herbal medicines," in *Cell and Developmental Biology of Arabinogalactan-proteins*, eds E. A. Nothnagel, A. Bacic, and A. E. Clarke (New York, NY: Kluwer Academic/Plenum), 221–229.

Yamada, H., Kiyohara, H., Cyong, J.-C., and Otsuka, Y. (1987). Structural characterisation of an anti-complementary arabinogalactan from the roots of *Angelica acutiloba* kitagawa. *Carbohydr. Res.* 159, 275–291.

Yang, J., Zhang, Y., Liang, Y., and Showalter, A. M. (2011). Expression analyses of AtAGP17 and AtAGP19, two lysine-rich arabinogalactan proteins, in *Arabidopsis*. *Plant Biol.* 13, 431–438.

York, W. S., Darvill, A. G., Mcneil, M., Stevenson, T. T., and Albersheim, P. (1986). "Isolation and characterization of plant cell walls and cell wall components," in *Methods in Enzymology*, eds A. Weissbach and H. Weissbach (Orlando, FL: Academic Press), 3–40.

Zhang, Y., Yang, J., and Showalter, A. M. (2011a). AtAGP18 is localized at the plasma membrane and functions in plant growth and development. *Planta* 233, 675–683.

Zhang, Y., Yang, J., and Showalter, A. M. (2011b). AtAGP18, a lysine-rich arabinogalactan protein in *Arabidopsis thaliana*, functions in plant growth and development as a putative co-receptor for signal transduction. *Plant Signal. Behav.* 6, 855–857.

Zhao, Z. D., Tan, L., Showalter, A. M., Lamport, D. T. A., and Kieliszewski, M. J. (2002). Tomato LeAGP-1 arabinogalactan-protein purified from transgenic tobacco corroborates the Hyp contiguity hypothesis. *Plant J.* 31, 431–444.

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Fluorescent tags to explore cell wall structure and dynamics

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Plant cell walls are highly dynamic and heterogeneous structures, which vary between cell types, growth stages but also between microdomains within a single cell wall. In this review, we summarize the imaging techniques using fluorescent tags that are currently being used and which should in the coming years revolutionize our understanding of the dynamics of cell wall architecture and the cellular processes involved in the synthesis of cell wall components.

Keywords: plant cell-wall, fluorescence microscopy

INTRODUCTION

We currently have a reasonably good understanding of the structure of polysaccharides and, in large part thanks to the use of genetics, rapid progress is being made in the understanding of the mechanisms and regulation of wall synthesis. A major challenge for the future will be to understand cell wall architecture and its dynamics during growth. Here, we will review recent progress in fluorescence microscopy, which should largely contribute to reaching this goal.

CELL WALL STRUCTURE AND DYNAMICS

Plant cell walls are highly dynamic and heterogeneous structures, which vary between cell types, growth stages but also between microdomains within a single cell wall. Tools are needed to visualize this heterogeneity in living cells with a high spatial and temporal resolution.

Electron microscopy techniques [transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM)] have been instrumental in obtaining information on wall architecture. However, these techniques require fixed material, which is not always representative for native hydrated cell walls and provide little or no information on cell wall dynamics. The use of fluorescent probes should provide access to dynamic aspects. A large number of anti-glycan antibodies are available (Knox, 2008; Moller et al., 2008; Pattathil et al., 2010; Ralet et al., 2010, see the review Lee et al., 2011; PlantProbes[1]; CarboSource Services[2]). In addition, the increasing number of sequenced genomes of plants and microorganisms provide a virtually unlimited supply of often highly specific carbohydrate binding domains (Guillen et al., 2010). These probes in general provide a static picture but labeling of living cells is also possible. For instance, Domozych et al. (2009) used antibodies to study pectin maturation in the single celled Charophycean alga *Penium margaritaceum*. It will be interesting to explore similar approaches to study wall dynamics in higher plants. Caution is required in using these probes since polysaccharide epitopes are frequently masked by other polymers (Marcus et al., 2008, 2010).

Recently, significant progress was also made in the visualization of cellulose dynamics in living cells using the cellulose-specific dye Pontamine Fast Scarlet 4B (S4B; Anderson et al., 2010). Confocal microscopy of S4B-stained roots revealed the orientation of cellulose microfibrils. Interestingly, the staining did not interfere with growth and a rotation of the microfibrils, from transverse to longitudinal to the elongation axis, could be observed during cell elongation. This is the first *in vivo* validation of the 57-year-old "multinet growth" hypothesis (Roelofsen and Houwink, 1953).

Enzyme activity can also be visualized in the wall using fluorescent donor substrates. Sulforhodamine conjuguates of xyloglucan oligosaccharides (XGO-SRs) were used to visualize endogenous xyloglucan endo-transglycosylase (XET) activity (of the xyloglucan transglycosylase/hydrolase enzyme, XTH) in *Arabidopsis* and

Abbreviations: BiFC, bimolecular fluorescence complementation; GFP, green fluorescent protein; FESEM, field emission scanning electron microscopy; FLIP, fluorescence loss in photobleaching; FPALM, fluorescence photoactivated localization microscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; LSCM, laser scanning confocal microscopy; LSFM, light-sheet-based fluorescence microscopy; PALM, photoactivated localization microscopy; SDCM, spinning disk confocal microscopy; SIM, structured illumination microscopy; SPIM, selective plane illumination; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TEM, transmission electron microscopy; TIRFM, total internal reflection fluorescence microscopy; VAEM, variable-angle epifluorescence microscopy; YFP, yellow fluorescent protein.

[1] http://www.plantprobes.net/

[2] http://www.ccrc.uga.edu/services/

Tobacco roots and cultured cells (Vissenberg et al., 2005) as well as in poplar wood (Nishikubo et al., 2011). XGO-SRs are infiltrated into the tissue and incorporated into xyloglucan in the cell wall yielding an orange fluorescence indicative of the simultaneous colocalization in the same compartment of active XET and acceptor xyloglucan chains. Interestingly, a fibrillar pattern associated with cellulose microfibrils was observed in elongating cells, suggesting that XTHs act on xyloglucans attached to cellulose microfibrils. The XET activity found in elongating cells may play a role in the incorporation of newly synthesized xyloglucan molecules into the cell wall and/or the remodeling of the existing cellulose/xyloglucan network. Glycosyl hydrolase activity also can be visualized *in situ*. For instance, endo-β-1,4-xyloglucanase activity was detected *in muro* using a resorufin β-glycoside of a xylogluco-oligosaccharide (XXXG-β-Res; Ibatullin et al., 2009) and cellulase activity using a resorufinyl-β-D-cellobioside (Glc-2-β-Res; Takahashi et al., 2009).

Another major advance was made with the use of click chemistry to study the metabolism of glycans *in vivo*. The strategy is based upon the metabolic incorporation of azide sugars into target molecules, priming them for selective covalent reaction with fluorescent probes. The azide is small, stable in biological systems, and selectively reactive with phosphines or activated alkynes. Previously, the Staudinger ligation has been used to tag azido sugar-labeled glycoconjugates on cultured cells and in live mice (Prescher et al., 2004). The copper-catalyzed click chemistry has shown the incorporation of an azido-containing fucose analog into glycoproteins via the fucose salvage pathway in jurkat cells (Sawa et al., 2006). Since these first experiments, the method has been further developed and adapted for plant cells. Anderson et al. (2012) used fucose alkyne (FucAI) to label pectic rhamnogalacturonan-I (RG-I) in roots of *Arabidopsis* seedlings. Using pulse-chase experiments, they were able to visualize the deposition of pectin and the reorientation of the pectin network during the elongation of epidermal root cells. A problem with this method is that Cu(I) catalyst formulations are toxic preventing their use in living cells. Different approaches have been developed to overcome this problem. Soriano Del Amo et al. (2010) reported that BTTES, a tris(triazolylmethyl)amine-based ligand for Cu(I), promotes the cycloaddition reaction rapidly in living systems without apparent toxicity. This catalyst allowed, for the first time, non-invasive imaging of fucosylated glycans during zebrafish early embryogenesis. Recent alternative approaches are based on "Cu-free click chemistry" (for review, see Chang et al., 2010; Jewett and Bertozzi, 2010) to increase the rate of the cycloaddition without the need of a catalyst. These methods have been used to label biomolecules in zebrafish (Laughlin et al., 2008) and in living mice (Chang et al., 2010). It will be extremely interesting to use similar methods to visualize the sites of synthesis, deposition, and turnover of different polysaccharides in plants (Wallace and Anderson, 2012).

FLUORESCENCE MICROSCOPY TO STUDY IN LIVING CELLS THE INTRACELLULAR DYNAMICS AND STOICHIOMETRY OF PROTEIN COMPLEXES INVOLVED IN CELL WALL BIOSYNTHESIS

Laser scanning confocal microscopy (LSCM) and spinning disk confocal microscopy (SDCM) technologies have been used to study, often in great detail, the dynamics of proteins involved in cell wall synthesis. Whereas LSCM uses a single pinhole for optical sectioning, SDCM uses an array of excitation and emission pinhole apertures (one or two pinhole arrays can be used) on a rapidly spinning disk, such that the pinhole array sweeps the entire field of view over 1,000 times/s. The high scan speed not only improves image acquisition rate (up to 360 frames/s), it also has the effect of lowering the peak excitation light density down to a few $\mu W/\mu m^2$, thereby increasing fluorescence efficiency and decreasing photobleaching and photodamage effects compared to point scanning. Perhaps most importantly, because the entire confocal field of view can be captured by a high-quantum efficiency, low-noise camera instead of a photomultiplier tube (PMT), SDCM systems have a more than 50-fold increase in light capture efficiency (i.e., average number of photons acquired from a single bead plotted against exposure index which is determined by measuring photobleaching rates and not the actual fluorescence at each analyzed location in the specimen), resulting in a several-fold increase in signal-to-noise ratio relative to LSCM. This unexpected difference is accounted for by the large difference in quantum efficiency (percentage of photons hitting the photoreactive surface that will produce an electron-hole pair) between the CCD camera of SDCM (around 60%) and the PMT of LSCM (around 10%). However, the reasons for the remainder of the efficiency gap remain unclear (Murray et al., 2007). Gräf et al. (2005) propose an explanation based on the fact that scanning systems, in contrast to SDCM, are often operated at or near saturation for fluorescence, thus a situation in which no new emitted photons are produced while photobleaching increases.

Using SDCM, fluorescent punctae could be observed in the plasma membrane of *Arabidopsis* hypocotyl cells expressing XFP fused to distinct components of the cellulose synthase complex (CSC; CESA3, CESA6, CESA5, or CSI1/POM2; Paredez et al., 2006; Desprez et al., 2007; Gu et al., 2010; Bischoff et al., 2011; Bringmann et al., 2012; Sanchez-Rodriguez et al., 2012). These punctae migrate bidirectionally following linear trajectories and most likely correspond to individual CSCs. Since the CSCs are propelled by the polymerization of the glucan chains, the rate of the CSCs can be used to quantify cellulose synthesis *in vivo*. These tools have been extensively used to study the role of cortical microtubules in guiding the CSC trajectories, their insertion into the plasma membrane, the regulation of CSC movement by phosphorylation, as well as the action of small inhibitors of cellulose biosynthesis (DeBolt et al., 2007a,b; Crowell et al., 2009; Gutierrez et al., 2009; Chen et al., 2010; Bischoff et al., 2011).

The intracellular trafficking and the lifetime of proteins can be conveniently studied using photoactivatable or photoconvertible fluorescent proteins (Patterson et al., 2010). Photoactivatable fluorescent proteins have been used in the plant field to analyze the dynamic relationship between the ER and Golgi stacks in tobacco leaf epidermal cells (Runions et al., 2006) or the distribution of KAT1 K^+ channels in tobacco leaves (Sutter et al., 2006). The property of photoconvertible fluorescent proteins to show pronounced light-induced spectral changes allows pulse-chase analysis of protein trafficking. For instance, UV-induced

green-to-red photoconversion of mEosFP was used to study the translocation of PIN2-mEosFP from one intracellular location to another (Dhonukshe et al., 2007). Such tools will be very useful to analyze the trafficking of CSC components or other cell wall biosynthetic enzymes.

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are complementary tools for imaging cell wall proteins. Using FRAP, the delivery frequency of CESA complexes to the plasma membrane was estimated at around 5 events/μm^2 h (Gutierrez et al., 2009). In this case, a short bleaching period followed by post-bleach acquisitions is used to measure any recovery of fluorescence into the bleached area by non-bleached CESA particles. FLIP has been used to study the distribution of CSCs in developing xylem vessels. Using a confocal microscope, yellow fluorescent protein (YFP)–CSCs appear as transverse bands in developing xylem vessels (Wightman and Turner, 2008). To overcome the problem of the high density of YFP–CSCs and the focal depth necessary for imaging deeper layers of the organ, FLIP was used. With this approach, the movement of bleached particles outwards from the bleached region was measured (White and Stelzer, 1999). The identical rates of fluorescence loss on either side of the bleached zone suggested that these CSCs, like the primary cell wall CSCs, also migrate bidirectionally. The motility of YFP–CSC complexes was estimated at around 7 μm/s far higher than those previously reported for primary wall CSCs. It is unlikely, however, that such a high velocity reflects the cellulose synthesis rate (which would be some 14,000 glucose residues/s.chain against 10 residues/s.chain for primary cell walls). It is more likely that the authors have measured the much faster movement of intracellular CSC-containing compartments (Wightman et al., 2009).

Fluorescence microscopy is also commonly used to study protein–protein interactions in plants. Techniques used are fluorescence resonance energy transfer (FRET) or bimolecular fluorescence (BiFC) or luciferase complementation (Jares-Erijman and Jovin, 2003; Bracha-Drori et al., 2004; Fujikawa and Kato, 2007). BiFC strategies have been used to determine specific interactions between the different CESA isoforms (Desprez et al., 2007; Timmers et al., 2009). The N-terminus half of YFP is fused to a first CESA isoform and the C-terminus to a second isoform. Detection of YFP signal signifies protein–protein interaction events since the YFP halves are in sufficient proximity to form an intact YFP molecule. It is an easy and rapid method, however, it is important to note that it creates irreversible interactions and appropriate controls need to be included such as proteins present in the same compartment that do not interact with the tested protein.

A recent improvement in the detection of molecules at the surface of plant cells is the use of total internal reflection fluorescence microscopy (TIRFM). This technique is based on the physical phenomenon of total internal reflection (TIR), which occurs when a ray of light strikes a boundary between two materials with different refractive indices (n) and the incident angle is greater than the critical angle of incidence. Under these conditions, all of the light is reflected back into the medium with the higher n value. When TIR occurs, a near-field light wave forms at the boundary; this "evanescent wave" (EW) can penetrate the surface of the medium to a depth approximately equal to 1/3 of the wavelength of the incident light. The TIRFM can provide around 100 nm of excitation volume near the plasma membrane. Interestingly despite the presence of the several 100-nm thick cell wall, a TIRF-derived method, known as variable-angle epifluorescence microscopy (VAEM), was recently developed in plants to visualize vesicle trafficking and fusion events at the plasma membrane. The technique uses a laser beam with a suboptimal incident angle, slightly smaller than the critical angle. Konopka *et al.* were able to visualize the dynamics of membrane proteins, organelles, and cytoskeleton in various plant cells (Konopka and Bednarek, 2008b; Wan et al., 2011) and validated the optical principles underlying VAEM in plant cells. They analyzed in detail the motility of clathrin light chains (CLCs) during endocytosis (Konopka et al., 2008). In both tip growing root hairs and diffusely expanding epidermal cells, a CLC-XFP fusion protein colocalized with the dynamin-related proteins DRP1C-GFP in dynamic foci at the cell cortex. The authors were able to follow appearance and disappearance at the plasma membrane of the proteins and differentiate the behavior of two distinct DRPs (Konopka and Bednarek, 2008a). The results demonstrate the involvement of DRPs in clathrin-mediated membrane dynamics. Since GFP-CESA3 can be visualized in living cells by VAEM (**Figure 1**), it will be interesting to see if the internalization of CSCs is also mediated by this pathway. Using VAEM, the organization and dynamics of actin filaments were studied in detail in epidermal cells of *Arabidopsis* hypocotyls and it has also been shown that there is a gradual appearance of the auxin efflux carrier PIN2-GFP at the outer lateral plasma membrane of root epidermal cells (Staiger et al., 2009; Kleine-Vehn et al., 2011). Interestingly, the VAEM method can also be used to analyze the oligomeric state of proteins. The subunit number and

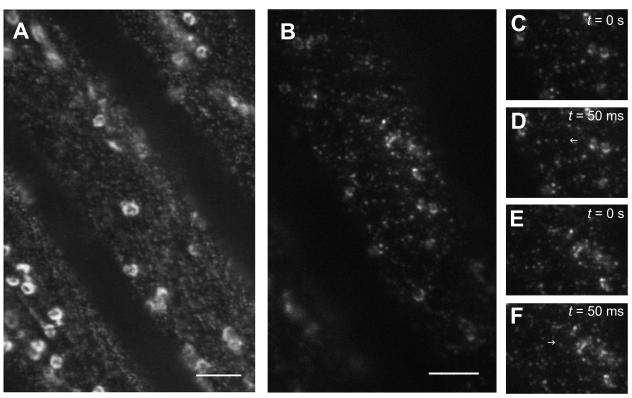


FIGURE 1 | Visualization of GFP-CESA3 using different microscopy techniques. *Arabidopsis* dark-grown hypocotyl cells expressing the cellulose synthase catalytic subunit CESA3 fused to GFP were imaged using spinning disk confocal microscopy **(A)** or variable-angle epifluorescence microscopy **(B)**. Note that in **(B)**, only the vesicles at the cell surface are well defined and thanks to the use of an objective ×100 with a numerical aperture of 1.49, the xy resolution and the signal-to-noise ratio near the cell edge are improved. Scale bars: 10 μM. Images from a time series **(C–D)**. Note the appearance of a GFP-CESA3 vesicle in the second image. Images from a time series **(E–F)**. Note the disappearance of a GFP-CESA3 vesicle. Blue arrows indicate the presence or the absence of GFP-CESA3 in successive pictures.

stoichiometry of membrane-bound proteins can be determined through the statistical analysis of bleaching steps of GFP fused to the proteins. Li et al. (2011) imaged GFP-labeled aquaporin PIP2;1 in the plasma membrane of root cells. The genetic attachment of GFP to PIP2;1 guaranteed that each PIP2;1 polypeptide was labeled with only one fluorophore. To determine the oligomeric state of PIP2;1, they counted the photobleaching steps for chosen fluorescent spots. The maximum number of elemental GFP photobleaching steps in an individual punctum was four, consistent with the aquaporin tetramer observed in crystallographic studies (Tornroth-Horsefield et al., 2006). This approach will be very helpful to study the stoichiometry of cell wall biosynthetic complexes.

Recently, new high-resolution techniques allow imaging beyond the diffraction resolution barrier. Several strategies are being used: stimulated emission depletion (STED) microscopy, linear or non-linear structured illumination microscopy (SIM), and the pointillist techniques: photoactivated localization microscopy (PALM), fluorescence photoactivated localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM). While they show great promise for cell wall research, these techniques all have individual strengths and weaknesses. For a detailed discussion, see Leung and Chou (2011). Such methods will be of great value to further analyze the organization of CSCs and their link with cortical microtubules. STED already has been used to show that PIN1 and PIN2 auxin efflux carriers cluster in microdomains in the plasma membrane (Kleine-Vehn et al., 2011).

IMAGING PLANT GROWTH IN 4D

Major obstacles for 4D (3D + time) imaging are the inherent speed limitation of point-scanning techniques for optical sectioning and the photobleaching that accompanies repeated excitation. The use of light-sheet-based fluorescence microscopy (LSFM), known in different implementations as selective plane illumination (SPIM) or digital scanned laser light-sheet microscopy (DSLM) overcomes these limitations (Huisken et al., 2004; Keller and Stelzer, 2008). The technique is based on the use of a thin sheet of light, which illuminates a single X-Y plane of the specimen, combined with wide-field fluorescence detection with a CCD camera in the direction perpendicular to the excitation plane. With this technique, only a narrow region near the plane of observation is illuminated, as a result fluorophores outside this plane are not excited which reduces background signals and photodamage. These instrumental characteristics produce an optical sectioning effect, improved contrast, and allow long observations of sensitive samples. Three-dimensional recording is performed by moving the specimen through the illuminated plane. The acquisition of series of images from three different angles, combined with a processing algorithm, allows the signal fluorescence to be discriminated from the scattered background light. This technique has recently been used to record the growth of vertically oriented primary root tips and lateral root primordia for up to 38 h without any obvious damage (Maizel et al., 2011). In addition, the dynamics of the endosomal compartments could be visualized for over 40 s with a speed of 4 frames per second. This technique will allow 3D reconstructions to be made of entire organs and follow the growth of each individual cell over long time periods.

CONCLUSION

A large panel of fluorescent imaging techniques is now available for the study of tissues, cells, and cellular components in 3D or 4D in plants. These imaging techniques most likely will revolutionize in the coming years our understanding of the dynamics of cell wall architecture and the cellular processes involved in synthesis of cell wall components. However, each imaging modality has relative strengths and weaknesses that need to be taken into account for a given biological problem.

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REFERENCES

Anderson, C. T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. *Plant Physiol.* 152, 787–796.

Anderson, C. T., Wallace, I. S., and Somerville, C. R. (2012). Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in *Arabidopsis* cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1329–1334.

Bischoff, V., Desprez, T., Mouille, G., Vernhettes, S., Gonneau, M., and Hofte, H. (2011). Phytochrome regulation of cellulose synthesis in *Arabidopsis*. *Curr. Biol.* 21, 1822–1827.

Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S., and Ohad, N. (2004). Detection of protein-protein interactions in plants using bimolecular fluorescence complementation. *Plant J.* 40, 419–427.

Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M. T., and Persson, S. (2012). POM-POM2/cellulose Synthase Interacting1 is essential for the functional association of cellulose synthase and microtubules in *Arabidopsis*. *Plant Cell* 24, 163–177.

Chang, P. V., Prescher, J. A., Sletten, E. M., Baskin, J. M., Miller, I. A., Agard, N. J., Lo, A., and Bertozzi, C. R. (2010). Copper-free click chemistry in living animals. *Proc. Natl. Acad. Sci. U.S.A.* 107, 1821–1826.

Chen, S., Ehrhardt, D. W., and Somerville, C. R. (2010). Mutations of cellulose synthase (CESA1) phosphorylation sites modulate anisotropic cell expansion and bidirectional mobility of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17188–17193.

Crowell, E. F., Bischoff, V., Desprez, T., Rolland, A., Stierhof, Y. D., Schumacher, K., Gonneau, M., Hofte, H., and Vernhettes, S. (2009). Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell* 21, 1141–1154.

DeBolt, S., Gutierrez, R., Ehrhardt, D. W., Melo, C. V., Ross, L., Cutler, S. R., Somerville, C., and Bonetta, D. (2007a). Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5854–5859.

DeBolt, S., Gutierrez, R., Ehrhardt, D. W., and Somerville, C. (2007b). Non-motile cellulose synthase subunits repeatedly accumulate within localized regions at the plasma membrane in *Arabidopsis* hypocotyl cells following 2,6-dichlorobenzonitrile treatment. *Plant Physiol.* 145, 334–338.

Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15572–15577.

Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D. G., Mravec, J., Stierhof, Y. D., and Friml, J. (2007). Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in *Arabidopsis*. *Curr. Biol.* 17, 520–527.

Domozych, D. S., Lambiasse, L., Kiemle, S. N., and Gretz, M. R. (2009). Cell-wall development and bipolar growth in the Desmid *Penium margaritaceum* (Zygnematophyceae, Streptophyta). Asymmetry in a symmetric world. *J. Phycol.* 45, 879–893.

Fujikawa, Y., and Kato, N. (2007). Split luciferase complementation assay to

study protein-protein interactions in *Arabidopsis* protoplasts. *Plant J.* 52, 185–195.

Gräf R., Rietdorf, J., and Zimmermann, T. (2005). Live cell spinning disk microscopy. *Adv. Biochem. Eng. Biotechnol.* 95, 57–75.

Gu, Y., Kaplinsky, N., Bringmann, M., Cobb, A., Carroll, A., Sampathkumar, A., Baskin, T., Persson, S., and Somerville, C. (2010). Identification of a novel CESA-associated protein required for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12866–12871.

Guillen, D., Sanchez, S., and Rodriguez-Sanoja, R. (2010). Carbohydrate-binding domains: multiplicity of biological roles. *Appl. Microbiol. Biotechnol.* 85, 1241–1249.

Gutierrez, R., Lindeboom, J. J., Paredez, A. R., Emons, A. M., and Ehrhardt, D. W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nat. Cell Biol.* 11, 797–806.

Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J., and Stelzer, E. H. (2004). Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* 305, 1007–1009.

Ibatullin, F. M., Banasiak, A., Baumann, M. J., Greffe, L., Takahashi, J., Mellerowicz, E. J., and Brumer, H. (2009). A real-time fluorogenic assay for the visualization of glycoside hydrolase activity in planta. *Plant Physiol.* 151, 1741–1750.

Jares-Erijman, E. A., and Jovin, T. M. (2003). FRET imaging. *Nat. Biotechnol.* 21, 1387–1395.

Jewett, J. C., and Bertozzi, C. R. (2010). Cu-free click cycloaddition reactions in chemical biology. *Chem. Soc. Rev.* 39, 1272–1279.

Keller, P. J., and Stelzer, E. H. (2008). Quantitative in vivo imaging of entire embryos with Digital Scanned Laser Light Sheet Fluorescence Microscopy. *Curr. Opin. Neurobiol.* 18, 624–632.

Kleine-Vehn, J., Wabnik, K., Martiniere, A., Langowski, L., Willig, K., Naramoto, S., Leitner, J., Tanaka, H., Jakobs, S., Robert, S., Luschnig, C., Govaerts, W., Hell, S. W., Runions, J., and Friml, J. (2011). Recycling, clustering, and endocytosis jointly maintain PIN auxin carrier polarity at the plasma membrane. *Mol. Syst. Biol.* 7, 540.

Knox, J. P. (2008). Revealing the structural and functional diversity of plant cell walls. *Curr. Opin. Plant Biol.* 11, 308–313.

Konopka, C. A., Backues, S. K., and Bednarek, S. Y. (2008). Dynamics of *Arabidopsis* dynamin-related protein 1C and a clathrin light chain at the plasma membrane. *Plant Cell* 20, 1363–1380.

Konopka, C. A., and Bednarek, S. Y. (2008a). Comparison of the dynamics and functional redundancy of the *Arabidopsis* dynamin-related isoforms DRP1A and DRP1C during plant development. *Plant Physiol.* 147, 1590–1602.

Konopka, C. A., and Bednarek, S. Y. (2008b). Variable-angle epifluorescence microscopy: a new way to look at protein dynamics in the plant cell cortex. *Plant J.* 53, 186–196.

Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008). In vivo imaging of membrane-associated glycans in developing zebrafish. *Science* 320, 664–667.

Lee, K. J., Marcus, S. E., and Knox, J. P. (2011). Cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219.

Leung, B. O., and Chou, K. C. (2011). Review of super-resolution fluorescence microscopy for biology. *Appl. Spectrosc.* 65, 967–980.

Li, X., Wang, X., Yang, Y., Li, R., He, Q., Fang, X., Luu, D. T., Maurel, C., and Lin, J. (2011). Single-molecule analysis of PIP2;1 dynamics and partitioning reveals multiple modes of *Arabidopsis* plasma membrane aquaporin regulation. *Plant Cell* 23, 3780–3797.

Maizel, A., von Wangenheim, D., Federici, F., Haseloff, J., and Stelzer, E. H. (2011). High-resolution live imaging of plant growth in near physiological bright conditions using light sheet fluorescence microscopy. *Plant J.* 68, 377–385.

Marcus, S. E., Blake, A. W., Benians, T. A., Lee, K. J., Poyser, C., Donaldson, L., Leroux, O., Rogowski, A., Petersen, H. L., Boraston, A., Gilbert, H. J., Willats, W. G., and Knox, J. P. (2010). Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *Plant J.* 64, 191–203.

Marcus, S. E., Verhertbruggen, Y., Herve, C., Ordaz-Ortiz, J. J., Farkas, V., Pedersen, H. L., Willats, W. G., and Knox, J. P. (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biol.* 8, 60. doi: 10.1186/1471-2229-8-60

Moller, I., Marcus, S. E., Haeger, A., Verhertbruggen, Y., Verhoef, R., Schols, H., Ulvskov, P., Mikkelsen, J. D., Knox, J. P., and Willats, W. (2008). High-throughput screening of monoclonal antibodies against plant cell wall glycans by hierarchical clustering of their carbohydrate microarray binding profiles. *Glycoconj. J.* 25, 37–48.

Murray, J. M., Appleton, P. L., Swedlow, J. R., and Waters J. C. (2007). Evaluating performance in three-dimensional fluorescence microscopy. *J. Microsc.* 228, 390–405.

Nishikubo, N., Takahashi, J., Roos, A. A., Derba-Maceluch, M., Piens, K., Brumer, H., Teeri, T. T., Stalbrand, H., and Mellerowicz, E. J. (2011). Xyloglucan endo-transglycosylase-mediated xyloglucan rearrangements in developing wood of hybrid aspen. *Plant Physiol.* 155, 399–413.

Paredez, A. R., Somerville, C. R., and Ehrhardt, D. W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. *Science* 312, 1491–1495.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Patterson, G., Davidson, M., Manley, S., and Lippincott-Schwartz, J. (2010). Superresolution imaging using single-molecule localization. *Annu. Rev. Phys. Chem.* 61, 345–367.

Prescher, J. A., Dube, D. H., and Bertozzi, C. R. (2004). Chemical remodelling of cell surfaces in living animals. *Nature* 430, 873–877.

Ralet, M. C., Tranquet, O., Poulain, D., Moise, A., and Guillon, F. (2010). Monoclonal antibodies to rhamnogalacturonan I backbone. *Planta* 231, 1373–1383.

Roelofsen, P. A., and Houwink, A. L. (1953). Architecture and growth of the primary cell wall in some plant hairs and in the Phycomyces sporangiophore. *Acta Bot. Neerland* 2, 218–225.

Runions, J., Brach, T., Kuhner, S., and Hawes, C. (2006). Photoactivation of GFP reveals protein dynamics within the endoplasmic reticulum membrane. *J. Exp. Bot.* 57, 43–50.

Sanchez-Rodriguez, C., Bauer, S., Hematy, K., Saxe, F., Ibanez, A. B., Vodermaier, V., Konlechner, C., Sampathkumar, A., Ruggeberg, M., Aichinger, E., Neumetzler, L., Burgert, I., Somerville, C., Hauser, M. T., and Persson, S. (2012). Chitinase-like1/pom-pom1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in *Arabidopsis*. *Plant Cell* 24, 589–607.

Sawa, M., Hsu, T. L., Itoh, T., Sugiyama, M., Hanson, S. R., Vogt, P. K., and Wong, C. H. (2006). Glycoproteomic probes for fluorescent imaging of fucosylated glycans in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12371–12376.

Soriano Del Amo, D., Wang, W., Jiang, H., Besanceney, C., Yan, A. C., Levy, M., Liu, Y., Marlow, F. L., and Wu, P. (2010). Biocompatible copper(I) catalysts for in vivo imaging of glycans. *J. Am. Chem. Soc.* 132, 16893–16899.

Staiger, C. J., Sheahan, M. B., Khurana, P., Wang, X., McCurdy, D. W., and Blanchoin, L. (2009). Actin filament dynamics are dominated by rapid growth and severing activity in the *Arabidopsis* cortical array. *J. Cell Biol.* 184, 269–280.

Sutter, J. U., Campanoni, P., Tyrrell, M., and Blatt, M. R. (2006). Selective mobility and sensitivity to SNAREs is exhibited by the *Arabidopsis* KAT1 K^+ channel at the plasma membrane. *Plant Cell* 18, 935–954.

Takahashi, J., Rudsander, U. J., Hedenstrom, M., Banasiak, A., Harholt, J., Amelot, N., Immerzeel, P., Ryden, P., Endo, S., Ibatullin, F. M., Brumer, H., del Campillo, E., Master, E. R., Scheller, H. V., Sundberg, B., Teeri, T. T., and Mellerowicz, E. J. (2009). KORRIGAN1 and its aspen homolog PttCel9A1 decrease cellulose crystallinity in *Arabidopsis* stems. *Plant Cell Physiol.* 50, 1099–1115.

Timmers, J., Vernhettes, S., Desprez, T., Vincken, J. P., Visser, R. G., and Trindade, L. M. (2009). Interactions between membrane-bound cellulose synthases involved in the synthesis of the secondary cell wall. *FEBS Lett.* 583, 978–982.

Tornroth-Horsefield, S., Wang, Y., Hedfalk, K., Johanson, U., Karlsson, M., Tajkhorshid, E., Neutze, R., and Kjellbom, P. (2006). Structural mechanism of plant aquaporin gating. *Nature* 439, 688–694.

Vissenberg, K., Fry, S. C., Pauly, M., Hofte, H., and Verbelen, J. P. (2005). XTH acts at the microfibril-matrix interface during cell elongation. *J. Exp. Bot.* 56, 673–683.

Wallace, I., and Anderson, C. T. (2012). Small molecule probes for plant cell wall polysaccharide imaging. *Front. Plant Physiol.* 3:89. doi: 10.3389/fpls.2012.00089

Wan, Y., Ash, W. M. III., Fan, L., Hao, H., Kim, M. K., and Lin, J. (2011). Variable-angle total internal

reflection fluorescence microscopy of intact cells of *Arabidopsis thaliana*. *Plant Methods* 7, 27.

White, J., and Stelzer, E. (1999). Photobleaching GFP reveals protein dynamics inside live cells. *Trends Cell Biol.* 9, 61–65.

Wightman, R., Marshall, R., and Turner, S. R. (2009). A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. *Plant Cell Physiol.* 50, 584–594.

Wightman, R., and Turner, S. R. (2008). The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. *Plant J.* 54, 794–805.

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Analytical technologies for identification and characterization of the plant *N*-glycoproteome

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N-glycosylation is one of the most common and complex post-translational modifications of eukaryotic proteins and one that has numerous roles, such as modulating protein stability, sorting, folding, enzyme activity, and ligand interactions. In plants, the functional significance of *N*-glycosylation is typically obscure, although it is a feature of most secreted proteins and so is potentially of considerable interest to plant cell wall biologists. While analytical pipelines have been established to characterize yeast, mammalian, and bacterial *N*-glycoproteomes, such large-scale approaches for the study of plant glycoproteins have yet to be reported. Indeed, the *N*-glycans that decorate plant and mammalian or yeast proteins are structurally distinct and so modification of existing analytical approaches are needed to tackle plant *N*-glycoproteomes. In this review, we summarize a range of existing technologies for large-scale *N*-glycoprotein analysis and highlight promising future approaches that may provide a better understanding of the plant *N*-glycoproteome, and therefore the cell wall proteome and other proteins associated with the secretory pathway.

Keywords: glycoproteins, *N*-glycans, glycopeptides, lectins, mass spectrometry

INTRODUCTION

Characterization of the detailed structures of *N*-glycoproteins can provide valuable insights into basics aspects of cell and organismal biology and is also becoming increasingly important for pharmaceutical development and production (De Marchis et al., 2011; Xu et al., 2011; Yang et al., 2012). In plants, *N*-glycosylation is a common feature of secreted proteins and there is considerable interest in developing more advanced analytical platforms to characterize *N*-glycosylation in order to provide new insights in the plant cell wall proteome (Minic et al., 2007; Marino et al., 2010; Zhang et al., 2010; Catala et al., 2011; Ruiz-May et al., 2012). However, structural elucidation of glycans, glycopeptides, and glycoproteins is notoriously difficult as glycans are structurally complex, being branched, and containing a variety of types of intersaccharide linkages, and heterogeneous, with a wide range of abundance between glycoforms and occupancy of glycosylated sites. Ideally, a comprehensive analysis of a plant glycoprotein involves the identification of glycosylated peptides, determination of the location of the glycosylation sites, and elucidation of the glycan structure (Fitchette et al., 2007), each of which presents a specific set of technical challenges.

In the past decade, biological mass spectrometry (MS) has become established as a core analytical tool for proteome profiling (Thelen and Peck, 2007; Yates et al., 2009; Ytterberg and Jensen, 2010; Song et al., 2011) as well as characterization of protein glycosylation patterns and glycan analysis (Harvey, 2005; Park and Lebrilla, 2005). However, while advanced tandem MS (MS/MS) technologies provide an effective means for glycopeptide amino acid sequencing and obtaining structural information from oligosaccharides (e.g., sequence, linkages, and branching), the high degree of complexity and variability in glycoprotein populations from any eukaryotic source still poses significant analytical obstacles. Sample preparation that allows enrichment of the targeted glycoproteins/glycopeptides is therefore often an important step as it increases the concentration of the desired analyte while reducing sample complexity. A second technical hurdle is the hydrophilic nature of glycans and their relatively larger masses compared with the native tryptic peptides to which they are attached. This often results in poor retention on the reverse phase chromatographic materials that are typically used in peptide separations, reduced ionization efficiency and larger than optimal *m*/*z* detection range in MS analysis, all of which can contribute to poor sensitivity (An et al., 2009; Ytterberg and Jensen, 2010). Furthermore, the labile nature of the glycan–peptide bond often causes the neutral loss of individual carbohydrates and few fragmented ions from the peptide during the collision induced dissociation (CID), yielding little or no information for peptide identification, glycol-site determination, and full glycan sequence. As a result, deglycosylation is often required and the free peptide and glycan are subjected separately to tandem MS (MS/MS) analysis. Finally, due to the high glycan heterogeneity, determination of the glycan structure through bioinformatics tool-based database searching is often difficult and requires time-consuming manual interpretation.

To overcome these analytical challenges, many new strategies have been developed and implemented over the past decade involving the development of three major tools: (1) selective enrichment technologies for glycoproteins, glycopeptides, and

released glycans, and improvements in separation science including the miniaturization of chromatographic formats, capillary electrophoresis (CE), and ion mobility technologies; (2) enhanced MS technology and development of more effective analysis workflows; and (3) newly developed software packages and algorithms for interpretation of MS fragmentation spectra. Most of these technologies have been initially developed to study mammalian glycoproteins and there are only a few reported examples of their successful application to characterize plant *N*-glycosylation (Fitchette et al., 2007; Bardor et al., 2009; Zhang et al., 2012), which is structurally distinct from that of mammalian or yeast proteins (Ruiz-May et al., 2012). Key structural differences, such as the absence of non-reducing terminal sialic acid in plant *N*-glycans and the presence of α-1,3-fucose (Fuc) in the innermost GlcNAc residue necessitate tailoring of existing analytical workflows.

In this review we present some of the analytical platforms or strategies, including new developments in MS instrumentation, which can be used for the systematic characterization of *N*-glycoproteins, with particular reference to the challenges of studying those from plants, as well as a perspective of future developments in the field. To our knowledge there are no published reports describing large- or even intermediate-scale analysis of plant protein glycosylation together with associated *N*-glycans analysis. However, given the new and emerging analytical pipelines we are soon likely to see a dramatic expansion in the identification and functional characterization of plant *N*-glycoproteomes, which in turn will be of great value for researchers studying the plant cell wall and associated secretory pathways.

GLYCOPROTEIN DETECTION

A summary of a typical workflow for characterizing *N*-glycoproteins/larger scale *N*-glycoproteomes is shown in **Figure 1**. A valuable first step is to confirm the presence of glycoproteins in a crude extract using 1D or 2D gel-based electrophoretic separation, followed by immunoblot analysis or direct visualization using a commercially available sugar detection stain (Fitchette et al., 2007; Bardor et al., 2009). Antibodies to horseradish peroxidase (HRP) can recognize glycoproteins with an epitope associated with β1→2 xylose and/or α1→3 fucose structures on complex-type *N*-glycans (Fitchette et al., 2007; Bardor et al., 2009; **Figure 2**). They are therefore useful for detecting plant *N*-glycoproteins since plant complex-type glycans carry β1→2 xylose and/or α1→3 core fucose structures (Strasser et al., 2008). This is a relatively straightforward technique, but does not distinguish between different types of *N*-glycans, or between *N*- and *O*-linked glycans. The presence of glycoproteins can also be suggested by the presence of smeared bands on SDS-PAGE gels (Zhang et al., 2010; Catala et al., 2011), resulting from the presence of heterogonous glycans. Another practical, but sometimes less informative means to assess the presence of glycoproteins in a sample is by comparison of changes in the electrophoretic mobility of the constituent proteins following specific deglycosylation steps (Fitchette et al., 2007; Bardor et al., 2009). It is important to note that that the enzyme PNGase-F, which is typically use to deglycosylate mammalian proteins does not process *N*-glycans that contain an α-1,3-fucose residue that is a common component of plant *N*-glycans, although enzymes such as PNGase-A can be used as an alternative (Ytterberg and Jensen, 2010).

GLYCOPROTEIN/GLYCOPEPTIDE ENRICHMENT AND PREPARATION

Depending on the protein complexity in the initial samples, the analysis of glycoproteins then often proceeds with the enrichment of glycoproteins or glycopeptides by specific lectins or immunoaffinity chromatography (Rudiger and Gabius, 2001) to remove non-glycosylated forms. Lectin chromatography using Concanavalin A (Con A) has been reported for the enrichment of *N*-glycoproteins from diverse sources including human serum, *Caenorhabditis elegans* plasma membrane glycoproteins (Bunkenborg et al., 2004; Fan et al., 2004), and plant tissues (Minic et al., 2007; Zhang et al., 2010; Catala et al., 2011). Various plants lectins are available with affinities for different sugars (Gabius et al., 2004) and such lectins have been used sequentially (Yamamoto et al., 1995, 1998), in parallel (Yang et al., 2006; Lee et al., 2009; Zielinska et al., 2010), and as mixtures (Li et al., 2004) to increase the range of identified glycoproteins. When working with limited amounts of sample it is often necessary to miniaturize the chromatography. Both silica-based and monolithic materials incorporating a lectin-containing stationary phase are now commercially available that can be packed into micro or capillary columns, greatly increasing the number of glycoproteins that can be detected in low concentrations compared with standard column formats (Madera et al., 2006; Feng et al., 2009).

Once an enriched glycoprotein sample has been prepared, a typical next step is to fragment the parent protein backbone and a potential technical challenge is that the glycan moiety may spatially interfere with proteolysis, particularly near the glycosylation site. This can result in missed cleavages, yielding glycosylated polypeptides that are too large to be detected by some MS instruments. However, the use of less specific proteases, such as pepsin, thermolysin, Proteinase K, either alone or in combination, can reduce this problem (Chen et al., 2009). Nevertheless, no matter what the digestion procedure, the degree of heterogeneity of the resulting mixture of peptides and glycopeptides can complicate the subsequent MS analysis as glycopeptides often ionize less efficiently than non-glycopeptides and may be less abundant than their unmodified counterparts. Therefore, in some cases, deglycosylation prior to or concurrent with enzymatic digestion is desirable (see below). If deglycosylation is not employed, an additional enrichment of glycopeptides following glycoprotein digestion is often implemented to improve specificity. An alternative chemical approach for glycoprotein/glycopeptides enrichment is the use of hydrazine resin. *N*-glycans bind covalently to this matrix after oxidation with periodic acid and can then be released with PNGase A, although some information related to the *N*-glycan structure is lost (Zhang et al., 2003). Other alternative chemical methods involve graphitized carbon (Larsen et al., 2005) or boronic acid-functionalized beads, since boric acid is able to form diesters with glycans that have *cis*-diol groups (Sparbier et al., 2005).

Several such enrichment methods have been successfully applied in large-scale *N*-glycoproteome analyses, such as the combined use of lectin (Con A) affinity chromatography and

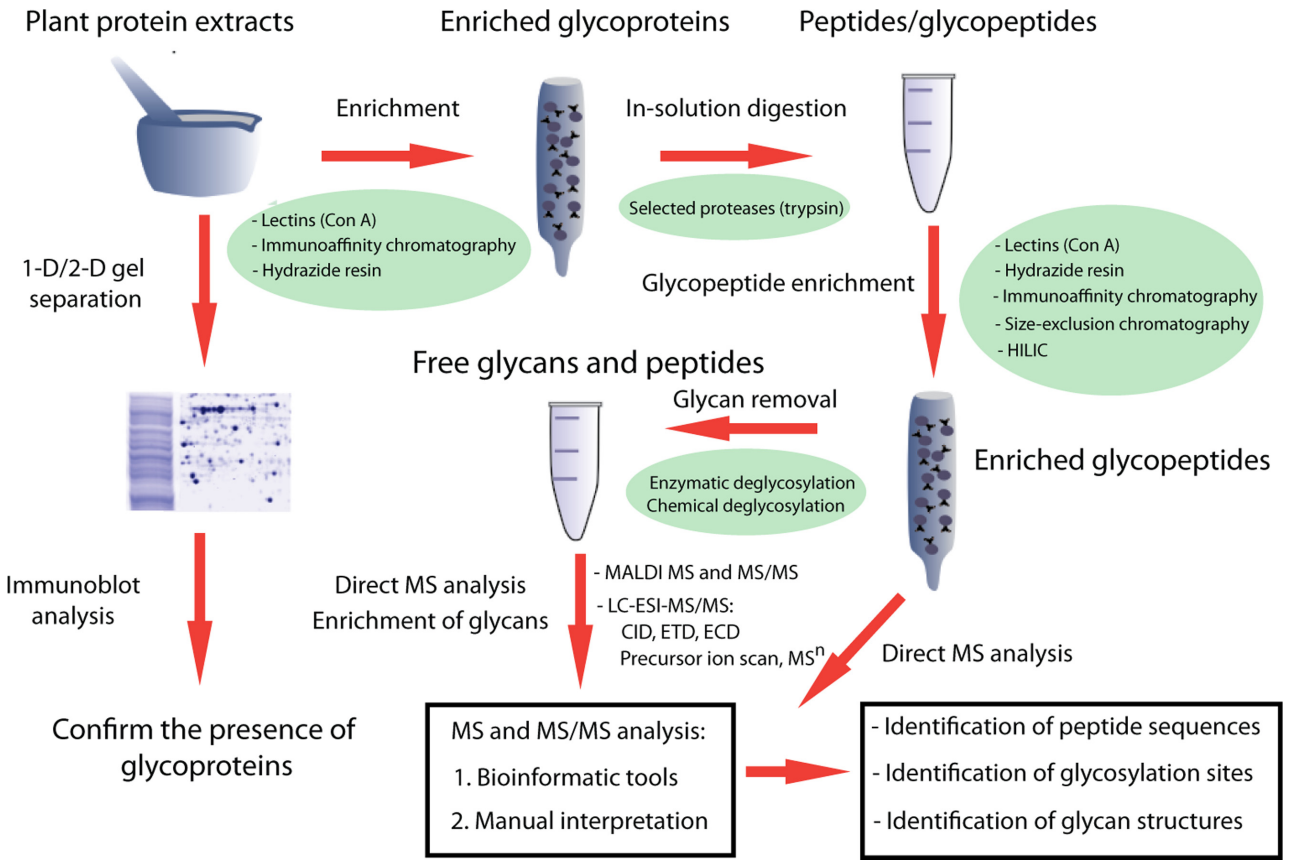


FIGURE 1 | A schematic overview of experimental approaches for the systematic characterization of glycoproteins by mass spectrometry-based proteomic analysis. Regardless of the sample sources, the general workflow includes three major steps: enrichment of glycoproteins, glycopeptides, and released glycans; mass spectrometry analysis of glycopeptides, glycans, and peptides; and interpretation of MS fragmentation spectra, allowing identification of core amino acid sequences of the glycopeptides, glycosylation sites, and potential glycan structures.

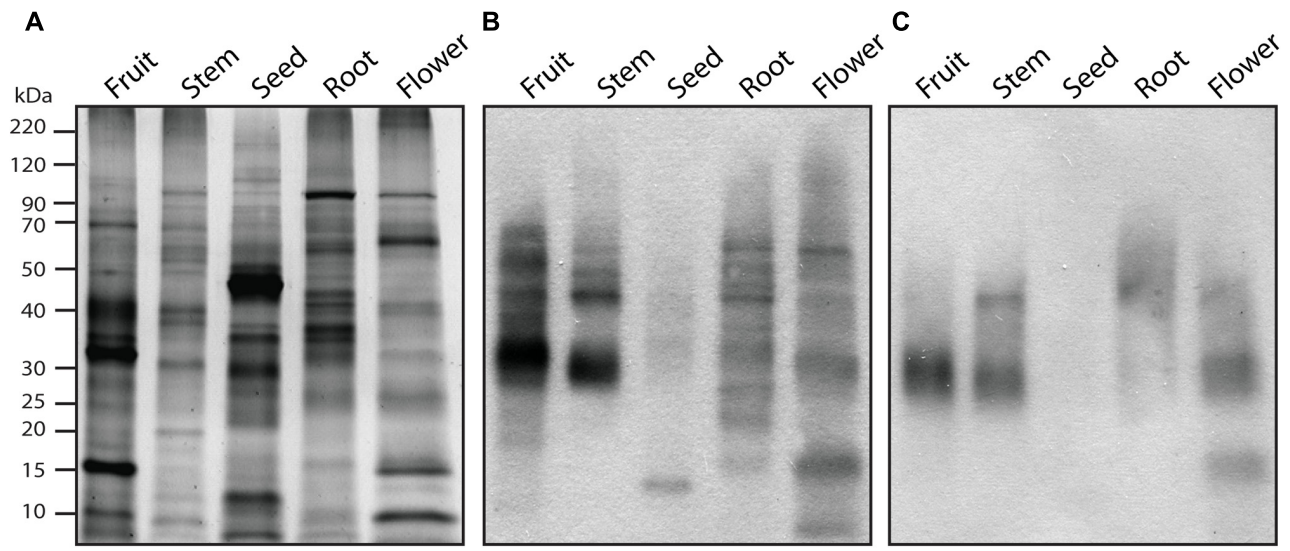


FIGURE 2 | Organ-specific immunodetection of glycan epitopes. Protein extracts from different tomato organs were fractionated 12% polyacrylamide gels and visualized by SYPRO Ruby protein staining **(A)** or transferred onto nitrocellulose membrane for immunoblotting using antisera that recognize β-1,2-xylose **(B)** or α-1,3-fucose **(C)** residues.

subsequent hydrophilic interaction chromatography (HILIC) enrichment of tryptic glycopeptides (Calvano et al., 2008; Selby et al., 2008), and size-exclusion chromatography has also been used to enrichment for tryptic glycopeptides (Alvarez-Manilla et al., 2006).

MS ANALYSIS

Once enriched glycopeptide samples have been isolated, they are then commonly subjected to MS/MS analysis, or to a deglycosylation step for simplifying MS analysis of individual peptides and glycans. However, to overcome the relatively poor sensitivity resulting from the hydrophilic nature of glycans, online nano-scale LC enrichment coupled with nano-electrospray ionization tandem MS (nanoESI-MS/MS; Bahr et al., 1997; Sandra et al., 2004; Zhang and Van Pelt, 2004) or additional derivatization (Zaia, 2004) is often used to improve glycopeptide ionization efficiency. Alternatively, matrix assisted laser desorption/ionization (MALDI) MS is used extensively for direct analysis of the released glycan classes or followed by glycan derivatization, as has been reviewed in detail (Zaia, 2010; Antonopoulos et al., 2011). Nano-scale liquid chromatography electrospray ionization (nanoLC-ESI) allows for low concentration of analytes, improves ionization efficiency and allows for low flow rates, which in turn makes it possible to use extended analysis times for MS/MS and multi-stage fragmentation (MS^n) oligosaccharide analyses (Bahr et al., 1997). Traditionally, low-energy CID MS/MS has been the method of choice for peptide and glycopeptides sequencing operating in either automated data-dependent acquisition mode, or manual selection for MS/MS through infusion analysis. Another unique feature of CID MS/MS analysis of glycopeptides or glycans is the production of oxonium ions, such as m/z 163 $(Hex + H)^+$, m/z 204 $(HexNAc + H)^+$, and m/z 366 $(Hex\text{-}HexNAc + H)^+$, which can be used as diagnostic marker ions, indicating the presence of specific sugar compositions. The oxonium ions are often monitored in precursor ion scanning mode for highly selective detection of glycopeptides in digestion mixture and subjected to MS/MS and MS/MS/MS of the selectively detected glycopeptides ions, yielding complete peptide and glycan sequences (Sandra et al., 2004; Zhang and Williamson, 2005). It should be note that highly selective precursor ion scanning for sugar oxonium ions also allows direct analysis of relatively less complex protein digests without further enrichment of glycopeptides (Sandra et al., 2004; Zhang and Williamson, 2005).

However, low-energy CID MS/MS can be rendered less effective by the labile nature of the glycosidic bond and often fails to produce fragment ions that can be used to determine both the peptide sequence and glycosylation site. In this regard, two new dissociation technologies, electron capture dissociation (ECD; Zubarev et al., 1998) and electron transfer dissociation (ETD; Syka et al., 2004), an analogue of ECD, provide valuable alternative approaches. Both ECD and ETD are non-ergodic fragmentation techniques that induce backbone fragmentation at N-Cα bonds and create complementary c- and z-type ion series, yielding information that is highly complementary to conventional CID fragmentation (Zubarev et al., 1998; Syka et al., 2004; Coon et al., 2005). ETD MS is becoming more widely accessible as it enables the incorporation of ECD-type fragmentation in more common RF ion trap mass spectrometers, thus eliminating the need for highly sophisticated higher-cost FTICR MS instruments. ETD cleaves the peptide backbone in a sequence-independent manner, leaving the glycan preserved on the peptide and is increasingly recognized as an important alternative dissociation technique to CID for glycosylation analysis (Catalina et al., 2007; Khidekel et al., 2007; Wuhrer et al., 2007b). With the combination of CID and ETD fragmentation available in single ion trap instruments, it is now possible to analyze intact glycopeptides and determine both peptide and glycan sequence information (Wuhrer et al., 2007a). To some degree the development of these non-ergodic fragmentation methods, which can be used to structurally characterize much larger analytes, has reduced the need to ensure that the proteolytic digestion proceeds to the point where the mass contribution of the peptidyl moiety is minimal. Wu et al. (2007) have successfully demonstrated the use of endoproteinase Lys-C to study the glycosylation of several model proteins using a combination of ETD and CID. This approach reduces the number of non-glycosylated peptides, thereby facilitating the characterization of the glycopeptides and reducing the need for additional chromatographic steps.

The development of ion mobility spectrometry (IMS) has extended the ability to characterize glycoprotein/glycopeptides to the realm of isomeric and conformational structure (Plasencia et al., 2008). For example, Olivova et al. (2008), using the high resolution provided by a recently developed quadrupole ion-mobility time-of-flight instrument, was able to determine the total glycosylation profile of a monoclonal antibody. The IMS function was used to resolve the light and heavy chains in the gas phase allowing the accurate mass measurement of each. Furthermore, the innovative dual-collision cell design of this instrument was leveraged in a two step fragmentation process to provide a detailed characterization of the glycan structures and the determination of the glycosylation site.

Other than direct MS analysis of enriched glycopeptides, deglycosylation steps can also be performed to gain additional information about peptide sequence and complete glycan structure. Three enzymes are commonly used for enzymatic deglycosylation. PNGase F, an amidase that hydrolyzes the amide bond between the Asn residue on the peptide backbone and the innermost HexNAc unit, is active on high mannose and complex plant *N*-glycans, except those with an α-1,3-fucose residue linked to the innermost HexNAc (Fitchette et al., 2007; Bardor et al., 2009). In this case, PNGase A can be used to release glycans from relatively short glycopeptides (Fitchette et al., 2007; Bardor et al., 2009). Alternatively, endoglycosidase H (Endo H) cleaves the glycosidic bond between the two GlcNAc residues on the high-mannose-type *N*-glycans of plant glycoproteins (Harvey, 2005). While these deglycosylation steps are time consuming it has been shown that pressure cycling and exposure to microwaves can greatly reduce deglycosylation times (Chen et al., 2009). Chemical deglycosylation can also be used, although enzymatic deglycosylation is generally favored as chemical approaches often introduce unexpected modifications of peptide side chains (Fitchette et al., 2007; Bardor et al., 2009).

After deglycosylation, LC-MS/MS is used to identify the amino acid sequence of the deglycosylated peptide. Alternatively, the

released glycans can be further purified and analyzed by MALDI-TOF or LC-ESI-MS/MS analysis to identify the glycan structure (An et al., 2009). In practice, MS/MS spectra derived from the glycosidic bond fragment ions, along with cross-ring fragmentation of glycans and treatments with different exo-hexosidases are required for complete structural elucidation (Zaia, 2004). In the past, per-derivation, such as permethylation, of glycans has often been used to improve sensitivity and has yielded more structurally relevant fragment ions during MS/MS. However, as new MS technologies are constantly being developed, the sensitivity improvements provided by derivatization has only been marginal and insufficient to compensate for the more complex sample preparation and the fact that derivatization makes it difficult to employ glycosidase treatment to determine linkages and identify saccharide units (Kang et al., 2007). Another technique, called isotope coded glycosylation site-specific tagging (IGOT), has been used for the large-scale identification of *N*-linked glycoproteins from complex biological samples of *C. elegans* (Kaji et al., 2003, 2007) and mouse (Zielinska et al., 2010). This approach involves protein tryptic digestion and enrichment of resulting glycopeptides followed by conversion of glycosylated Asn residues into ^{18}O-labeled aspartic acids by PNGase F digestion in ^{18}O water. The subsequent 3 Da mass difference created by this process is readily identified by LC-MS/MS analysis, indicating *N*-linked glycosylation sites. However, a potential pitfall of this approach is that a significantly high false positive rate (∼60%) has been reported (Angel et al., 2007), apparently due to the fact that the trypsin used for proteolysis was still active, leading to the incorporation of ^{18}O into the C-termini of the peptides during the deglycosylation step.

Capillary electrophoresis has been used to analyze the heterogeneity of isolated glycoproteins with MS detection (Balaguer and Neususs, 2006; Thakur et al., 2009; Szabo et al., 2010) and the incorporation of microfluidic devices can also greatly facilitate the analysis by increasing sensitivity and reducing analysis times (Zhuang et al., 2007, 2011). While these CE/MS methods have proven useful as a means to characterize glycoprotein heterogeneity and to investigate the structure of glycans at the *N*- and *O*-glycosylation sites of recombinant glycoproteins (Zaia, 2010), they have not yet proven sufficiently robust for the analysis of complex glycoprotein mixtures.

QUANTITATIVE *N*-GLYCOPROTEIN ANALYSIS BY MS

In principle quantitative glycoproteomics experiments may be carried out by the same assortment of techniques that have been developed and are currently in use for labeling and label-free approaches in other proteomics disciplines. There have been several excellent reviews (Bindschedler and Cramer, 2011; Matros et al., 2011, Pan et al., 2011) and the reader is referred to these for detailed descriptions of the various experimental strategies employed and methodologies in use.

Historically, the quantification of proteoglycans has been difficult as it involves both the structural elucidation of glycan and peptide moieties. While structural characterization of peptides is well developed and is facilitated by the existence of complete genomic databases for a growing number of species, the structural characterization of the glycan moiety remains a challenge as they are often exceedingly heterogeneous and chemically complex, with differing chemical configurations and abundant isoforms. Furthermore, glycans are frequently branched and contain an assortment of glycan linkages (Zaia, 2004). These complications combine in such a way that quantitative experiments must be carried out in different tiers with each providing a different level of quantitative information. The first tier involves determining glycosylation sites and their degree of occupancy. For *N*-linked glycans potential glycosylation sites can be recognized due to the presence of the consensus sequence Asn-X-Ser/Thr using any of several open access programs such as Net Glyc (http://www.cbs.dtu.dk/services/NetOGlyc/). However, not all potential sites are occupied and those that are occupied are often only partially so but by a variety of differing glycans. Determining the number and types of glycoforms and their relative proportion present at each site represents the second tier of quantifications. The third tier of glycoprotein quantification involves comparing the state of glycosylation between different samples, i.e., control verses treated, control verses diseased, etc. Of the methods available, only MS allows for quantitative characterization at all three tiers.

Two-dimensional gel electrophoresis with glycan-specific staining has been the traditional method used to identify and quantify glycoproteins (Bardor et al., 2009). When coupled with MS of tryptic digests of excised spots it can provide quantitative characterization on all three levels. However, the heterogeneity and structural complexity of the glycans has important consequences on a protein's mobility which complicate the interpretation of the electropherograms. Thus, LC-MS has emerged as the preeminent tool for the identification and quantitative characterization of glycoproteins and glycopeptides. The development of novel hybrid mass spectrometers incorporating ion mobility cells, high mass accuracy and resolution, improved sensitivity, and scanning capabilities has allowed detailed glycoprotein structural characterization, with accurate empirical information concerning the position and degree of occupancy of the glycosylation sites as well as the composition, structure, and distribution of glycoforms.

In the discovery phase of research, most quantitative MS strategies involve either a labeling approach (Gygi et al., 1999; Kaji et al., 2003; Zhang et al., 2003; Ross et al., 2004; Aggarwal et al., 2006; Atwood et al., 2008; Haqqani et al., 2008) or a label-free strategy (Hill et al., 2009; Rebecchi et al., 2009; Zhang et al., 2012). These non-targeted approaches allow one to quickly survey complex proteomes to determine the features that vary between samples and more precise quantitative information can be obtained through a targeted approach involving specific reaction monitoring (SRM) as recently described by Zhang et al. (2012). New developments involving data independent acquisition (DIA) using approaches such as MS^E or SWATH (Levin et al., 2011; Gillet et al., 2012; Hopfgartner et al., 2012) could potentially simplify these procedures further by allowing the discovery and targeted data to be collected simultaneously greatly reducing the instrument time required.

BIOINFORMATIC TOOLS

One of the biggest challenges in the field of glycoproteomics is the development of bioinformatic tools for glycan analysis. As a consequence of the complex fragmentation behaviors of glycopeptides and glycans, which tend to vary under different CID

conditions utilized by various MS instruments, and the high level of glycan heterogeneity, there are few tools for direct large-scale identification of glycoproteins through database searching. As a result, tedious manual data interpretation represents the major bottle-neck for glycoprotein characterization. However, several computer programs have been developed, such as GlysodeIQ (Joshi et al., 2004), GlyMod (Cooper et al., 2001), GlycoMiner (Ozohanics et al., 2008), and Peptoonist (Goldberg et al., 2007), to aid identification of intact *N*-linked glycopeptides and oligosaccharides, as well as CartoonistTwo (Goldberg et al., 2006) for automatic determination of the topology of *O*-glycan without linkage information from MS/MS spectra. Nevertheless, there is an unquestionable need for improved bioinformatic tools to keep pace with the rapid advances in MS technologies and strategies for sample preparation, such as software for the automatic determination of *N*-linked glycopeptides.

DISCUSSION AND PERSPECTIVES

As described above, the characterization of the plant *N*-glycoproteome is methodologically challenging due to the extreme structural heterogeneity and the often low concentration of several glycoforms attached to each glycopeptide residing in the total peptide pool. However, several approaches have been established to study specific population of glycoproteins from different tissues. Until now, there had not been a single enrichment approach that permits the analysis of the complete population of glycoprotein in a specific tissue, but lectin affinity chromatography is an effective option because it permits the purification and analysis of native glycoproteins, allowing the characterization of endogenous glycoforms without any modification of the *N*-glycans structure. Moreover, the availability of several lectins with different ligand affinities allows broader coverage of the *N*-glycoproteome, as has been described in mammals (Zielinska et al., 2010).

As far as we are aware, there have been no such reports describing the application of large-scale enrichment techniques in combination with cutting-edge MS/MS methods for the direct analysis of intact plant glycopeptides to gain information of both glycan and peptide moiety. However, the establishment of new MS platforms, such as the use of CID and ETD fragmentation in a single ion trap instrument for simultaneous analysis of the sugar and protein components of glycopeptides (Wuhrer et al., 2007a) is likely to revolutionize the study of plant glycoproteins. Rapid progress will depend on other technical advances such as solving the problems of the recalcitrance of plant proteins to deglycosylation by standard PNGase F treatments and the development of new, robust bioinformatics tools.

Taken together, such developments hold great promise for plant researchers in many fields, including those interested in the plant cell wall. Increasing numbers of wall localized proteins are being identified (Ruiz-May et al., 2012) and yet remarkably little is known about the functional significance of post-translational modifications such as glycosylation. New analytical pipelines such as those described here, perhaps coupled with other techniques that will likely result in glycoprotein enrichment (e.g., Parsons et al., 2012), hold great potential in this regard. This represents an important new frontier in plant cell wall biology and it can be argued that the advent of the plant *N*-glycoproteome starts now.

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REFERENCES

Aggarwal, K., Choe, L. H., and Lee, K. H. (2006). Shotgun proteomics using the iTRAQ isobaric tags. *Brief. Funct. Genomic. Proteomic.* 5, 112–120.

Alvarez-Manilla, G., Atwood, J. III, Guo, Y., Warren, N. L., Orlando, R., and Pierce, M. (2006). Tools for glycoproteomic analysis: size exclusion chromatography facilitates identification of tryptic glycopeptides with *N*-linked glycosylation sites. *J. Proteome Res.* 5, 701–708.

An, H. J., Froehlich, J. W., and Lebrilla, C. B. (2009). Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. *Curr. Opin. Chem. Biol.* 13, 421–426.

Angel, P. M., Lim, J. M., Wells, L., Bergmann, C., and Orlando, R. (2007). A potential pitfall in 18*O*-based *N*-linked glycosylation site mapping. *Rapid Commun. Mass Spectrom.* 21, 674–682.

Antonopoulos, A., North, S. J., Haslam, S. M., and Dell, A. (2011). Glycosylation of mouse and human immune cells: insights emerging from *N*-glycomics analyses. *Biochem. Soc. Trans.* 39, 1334–1340.

Atwood, J. A. III, Cheng, L., Alvarez-Manilla, G., Warren, N. L., York, W. S., and Orlando, R. (2008). Quantitation by isobaric labeling: applications to glycomics. *J. Proteome Res.* 7, 367–374.

Bahr, U., Pfenninger, A., Karas, M., and Stahl, B. (1997). High-sensitivity analysis of neutral underivatized oligosaccharides by nanoelectrospray mass spectrometry. *Anal. Chem.* 69, 4530–4535.

Balaguer, E., and Neususs, C. (2006). Glycoprotein characterization combining intact protein and glycan analysis by capillary electrophoresis-electrospray ionization-mass spectrometry. *Anal. Chem.* 78, 5384–5393.

Bardor, M., Cabrera, G., Stadlmann, J., Lerouge, P., Cremata, J. A., Gomord, V., and Fitchette, A. C. (2009). *N*-glycosylation of plant recombinant pharmaceuticals. *Methods Mol. Biol.* 483, 239–264.

Bindschedler, L. V., and Cramer, R. (2011). Quantitative plant proteomics. *Proteomics* 11, 756–775.

Bunkenborg, J., Pilch, B. J., Podtelejnikov, A. V., and Wiśniewski, J. R. (2004). Screening for *N*-glycosylated proteins by liquid chromatography mass spectrometry. *Proteomics* 4, 454–465.

Calvano, C. D., Zambonin, C. G., and Jensen, O. N. (2008). Assessment of lectin and HILIC based enrichment protocols for characterization of serum glycoproteins by mass spectrometry. *J. Proteomics* 71, 304–317.

Catala, C., Howe, K. J., Hucko, S., Rose, J. K., and Thannhauser, T. W. (2011). Towards characterization of the glycoproteome of tomato (*Solanum lycopersicum*) fruit using Concanavalin A lectin affinity chromatography and LC-MALDI-MS/MS analysis. *Proteomics* 11, 1530–1544.

Catalina, M. I., Koeleman, C. A., Deelder, A. M., and Wuhrer, M. (2007). Electron transfer dissociation of *N*-glycopeptides: loss of the entire *N*-glycosylated asparagine side chain. *Rapid Commun. Mass Spectrom.* 21, 1053–1061.

Chen, R., Jiang, X., Sun, D., Han, G., Wang, F., Ye, M., Wang, L., and Zou, H. (2009). Glycoproteomics analysis of human liver tissue by combination of multiple enzyme digestion and hydrazide chemistry. *J. Proteome Res.* 8, 651–661.

Coon, J. J., Ueberheide, B., Syka, J. E., Dryhurst, D. D., Ausio, J., Shabanowitz, J., and Hunt, D. F. (2005). Protein identification using sequential ion/ion reactions and tandem mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9463–9468.

Cooper, C. A., Gasteiger, E., and Packer, N. (2001). GlycoMod – a software tool for determining glycosylation compositions from mass spectrometric data. *Proteomics* 1, 340–349.

De Marchis, F., Balducci, C., Pompa, A., Riise Stensland, H. M., Guaragno, M., Pagiotti, R., Menghini, A. R.,

Persichetti, E., Beccari, T., and Bellucci, M. (2011). Human alpha-mannosidase produced in transgenic tobacco plants is processed in human alpha-mannosidosis cell lines. *Plant Biotechnol. J.* 9, 1061–1073.

Fan, X., She, Y. M., Bagshaw, R. D., Callahan, J. W., Schachter, H., and Mahuran, D. J. (2004). A method for proteomic identification of membrane-bound proteins containing Asn-linked oligosaccharides. *Anal. Biochem.* 332, 178–186.

Feng, S., Yang, N., Pennathur, S., Goodison, S., and Lubman, D. M. (2009). Enrichment of glycoproteins using nanoscale chelating concanavalin A monolithic capillary chromatography. *Anal. Chem.* 81, 3776–3783.

Fitchette, A. C., Dinh, O. T., Faye, L., and Bardor, M. (2007). Plant proteomics and glycosylation. *Methods Mol. Biol.* 355, 317–342.

Gabius, H. J., Siebert, H. C., André, S., Jiménez-Barbero, J., and Rüdiger, H. (2004). Chemical biology of the sugar code. *Chembiochem* 5, 740–764.

Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., Bonner, R., and Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol. Cell. Proteomics* 11, O111.016717.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999). Quantitative analysis of mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994–999.

Goldberg, D., Bern, M., Li, B., and Lebrilla, C. B. (2006). Automatic determination of *O*-glycan structure from fragmentation spectra. *J. Proteome Res.* 5, 1429–1434.

Goldberg, D., Bern, M., Parry, S., Sutton-Smith, M., Panico, M., Morris, H. R., and Dell, A. (2007). Automated *N*-glycopeptide identification using a combination of single- and tandem-MS. *J. Proteome Res.* 6, 3995–4005.

Haqqani, A. S., Kelly, J. F., and Stanimirovic, D. B. (2008). Quantitative protein profiling by mass spectrometry using isotope-coded affinity tags. *Methods Mol. Biol.* 439, 225–240.

Harvey, D. J. (2005). Proteomic analysis of glycosylation: structural determination of *N*- and *O*-linked glycans by mass spectrometry. *Exp. Rev. Proteomics* 2, 87–101.

Hill, J. J., Tremblay, T.-L., Cantin, C., O'Connor-McCourt, M., Kelly, J. F., and Lenferink, A. E. (2009). Glycoproteomic analysis of two mouse mammary cell lines during transforming growth factor (TGF)-β induced epithelial to mesenchymal transition. *Proteome Sci.* 7, 2. doi: 10.1186/1477-5956-7-2

Hopfgartner, G., Tonoli, D., and Varesio, E. (2012). High-resolution mass spectrometry for integrated qualitative and quantitative analysis of pharmaceuticals in biological matrices. *Anal. Bioanal. Chem.* 402, 2587–2596.

Joshi, H. J., Harrison, M. J., Schulz, B. L., Cooper, C. A., Packer, N. H., and Karlsson, N. G. (2004). Development of a mass fingerprinting tool for automated interpretation of oligosaccharide fragmentation data. *Proteomics* 4, 1650–1664.

Kaji, H., Kamiie, J., Kawakami, H., Kido, K., Yamauchi, Y., Shinkawa, T., Taoka. M., Takahashi, N., and Isobe, T. (2007). Proteomics reveals *N*-linked glycoprotein diversity in *Caenorhabditis elegans* and suggests an atypical translocation mechanism for integral membrane proteins. *Mol. Cell Proteomics* 6, 2100–2109.

Kaji, H., Saito, H., Yamauchi, Y., Shinkawa, T., Taoka, M., Hirabayashi, J., Kasai, K.-I., Takahashi, N., and Isobe, T. (2003). Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify *N*-linked glycoproteins. *Nat. Biotechnol.* 21, 667–672.

Kang, P., Mechref, Y., Kyselova, Z., Goetz, J. A., and Novotny, M. V. (2007). Comparative glycomic mapping through quantitative permethylation and stable-isotope labeling. *Anal. Chem.* 79, 6064–6073.

Khidekel, N., Ficarro, S. B., Clark, P. M., Bryan, M. C., Swaney, D. L., Rexach, J. E., Sun, Y. E., Coon, J. J., Peters, E. C., and Hsieh-Wilson, L. C. (2007). Probing the dynamics of O-GlcNAc glycosylation in the brain using quantitative proteomics. *Nat. Chem. Biol.* 3, 339–348.

Larsen, M. R., Hojrup, P., and Roepstorff, P. (2005). Characterization of gel-separated glycoproteins using two-step proteolytic digestion combined with sequential microcolumns and mass spectrometry. *Mol. Cell Proteomics* 4, 107–119.

Lee, A., Kolarich, D., Haynes, P. A., Jensen, P. H., Baker, M. S., and Packer, N. H. (2009). Rat liver membrane glycoproteome: enrichment by phase partitioning and glycoprotein capture. *J. Proteome Res.* 8, 770–781.

Levin, Y., Hradetzky, E., and Bahn, S. (2011). Quantification of proteins using data-independent analysis (MSE) in simple andcomplex samples: a systematic evaluation. *Proteomics* 11, 3273–3287.

Li, L., Wang, L., Zhang, W., Tang, B., Zhang, J., Song, H., Yao, D., Tang, Y., Chen, X., Yang, Z., Wang, G., Li, X., Zhao, J., Ding, H., Reed, E., and Li, Q. Q. (2004). Correlation of serum VEGF levels with clinical stage, therapy efficacy, tumor metastasis and patient survival in ovarian cancer. *Anticancer Res.* 24, 1973–1979.

Madera, M., Mechref, Y., Klouckova, I., and Novotny, M. V. (2006). Semi-automated high-sensitivity profiling of human blood serum glycoproteins through lectin preconcentration and multidimensional chromatography/tandem mass spectrometry. *J. Proteome Res.* 5, 2348–2363.

Marino, K., Bones, J., Kattla, J. J., and Rudd, P. M. (2010). A systematic approach to protein glycosylation analysis: a path through the maze. *Nat. Chem. Biol.* 6, 713–723.

Matros, A., Kaspar, S., Witzel, K., and Mock, H. P. (2011). Recent progress in liquid chromatography-based separation and label-free quantitative plant proteomics. *Phytochemistry* 72, 963–974.

Minic, Z., Jamet, E., Négroni, L., der Garabedian, P. A., Zivy, M., and Jouanin, L. (2007). A sub-proteome of *Arabidopsis thaliana* mature stems trapped on Concanavalin A is enriched in cell wall glycoside hydrolases. *J. Exp. Bot.* 58, 2503–2512.

Olivova, P., Chen, W., Chakraborty, A. B., and Gebler, J. C. (2008). Determination of *N*-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 22, 29–40.

Ozohanics, O., Krenyacz, J., Ludányi, K., Pollreisz, F., Vékey, K., and Drahos, L. (2008). GlycoMiner: a new software tool to elucidate glycopeptide composition. *Rapid Commun. Mass Spectrom.* 22, 3245–3254.

Pan, S., Chen, R., Aebersold, R., and Brentnall, T. A. (2011). Mass spectrometry based glycoproteomics-from a proteomics perspective. *Mol. Cell Proteomics* 10, R110.003251.

Park, Y., and Lebrilla, C. B. (2005). Application of Fourier transform ion cyclotron resonance mass spectrometry to oligosaccharides. *Mass Spectrom. Rev.* 24, 232–264.

Parsons, H. T., Christiansen, K., Knierim, B., Carroll, A., Ito, J., Batth, T. S., Smith-Moritz, A. M., Morrison, S., McInerney, P., Hadi, M. Z., Auer, M., Mukhopadhyay, A., Petzold, C. J., Scheller, H. V., Loqué, D., and Heazlewood, J. L. (2012). Isolation and proteomic characterization of the *Arabidopsis* Golgi defines functional and novel components involved in plant cell wall biosynthesis. *Plant Physiol.* 159, 12–26.

Plasencia, M. D., Isailovic, D., Merenbloom, S. I., Mechref, Y., Novotny, M. V., and Clemmer, D. E. (2008). Resolving and assigning *N*-linked glycan structural isomers from ovalbumin by IMS-MS. *J. Am. Soc. Mass Spectrom.* 19, 1706–1715.

Rebecchi, K. R., Wenke, J. L., Go, E. P., and Desaire, H. (2009). Label-free quantitation: a new glycoproteomics approach. *J. Am. Soc. Mass Spectrom.* 20, 1048–1059.

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004). Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell Proteomics* 3, 1154–1169.

Rudiger, H., and Gabius, H. J. (2001). Plant lectins: occurrence, biochemistry, functions and applications. *Glycoconj. J.* 18, 589–613.

Ruiz-May, E., Kim, S. J., Brandizzi, F., and Rose, J. K. C. (2012). The secreted plant *N*-glycoproteome and associated secretory pathways. *Front. Plant Sci.* 3:117. doi: 10.3389/fpls.2012.00117

Sandra, K., Devreese, B., Van Beeumen, J., Stals, I., and Claeyssens, M. (2004). The Q-Trap mass spectrometer, a novel tool in the study of protein glycosylation. *J. Am. Soc. Mass Spectrom.* 15, 413–423.

Selby, D. S., Larsen, M. R., Calvano, C. D., and Jensen, O. N. (2008). Identification and characterization of *N*-glycosylated proteins using proteomics. *Methods Mol. Biol.* 484, 263–276.

Song, W., Henquet, M. G., Mentink, R. A., van Dijk, A. J., Cordewener, J. H., Bosch, D., America, A. H., and van der Krol, A. R. (2011). *N*-glycoproteomics in plants: perspectives and challenges. *J. Proteomics* 74, 1463–1474.

Sparbier, K., Koch, S., Kessler, I., Wenzel, T., and Kostrzewa, M. (2005). Selective isolation of glycoproteins and glycopeptides for MALDI-TOF MS detection supported by magnetic particles. *J. Biomol. Tech.* 16, 407–413.

Strasser, R., Stadlmann, J., Schähs, M., Stiegler, G., Quendler, H., Mach, L.,

Glössl, J., Weterings, K., Pabst, M., and Steinkellner, H. (2008). Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like *N*-glycan structure. *Plant Biotechnol. J.* 6, 392–402.

Syka, J. E., Coon, J. J., Schroeder, M. J., Shabanowitz, J., and Hunt, D. F. (2004). Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9528–9533.

Szabo, Z., Guttman, A., Rejtar, T., and Karger, B. L. (2010). Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. *Electrophoresis* 31, 1389–1395.

Thakur, D., Rejtar, T., Karger, B. L., Washburn, N. J., Bosques, C. J., Gunay, N. S., Shriver, Z., and Venkataraman, G. (2009). Profiling the glycoforms of the intact alpha subunit of recombinant human chorionic gonadotropin by high-resolution capillary electrophoresis-mass spectrometry. *Anal. Chem.* 81, 8900–8907.

Thelen, J. J., and Peck, S. C. (2007). Quantitative proteomics in plants: choices in abundance. *Plant Cell* 19, 3339–3346.

Wu, S. L., Huhmer, A. F., Hao, Z., and Karger, B. L. (2007). On-line LC-MS approach combining collision-induced dissociation (CID), electron-transfer dissociation (ETD), and CID of an isolated charge-reduced species for the trace-level characterization of proteins with post-translational modifications. *J. Proteome Res.* 6, 4230–4244.

Wuhrer, M., Catalina, M. I., Deelder, A. M., and Hokke, C. H. (2007a). Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 849, 115–128.

Wuhrer, M., Stam, J. C., van de Geijn, F. E., Koeleman, C. A., Verrips, C. T., Dolhain, R. J., Hokke, C. H., and Deelder, A. M. (2007b). Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. *Proteomics* 7, 4070–4781.

Xu, J., Ge, X., and Dolan, M. C. (2011). Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol. Adv.* 29, 278–299.

Yamamoto, K., Tsuji, T., and Osawa, T. (1995). Analysis of asparagine-linked oligosaccharides by sequential lectin affinity chromatography. *Mol. Biotechnol.* 3, 25–36.

Yamamoto, K., Tsuji, T., and Osawa, T. (1998). Analysis of asparagine-linked oligosaccharides by sequential lectin-affinity chromatography. *Methods Mol. Biol.* 76, 35–51.

Yang, Z., Drew, D. P., Jørgensen, B., Mandel, U., Bach, S. S., Ulvskov, P., Levery, S. B., Bennett, E. P., Clausen, H., and Petersen, B. L. (2012). Engineering mammalian mucin-type *O*-glycosylation in plants. *J. Biol. Chem.* 287, 11911–11912

Yang, Z., Harris, L. E., Palmer-Toy, D. E., and Hancock, W. S. (2006). Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. *Clin. Chem.* 52, 1897–1905.

Yates, J. R., Ruse, C. I., and Nakorchevsky, A. (2009). Proteomics by mass spectrometry: approaches, advances, and applications. *Annu. Rev. Biomed. Eng.* 11, 49–79.

Ytterberg, A. J., and Jensen, O. N. (2010). Modification-specific proteomics in plant biology. *J. Proteomics* 73, 2249–2266.

Zaia, J. (2004). Mass spectrometry of oligosaccharides. *Mass Spectrom. Rev.* 23, 161–227.

Zaia, J. (2010). Mass spectrometry and glycomics. *OMICS* 14, 401–418.

Zhang, H., Li, X. J., Martin, D. B., and Aebersold, R. (2003). Identification and quantification of *N*-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat. Biotechnol.* 21, 660–666.

Zhang, S., Sherwood, R. W., Yang, Y., Fish, T., Chen, W., McCardle, J. A., Jones, R, M., Yusibov, V., May, E. R., Rose, J. K., and Thannhauser, T. W. (2012). Comparative characterization of the glycosylation profiles of an influenza hemagglutinin produced in plant and insect hosts. *Proteomics* 12, 1269–1288.

Zhang, S., and Van Pelt, C. K. (2004). Chip-based nanoelectrospray mass spectrometry for protein characterization. *Exp. Rev. Proteomics* 1, 449–468.

Zhang, S., and Williamson, B. L. (2005). Characterization of protein glycosylation using chip-based nanoelectrospray with precursor ion scanning quadrupole linear ion trap mass spectrometry. *J. Biomol. Tech.* 16, 209–219.

Zhang, Y., Giboulot, A., Zivy, M., Valot, B., Jamet, E., and Albenne, C. (2010). Combining various strategies to increase the coverage of the plant cell wall glycoproteome. *Phytochemistry* 72, 1109–1123.

Zhuang, Z., Mitra, I., Hussein, A., Novotny, M. V., Mechref, Y., and Jacobson, S. C. (2011). Microchip electrophoresis of *N*-glycans on serpentine separation channels with asymmetrically tapered turns. *Electrophoresis* 32, 246–253.

Zhuang, Z., Starkey, J. A., Mechref, Y., Novotny, M. V., and Jacobson, S. C. (2007). Electrophoretic analysis of *N*-glycans on microfluidic devices. *Anal. Chem.* 79, 7170–7175.

Zielinska, D. F., Gnad, F., Wiśniewski, J. R., and Mann, M. (2010). Precision mapping of an *in vivo N*-glycoproteome reveals rigid topological and sequence constraints. *Cell* 141, 897–907.

Zubarev, R. A., Kelleher, N. L., and McLafferty, F. W. (1998). Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J. Am. Chem. Soc.* 120, 3265–3266.

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Cell wall evolution and diversity

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Plant cell walls display a considerable degree of diversity in their compositions and molecular architectures. In some cases the functional significance of a particular cell wall type appears to be easy to discern: secondary cells walls are often reinforced with lignin that provides durability; the thin cell walls of pollen tubes have particular compositions that enable their tip growth; lupin seed cell walls are characteristically thickened with galactan used as a storage polysaccharide. However, more frequently the evolutionary mechanisms and selection pressures that underpin cell wall diversity and evolution are unclear. For diverse green plants (chlorophytes and streptophytes) the rapidly increasing availability of transcriptome and genome data sets, the development of methods for cell wall analyses which require less material for analysis, and expansion of molecular probe sets, are providing new insights into the diversity and occurrence of cell wall polysaccharides and associated biosynthetic genes. Such research is important for refining our understanding of some of the fundamental processes that enabled plants to colonize land and to subsequently radiate so comprehensively. The study of cell wall structural diversity is also an important aspect of the industrial utilization of global polysaccharide bio-resources.

Keywords: biomechanics, carbohydrate microarrays, CAZy, diversity, monoclonal antibodies, evolution, glycome, plant cell wall

INTRODUCTION

Plant cell walls are multifunctional polysaccharide-rich fibrous composites in which polymers interact to form load-bearing structures embedded in a polysaccharide matrix (Bacic et al., 1988; Fry, 2004). Cells in the growing parts of plants are bound by "primary walls" in which the load bearing function is performed primarily by cellulose microfibrils. Models of the plant cell wall typically depict the microfibrils cross-linked with hemicelluloses, including mannans, xylans, mixed-linkage glucans (MLG), and xyloglucans. This network is then further embedded in a matrix of pectic polysaccharides including homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), and xylogalacturonan (Fry, 2004; Mohnen, 2008; Caffall and Mohnen, 2009; Harholt et al., 2010). However, this conventional description of primary walls that emphasizes tethering glycans as indispensible "load-bearing" structures may need revising as discussed in Scheller and Ulvskov (2010). Primary cell walls establish the foundations for cell shape and resist the tensile forces exerted by turgor pressure. They must also be capable of controlled expansion to enable cell growth. In non-growing plant tissues, some cells are typically surrounded by "secondary walls" whose primary role is to resist compressive force and since cell expansion is not required, these walls are often reinforced with lignin (Hepler et al., 1970; Carpita and Gibeaut, 1993; Boerjan et al., 2003; Cosgrove, 2005). Although these descriptions serve to describe many plant cell walls in broad terms, they are generalizations and are primarily based on investigations of the cell walls of flowering plants. However, cell walls display remarkable diversity at many levels and their constituent polysaccharides differ in fine structure, relative abundance, and molecular associations (Burton et al., 2010). The vast complexity and heterogeneity of cell wall glycomes is the product of the cooperative activities of prodigious numbers of biosynthetic enzymes. It is clear from genome sequencing that hundreds of glycosyltransferases (GTs) catalyze the formation of glycosidic linkages in polysaccharides — more than 50 for the pectic polymers alone (Scheible and Pauly, 2004; Mohnen, 2008; Yin et al., 2010; Dhugga, 2012). Most GTs act in the Golgi apparatus and their products are transported to cell walls in secretory vesicles. In contrast, cellulose- and callose synthases, and possibly the "D" class of cellulose synthase-like GTs, are embedded in the plasma membrane and their products are extruded directly into cell walls (Endler and Persson, 2011; Park et al., 2011). The large numbers of GT-encoding genes and their varied temporal and spatial expression profiles produce vast possibilities for cell wall variability. Further heterogeneity is generated by the availability of a wide range of activated sugar donors (Feingold and Avigad, 1980), methylation and acetylation, different enantiomer and the variety and number of possible glycosyl linkages as well as *in muro* modification of polysaccharides, e.g., by esterification/deesterification of pectins and transglycosylation between certain hemicelluloses (Fry et al., 2008; Burton et al., 2010). Collectively, these dynamic processes enable plants to generate cell walls that are exquisitely suited to prevailing functional requirements and that can respond to biotic and

abiotic stresses as well as developmental cues (Sarkar et al., 2009; Sørensen et al., 2010).

WHY STUDY CELL WALL DIVERSITY?

The study of cell wall glycomes across the plant kingdom is important for developing our understanding of cell wall structures and functions, for understanding cell wall and plant evolution, and for optimizing the utilization of the largest source of biomass on earth. Plants emerged onto land around 470 million years ago and have since colonized a large proportion of the Earth's surface (Kenrick and Crane, 1997; Waters, 2003; Niklas and Kutschera, 2010). The transition to land was a pivotal event in the history of life which resulted in the formation of new habitats and ecosystems and had profound effects on atmospheric chemistry. Cell walls have played significant roles in these epochal evolutionary events but our current understanding of many aspects of cell wall structures and their evolution is limited (Niklas, 2004; Popper and Tuohy, 2010; Sørensen et al., 2010). Improving our understanding will contribute to a wider understanding of plant evolution and phylogenetic relationships and may provide knowledge about past environments and insight into how plants might respond to predicted climate change scenarios.

The study of cell wall evolution is based largely upon the surveying of cell wall diversity (Popper, 2008; Sørensen et al., 2010; Popper et al., 2011). Only by doing this is it possible to correlate changes in plant physiology, morphology or habit with corresponding innovations in cell wall biology. A study of cell wall diversity across the plant kingdom also has other benefits. Cell wall polysaccharides are an immensely valuable renewable bio-resource and have numerous industrial applications. Timber, fibers, paper, functional ingredients (e.g., pectins from flowering plants and alginates and carrageenans from algae), and nutraceuticals, and first and second generation biofuels are predominantly cell wall-based (Bacic et al., 1988; Willats et al., 2006; Pauly and Keegstra, 2010). In contrast to nucleotide sequences and proteins, polysaccharides cannot readily be synthesized and so must be sourced from nature. Currently we use only a minute fraction of the global cell wall glycome and a comprehensive inventory of available polysaccharides may reveal valuable new molecules and materials with novel uses. The analysis of diverse cell wall compositions and architectures might also provide inspiration for current efforts aimed at the targeted modification of cell walls, notably for energy crops. However, surveying of cell walls across the plant kingdom is a daunting undertaking which as described below, entails many significant challenges and requires a multi-disciplinary approach. This is in large part because polysaccharides are not directly encoded by genome sequence; multiple enzymes are required to synthesize the activated sugar residues, linkages and many wall components undergo extensive modifications including methylation, esterification/deesterification, and acetylation as well as the addition of single or blocks of sugar residues.

CHALLENGES IN SURVEYING CELL WALL DIVERSITY

The specific genes and enzymes that lead to synthesis of specific cell wall components has yet to be fully elucidated. Furthermore, in the majority of cases several enzymes are required to synthesize a specific cell wall component which may additionally undergo subsequent modification *in muro*. Consequently we are not yet at the stage where it is possible to determine cell wall composition and diversity via a comparative genomics approach and much of the knowledge so far gleaned has relied on polymer analysis. One fundamental difficulty associated with this is that polysaccharides are not amenable to facile sequencing. Their structures can be determined by several well established chemical methods which have been developed and applied to cell wall studies over the last 50 or so years. Each method has both limitations and merits but they may be applied in concert to reveal and determine cell wall complexity and diversity. Few of the methods developed so far are amenable to high throughput screening, so wide surveys have to rely on partial characterization initially. Fourier Transform Infra-Red Spectroscopy (FTIR) requiring little sample preparation can be high throughput and is useful for determining differences in cell wall composition across samples but is rarely effective for precise compositional analysis as it does not yield sequence information (Mouille et al., 2003). Recently, methods based on carbohydrate microarrays probed with monoclonal antibodies (mAbs) and carbohydrate-binding modules (CBMs) with specificities for cell wall polysaccharide epitopes have been developed. This technology can enable analysis of the occurrence of 100 epitopes in 2–3 days and can reveal much about cell wall composition despite some limitations largely derived from the current, although increasing, availability of characterized mAbs and CBMs (Moller et al., 2007; Sørensen et al., 2009; Pattathil et al., 2010). Application of FTIR and carbohydrate microarrays can be used to indicate cell walls that have a composition that may merit further and detailed analysis because they appear to be significantly different from characterized cell walls. Thus, use of these methods facilitates the development of hypotheses regarding cell wall composition that can be further explored by more detailed analysis of a subset of the sample set. Polysaccharide gel electrophoresis (PACE) (Goubet et al., 2002), Oligosaccharide mass profiling (OLIMP; Lerouxel et al., 2002), paper chromatography, and thin layer chromatography (Fry, 2001) and related approaches are powerful tools for the next level operating on a subset of the original sample set. Glycosyl linkage analyses and NMR make up the final tier as these methods are time consuming and for NMR also quite insensitive. These techniques are thus unsuitable for wide scale sampling but are often indispensible for in-depth analysis of selected samples. Whatever method of analysis is chosen, sampling will always pose significant challenges. Given that it is not feasible to sample the walls of every extant plant then what plants and what organs or tissues should be chosen and is there sufficient tissue available? It seems reasonable to select species that are representative of taxonomic groups or morphotypes, but such prioritizations are not always straightforward. Once plants are chosen then one is faced with the further difficulty of properly sampling the individual cell walls within that plant. One option would be to simply homogenize the whole plant and extract as many cell wall polysaccharides as possible. This is feasible for say, small seedlings and microalgae, but not for woody species and trees. Clearly then, certain tissues, organs or developmental stages need to be selected – but on what basis? Equivalence can also be problematic because plants differ in the organs and tissues they

have. Some have flowers and leaves, some do not. Additionally, altered growth conditions may affect the expression and structure of cell wall components within the same species (Iraki et al., 1989a,b,c). Such considerations are important because we know that some cell wall components can be very selectively distributed throughout a plant (see section Fine mapping of cell wall diversity and heterogeneity at the cellular and subcellular levels) and can easily be missed. Interpretation of various analyses is a further important challenge. Considerable caution is required considering the near impossibility of truly inclusive sampling. If positively identified by multiple methods then a particular polysaccharide can be regarded as "present," but unless all parts of a plant have been sampled (including all developmental stages) then failure to identify a particular polysaccharide should be interpreted as the presence of that polysaccharide being "unknown" rather than "absent." When investigating cell wall evolution it is also important to consider polysaccharides that may occur at such low levels that they may be regarded as functionally unimportant in other studies. For example, although glycosyl linkage and ICP-MS data suggest RG-II or RG-II-like oligosaccharide occurs at very low levels in avascular bryophytes, less than 1% of that in angiosperms (Matsunaga et al., 2004), its presence in these plants would nevertheless be significant in terms of the evolution of underlying biosynthetic mechanisms.

A MULTI-LEVEL APPROACH TOWARDS UNDERSTANDING CELL WALL DIVERSITY AND EVOLUTION

The authors have adopted a multi-level strategy for mapping cell wall polysaccharide and genetic diversity in order to gain insight into underlying evolutionary mechanisms (**Figure 1A**). The first level consists of primary screens for cell wall polysaccharides based on carbohydrate microarrays probed with mAbs and CBMs (Moller et al., 2007). This level is limited by the availability of characterized mAbs and CBMs and their ability to recognize the numerous epitopes which occur in the various plant cell wall components. Obviously the ideal would be to have as much coverage as possible of all the epitopes that exist within cell wall components. However, this is not the current situation and there are some notable wall components, such as RG-II, for which an effective mAb has yet to be generated. In parallel to carbohydrate microarrays, genomes and transcriptomes are mined to identify cell wall-related GTs. The second level of analysis seeks to obtain more detailed information about certain polysaccharides and genes from subsets of plants. These analyses are performed using established methods for polysaccharide analysis and gene cloning and sequencing. A third level is aimed at obtaining definitive information and the functions of genes, protein, and polysaccharides. In some cases genes are expressed and biochemical activities of GTs determined. **Figure 1B** shows some preliminary data from primary screens of polysaccharides and cell wall-related genes. The combined analysis of data sets can provide insight into the timing and mechanism of certain evolutionary events. For example, Xue and Fry (2012) have suggested that MLGs are restricted to horsetails based on the results obtained when diverse monilophyte cell walls were treated with lichenase, an enzyme that specifically fragments these (1→3)(1→4)-β-D-glucans. In contrast, we have obtained evidence for MLGs in *Selaginella moellendorffii* and selected Charophycean green algae (CGA) using microarray-based polysaccharide screening and lichenase treatments (Harholt et al., 2012; Fangel and Willats, unpublished). Since a genome sequence is available for *S. moellendorffii* it is possible to establish with confidence that this plant does not contain orthologs of the *CSLF* and *CSLH* genes (Harholt et al., 2012) that are implicated in the synthesis of (1→3)(1→4)-β-D-glucan in Poales species (Burton et al., 2006; Doblin et al., 2009). These data provide good evidence that (1→3)(1→4)-β-D-glucan has evolved at least twice by convergent evolution.

GENOME AND TRANSCRIPTOME MINING FOR CELL WALL-RELATED GLYCOSYL TRANSFERASES

"At least one GT for each glycosidic linkage" is axiomatic for our bioinformatic analysis of cell wall biogenesis. Rare dual function GTs are known from animals and plants, with CslA as an example of a GT that can utilize two different GDP-sugars as substrate. Accepting the axiom of one GT per glycosidic linkage should permit inference of the minimum number of GTs involved in cell wall biosynthesis. However, this is not possible for two reasons. Firstly, the evolution of flowering plants was accompanied by very substantial gene duplication. Differentiation of complex tissues in angiosperms calling for separate regulation in space and time of the same catalytic activity is a likely contributing factor. Mitchell et al. (2007) combined this line of thinking with expression analysis and proposed that certain clades of family GT61 should comprise genes involved in synthesis of a polymer of particular importance in grasses, a prediction that was recently proven correct (Anders et al., 2012). The large repertoire of GTs of the moss *Physcomitrella patens*, despite being non-vascular (so having non-lignified tissues only) and also having diverged prior to the gene duplication events associated with the evolution of flowering plants, cautions us not to generalize this principle excessively. The second reason why the number of GTs cannot be inferred from the number of different linkages is that the biosynthesis of some polysaccharides has turned out to be much more complicated than anticipated from the polysaccharide structure; xylan biosynthesis, reviewed by Scheller and Ulvskov (2010), provides a striking example. In dicots,

BOX 1 | Text Box entitled CAZy.

The CAZy database, www.cazy.org, describes the families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds (Cantarel et al., 2009). GTs are classified to 94 families (and growing). The classification is partly based on sequence similarity, partly on 3D information of protein structure. The 94 families can be grouped into a small number of GT-folds creating a higher level in the hierarchy formalized as clans for glycoside hydrolases but not yet for GTs. Most families comprise several activities so assigning a GT to a family is rarely sufficient for deducing the precise catalytic activity of the GT. GTs may, however, with good precision be predicted to be inverting or retaining based on their CAZy family, where retaining refers to the situation where the transferred monosaccharide ends up in the same anomeric conformation as in the donor substrate and conversely for inverting GTs.

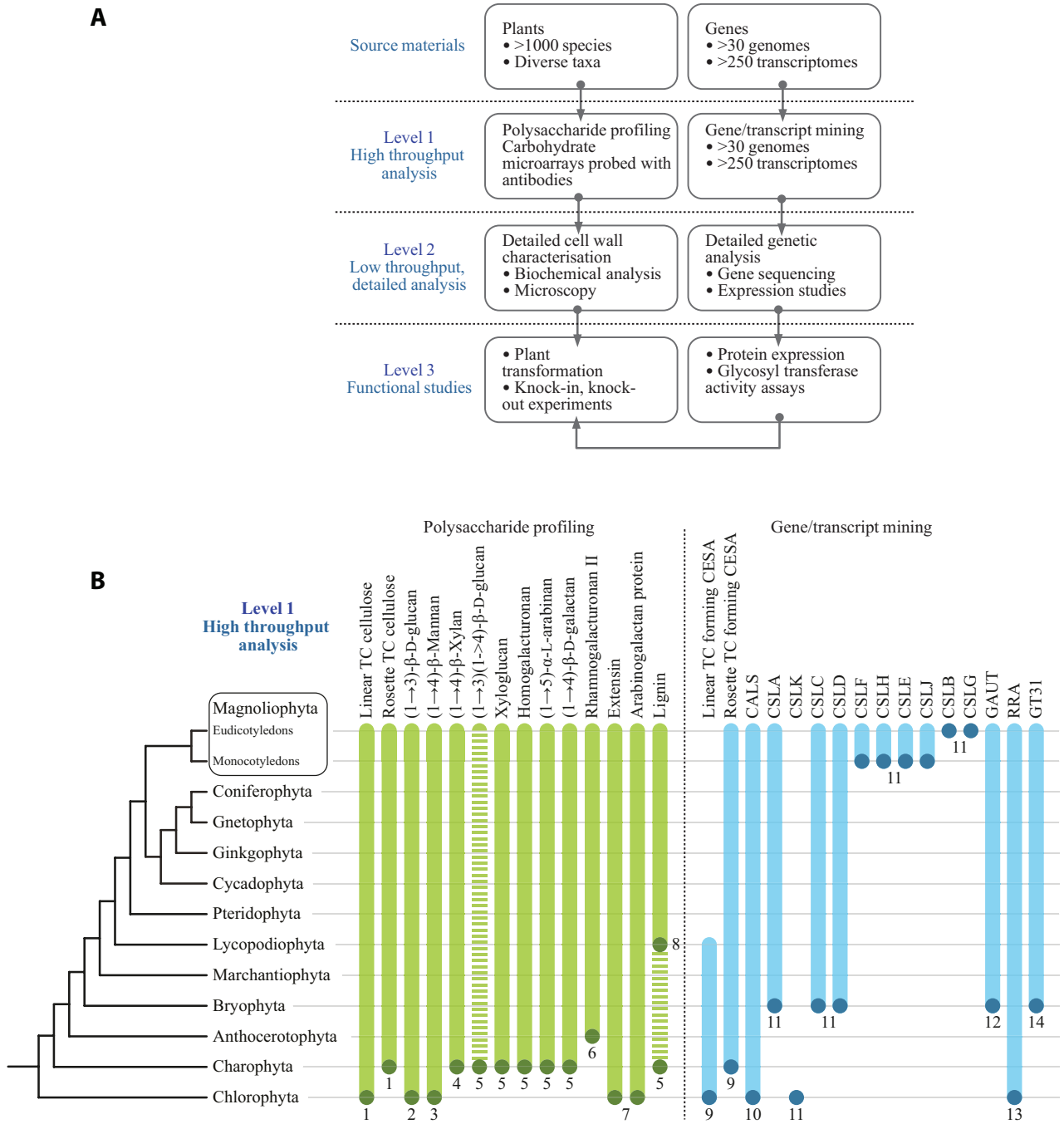


FIGURE 1 | (A) A multi-level approach is required to assess the occurrence of cell wall polysaccharides and related genes throughout the plant kingdom. **(B)** Selected data from primary screens of polysaccharide occurrence in diverse plant species and results from mining genomes and transcriptomes for cell wall-related GTs. The dots at the bottom of the bars indicate the most basal plant group in which that particular polysaccharide or gene sequence has so far been identified. The bars indicate that it is assumed in most cases, once evolved, a gene or polysaccharide persists throughout evolution and is present in later diverging species. However, this is by no means universal. For example, the dashed bar for (1→3)(1→4)-β-D-glucan indicates that the occurrence of this polysaccharide is intermittent throughout the plant kingdom and has arisen by convergent evolution more than once. In some cases the presence of cell wall components is equivocal. For example, lignin has been tentatively identified in certain Charophycean green algae but the most basal group in which it has been definitively identified is the Lycopodiophyta. A clade of putative ancestral CSLs that are common ancestors to the *CSLA*s and *CSLC*s has been identified in certain Chlorophyte algae by Yin et al. (2009). These genes share homology with both *CSLA*s and *CSLC*s but appear to differ sufficiently to warrant a new family name, which we have assigned *CSLK*s, consistent with the existing alphabetical sequence of the existing *CSL* family names. (1) Tsekos (1999); (2) Scherp et al. (2001); (3) Mackie and Sto (1968); (4) Domozych et al. (2009); (5) Sørensen et al. (2011); (6) Matsunaga et al. (2004); (7) Estevez et al. (2009); (8) Weng et al. (2008); (9) Roberts et al. (2002); (10) Abercrombie et al. (2011); (11) Yin et al. (2009); (12) Yin et al. (2010); (13) Egelund et al. (2007), Velasquez et al. (2011); (14) Qu et al. (2004), Harholt et al. (2012). TC, terminal complex.

GTs from two different families (GT43 and GT47) are implicated in synthesizing the xylan backbone totaling eight GTs for the synthesis of one linkage. Pectin biosynthesis is predicted to require at least 67 enzymes, GTs plus methyl- and acetyl transferases (Mohnen, 2008). Too few of these have been identified so far to judge whether Nature's approach to pectin biosynthesis is lean, or expansive as with xylan. These limitations notwithstanding, it has proven fruitful to mine genomes for their GT repertoire. This is usually done using the CAZy database as a foundation, See **Box 1**. The CAZy database is the most extensive database of GTs and contains GTs from all three kingdoms. By using a global approach including the whole CAZy database in the screen, more remote orthologies can be discovered, exemplified by the discovery of a mannosylglycerate synthase of GT78 in *S. moellendorffii* (Scheller et al., 2010). But CAZy is not complete and GTs may be found outside CAZy and certain activities are hard to account for within the limit of the present CAZy database (Hansen et al., 2012). The CAZy driven approach has been applied to poplar (Geisler-Lee et al., 2006) and most recently to *Brachypodium distachyon* (Vogel et al., 2010) and *S. moellendorffii* (Banks et al., 2011) also leading to a CAZy-based naming convention for putative GTs that can be assigned to a gene family but not to a function.

Applying the CAZy-based classification of putative GTs across large phylogenetic distances can yield evolutionary relevant information as exemplified by the case for convergent evolution of MLG. CAZy may also be employed as a support for gene discovery efforts as Egelund et al. (2007) did using homology between *Chlamydomonas* and *Arabidopsis* genes in clade A of family GT77 to infer the function of the Reduced Residual Arabinose (RRA) genes as encoding putative extensin arabinosyl transferases. This annotation is not proven but was corroborated by Velasquez et al. (2011). Extensin along with mannan are to the authors' knowledge the only known shared cell wall components between Chlorophycean green algae and *Arabidopsis* (**Figure 1**; Estevez et al., 2009).

No CGA has yet been sequenced which is unfortunate given that cell wall analysis strongly suggests that the common ancestor of all plants, with xyloglucan and the pectic polymers typical of vascular plants, was a CGA (**Figure 1**). A number of EST datasets (Timme and Delwiche, 2010; Wodniok et al., 2011; Timme et al., 2012) are available and may be subjected to the same CAZy-based analysis as full genomes, albeit less safely. Our unpublished observations of this nature, in combination with recent phylogenetic analyses based on genomic data, lead us to propose that genera like *Penium* and *Coleochaete* represent the earliest versions of a higher plant cell wall while species like *Chara*, which superficially looks more advanced, has diverged substantially from the main evolutionary path leading to terrestrial plants and hence is a less attractive model for tracing the evolutionary history of the plants cell wall (Timme et al., 2012).

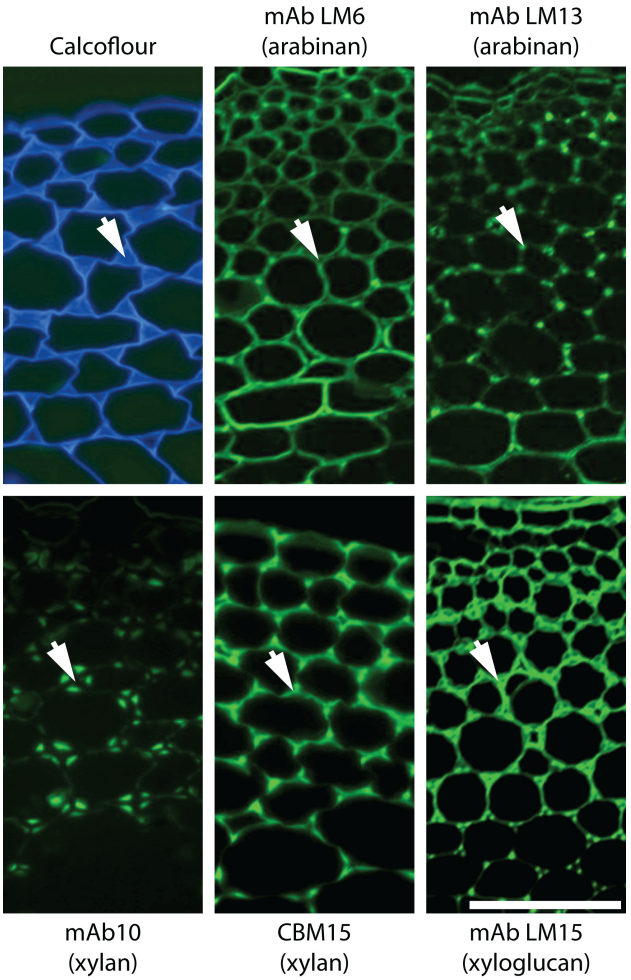


FIGURE 2 | Architectural heterogeneity in primary cell walls. Equivalent transverse sections of the cortical region of tobacco stems with immunofluorescence imaging of two arabinan epitopes (mAbs LM6 and LM13), two xylan epitopes (mAb LM10 and CBM15) and one xyloglucan epitope (mAb LM15). Arrows indicate intercellular matrix shared by adjacent cells. Xylan and xyloglucan epitopes are shown after removal of pectic homogalacturonan. Pectic arabinan, xylan, and xyloglucan structural features all display spatial heterogeneity in relation to cell wall thickening and intercellular regions. mAb, monoclonal antibody; CBM, carbohydrate-binding module. Scale bar = 100 μm.

FINE MAPPING OF CELL WALL DIVERSITY AND HETEROGENEITY AT THE CELLULAR AND SUBCELLULAR LEVELS

Cellulose is widely distributed in most cell walls but it is clear that the known structural diversity of the polysaccharides of the hemicellulose and pectic groups is regulated both taxonomically and also in relation to cell type and cell wall microstructures within tissues. The use of mAbs and CBMs for *in situ* analyses of cell walls remains a key approach to determine wall molecular architectures and their heterogeneities (Burton et al., 2010; Pattathil et al., 2010; Lee et al., 2011). The use of the same molecular probes in glycomic analysis is a very useful and complementary activity in which specific oligosaccharide structural features can be studied widely in terms of occurrence and biochemistry in addition to cellular locations.

It is a significant point that although we know some broad cellular occurrences of specific non-cellulosic polysaccharides – such as the abundance of xylan in secondary cell walls of dicotyledons,

of HG in primary cell walls and (an example from the subcellular level) the absence of pectic galactan from pit fields, we do not have a good understanding of the cellular distributions of all polymers for most plant cell types. Even for *Arabidopsis* a systematic *in situ* assessment of the major polymers in the cell walls of the major organs has not yet been achieved. Some can be predicted – such as those indicated above but the distributions of RG-I and other pectic epitopes or hemicelluloses cannot yet be predicted with certainty. *In situ* analyses of cell wall structures are made more complex but also more revealing in that as probe sets for specific polysaccharides are extended more cell wall diversity and heterogeneity can be uncovered. For example, this is the case for pectic HG (Willats et al., 2001; Parre and Geitmann, 2005; Wolf et al., 2009) and also for pectic arabinan (Verhertbruggen et al., 2009) where a mAb for linear arabinan detects arabinan substructures in restricted intercellular regions of parenchyma as shown in **Figure 2**. This also applies to xylan structures in the same parenchyma (Hervé et al., 2009). What is the functional basis for polysaccharide fine structure of both xylans and arabinans being so intimately spatially regulated in relation to factors such as cell adhesion (**Figure 2**)? Is this to be a paradigm for non-cellulosic wall polymers for which we currently have one or a limited number of probes such as pectic galactan and xyloglucan? The next few years will see more detailed systematic assessments of molecular architectures. This will be in conjunction with enzymatic and/or chemical pre-treatments that are in some cases required to optimize polysaccharide detection and in the case of polysaccharide masking (in which one polymer class blocks access to another polymer class) indicates important features of cell wall architectures reflecting protein access (Vreeland et al., 1984; Marcus et al., 2010; Davies et al., 2012).

Clearly we face many challenges in understanding cell wall evolution not least of which include sufficient sampling followed by appropriate synthesis and interpretation of large data sets including diverse information such as gene and protein sequence data as well as sugar linkages and epitope distributions. However, as cell and tissue molecular architectures are documented another major issue that is brought into focus is the function of individual wall components and of the entire cell walls, which can vary enormously with respect to quantitative and qualitative composition. Why do some cell walls have xyloglucan, xylan, and mannan hemicelluloses in distinct spatial distributions as for example in the extensively studied tobacco stem system as shown in **Figure 2**? How are these heterogeneities integrated into a functional whole in terms of wall properties and functions? *In vitro* analyses of composites formed from cellulose, pectins, and xyloglucans have yielded invaluable information about the properties of some wall components (Chanliaud et al., 2002). However, the structural and compositional complexity of naturally occurring cell walls means that an *in vitro* approach cannot reasonably be applied to investigate the functional properties of the full diversity of extant walls. *In vivo* methods of investigating wall biomechanics, at the tissue and lower levels, have been developed (Spatz et al., 1998; Burget, 2006) facilitating an improved understanding of how walls are assembled and the fine detail of wall domains and their nanomechanical properties are likely to emerge within the next few years as detailed *in situ* analyses are combined with genetic and enzymatic interventions. Integrating this knowledge will be a major challenge and is an exciting frontier for cell wall biology.

REFERENCES

Abercrombie, J. M., O'Meara, B. C., Moffatt, A. R., and Williams, J. H. (2011). Developmental evolution of flowering plant pollen tube cell walls: callose synthase (CalS) gene expression patterns. *EvoDevo* 2, 14.

Anders, N., Wilkinson, M. D., Lovegrove, A., Freeman, J., Tryfona, T., Pellny, T. K., Weimar, T., Mortimer, J. C., Stott, K., Baker, J. M., Defoin-Platel, M., Shewry, P. R., Dupree, P., and Mitchell, R. A. C. (2012). Glycosyl transferases in family 61 mediate arabinofuranosyl transfer onto xylan in grasses. *Proc. Natl. Acad. Sci. U.S.A.* 17, 989–993.

Bacic, A., Harris, A. J., and Stone, B. A. (1988). "Structure and function of plant cell walls," in *The Biochemistry of Plants*, ed J. Preiss (New York: Academic Press), 297–371.

Banks, J. A., Nishiyama, T., Hasebe, M., Bowman, J. L., Gribskov, M., dePamphilis, C., Albert, V. A., Aono, N., Aoyama, T., Ambrose, B. A., Ashton, N. W., Axtell, M. J., Barker, E., Barker, M. S., Bennetzen, J. L., Bonawitz, N. D., Chapple, C., Cheng, C., Correa, L. G., Dacre, M., DeBarry, J., Dreyer, I., Elias, M., Engstrom, E. M., Estelle, M., Feng, L., Finet, C., Floyd, S. K., Frommer, W. B., Fujita, T., Gramzow, L., Gutensohn, M., Harholt, J., Hattori, M., Heyl, A., Hirai, T., Hiwatashi, Y., Ishikawa, M., Iwata, M., Karol, K. G., Koehler, B., Kolukisaoglu, U., Kubo, M., Kurata, T., Lalonde, S., Li, K., Li, Y., Litt, A., Lyons, E., Manning, G., Maruyama, T., Michael, T. P., Mikami, K., Miyazaki, S., Morinaga, S., Murata, T., Mueller-Roeber, B., Nelson, D. R., Obara, M., Oguri, Y., Olmstead, R. G., Onodera, N., Petersen, B. L., Pils, B., Prigge, M., Rensing, S. A., Riaño-Pachón, D. M., Roberts, A. W., Sato, Y., Scheller, H. V., Schulz, B., Schulz, C., Shakirov, E. V., Shibagaki, N., Shinohara, N., Shippen, D. E., Sørensen, I., Sotooka, R., Sugimoto, N., Sugita, M., Sumikawa, N., Tanurdzic, M., Theissen, G., Ulvskov, P., Wakazuki, S., Weng, J. K., Willats, W. W., Wipf, D., Wolf, P. G., Yang, L., Zimmer, A. D., Zhu, Q., Mitros, T., Hellsten, U., Loqué, D., Otillar, R., Salamov, A., Schmutz, J., Shapiro, H., Lindquist, E., Lucas, S., Rokhsar, D., and Grigoriev, I. V. (2011). The *Selaginella* genome identifies genetic changes associated with the evolution of vascular plants. *Science* 332, 960–963.

Boerjan, W., Ralph, J., and Baucher, M. (2003). Lignin biosynthesis. *Annu. Rev. Plant. Biol.* 54, 519–546.

Burget, I. (2006). Exploring the micromechanical design of plant cell walls. *Am. J. Bot.* 93, 1391–1401.

Burton, R. A., Wilson, S. M., Hrmova, M., Harvey, A. J., Shirley, N. J., Medhurst, A., Stone, B. A., Newbigin, E. J., Bacic, A., and Fincher, G. B. (2006). Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1,3;1,4)-β-D-glucans. *Science* 311, 1940–1942.

Burton, R. A., Gidley, M. J., and Fincher, G. B. (2010). Heterogeneity in the chemistry, structure and function of plant cell walls. *Nat. Chem. Biol.* 6, 724–732.

Caffall, K. H., and Mohnen, D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydr. Res.* 344, 1879–1900.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and, Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, 233–238.

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30.

Chanliaud, E., Burrows, K. M., Jeronimidis, G., and Gidley, M. J. (2002). Mechanical properties of primary cell wall analogues. *Planta* 215, 989–996.

Cosgrove, D. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell. Biol.* 6, 850–861.

Davies, L. J., Lilley, C. J., Knox, J. P., and Urwin, P. E. (2012). Syncytia formed by adult female *Heterodera schachtii* on *Arabidopsis thaliana* roots have a distinct cell wall molecular architecture. *New Phytol.* (in press).

Dhugga, K. S. (2012). Biosynthesis of non-cellulosic polysaccharides of plant cell walls. *Phytochemistry* 74, 8–19.

Doblin, M. S., Pettolino, F. A., Wilson, S. M., Campbell, R., Burton, R. A., Fincher, G. B., Newbigin, E., and Bacic, A. (2009). A barley cellulose synthase-like CSLH gene mediates (1,3;1,4)-β-D-glucan synthesis in

transgenic *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5996–6001.

Domozych, D. S., Sørensen, I., and Willats, W. G. T. (2009). The distribution of cell wall polymers during antheridium development and spermatogenesis in the Charophycean green alga, *Chara corallina*. *Ann. Bot.* 104, 1045–1056.

Egelund, J., Obel, N., Ulvskov, P., Geshi, N., Pauly, M., Bacic, A., and Petersen, B. L. (2007). Molecular characterization of two *Arabidopsis thaliana* glycosyltransferase mutants, rra1 and rra2, which have a reduced residual arabinose content in a polymer tightly associated with the cellulosic wall residue. *Plant Mol. Biol.* 64, 439–451.

Endler, A., and Persson, S. (2011). Cellulose synthases and synthesis in *Arabidopsis*. *Mol. Plant* 4, 199–211.

Estevez, J. M., Fernández, P. V., Kasulin, L., Dupree, P., and Ciancia, M. (2009). Chemical and *in situ* characterization of macromolecular components of the cell walls from the green seaweed *Codium fragile*. *Glycobiology* 19, 212–228.

Feingold, D. S., and Avigad, G. (1980). "Sugar nucleotide transformations in plants," in *The Biochemistry of Plants: A Comprehensive Treatise*, eds P. K. Stumpf and E. E. Conn (New York: Academic Press), 101–170.

Fry, S. C. (2001). *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*, Reprint edition. New Jersey: The Blackburn Press.

Fry, S. C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. *New Phytol.* 161, 641–675.

Fry, S. C., Mohler, K. E., Nesselrode, B. H. W. A., and Franková, L. (2008). Mixed-linkage β-glucan: xyloglucan endotransglucosylase, a novel wall-remodelling enzyme from Equisetum (horsetails) and charophytic algae. *Plant J.* 55, 240–252.

Geisler-Lee, J., Geisler, M., Coutinho, P. M., Segerman, B., Nishikubo, N., Takahashi, J., Aspeborg, H., Djerbi, S., Master, E., Andersson-Gunneras, S., Sundberg, B., Karpinski, S., Teeri, T. T., Kleczkowski, L. A., Henrissat, B., and Mellerowicz, E. J. (2006). Poplar carbohydrate-active enzymes. Gene identification and expression analyses. *Plant Physiol.* 140, 946–962.

Goubet, F., Jackson, P., Deery, M. J., and Dupree, P. (2002). Polysaccharide analysis using carbohydrate gel electrophoresis: a method to study plant cell wall polysaccharides and polysaccharide hydrolases. *Anal. Biochem.* 300, 53–68.

Hansen, S. F., Harholt, J., Oikawa, A., and Scheller, H. V. (2012). Plant glycosyltransferases beyond CAZy: a perspective on DUF families. *Front. Plant Sci.* 3:59. doi: 10.3389/fpls.2012.00059

Harholt, J., Suttangkakul, A., and Vibe Scheller, H. (2010). Biosynthesis of pectin. *Plant Physiol.* 153, 384–395.

Harholt, J., Sørensen, I., Fangel, J. U., Roberts, A., Willats, W. G. T., Scheller, H. V., Bent Larsen Petersen, B. L., Banks, J. A., and Ulvskov, P. (2012). The glycosyltransferase repertoire of the spikemoss *Selaginella moellendorffii* and a comparative study of the cell wall structure. *PLoS ONE* 7, e35846. doi:10.1371/journal.pone.0035846

Hepler, P. K., Fosket, D. E., and Newcomb, E. H. (1970). Lignification during secondary wall formation in *Coleus*: an electron microscopy study. *Am. J. Bot.* 57, 85–96.

Hervé, C., Rogowski, A., Gilbert, H. J., and Knox, J. P. (2009). Enzymatic treatments reveal differential capacities for xylan recognition and degradation in primary and secondary plant cell walls. *Plant J.* 58, 413–422.

Iraki, N. M., Bressan, R. A., Hasegawa, P. M., and Carpita, N. C. (1989a). Alteration of the physical and chemical structure of the primary cell wall of growth-limited plant cells adapted to osmotic stress. *Plant Physiol.* 91, 39–47.

Iraki, N. M., Singh, N., Bressan, R. A., and Carpita, N. C. (1989b). Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. *Plant Physiol.* 91, 48–53.

Iraki, N. M., Bressan, R. A., and Carpita, N. C. (1989c). Extracellular polysaccharides and proteins of tobacco cell cultures and changes in composition associated with growth-limiting adaptation to water and saline stress. *Plant Physiol.* 91, 54–61.

Kenrick, P., and Crane, P. R. (1997). The origin and early evolution of plants on land. *Nature* 389, 33–39.

Lee, K. J. D., Marcus, S. E., and Knox, J. P. (2011). Plant cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219.

Lerouxel, O., Choo, T. S., Seveno, M., Usadel, B., Faye, L., Lerouge, P., and Pauly, M. (2002). Rapid structural phenotyping of plant cell wall mutants by enzymatic oligosaccharide fingerprinting. *Plant Physiol.* 130, 1754–1763.

Mackie, W., and Sto, R. D. P. R. (1968). The occurrence of mannan microfibrils in the green algae *Codium fragile* and *Acetabularia crenulata*. *Planta* 253, 249–253.

Marcus, S. E., Blake, A. W., Benians, T. A. S., Lee, K. J. D., Poyser, C., Donaldson, L., Leroux, O., Rogowski, A., Petersen, H. L., Boraston, A., Gilbert, H. J., Willats, W. G. T., and Knox, J. P. (2010). Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *Plant J.* 64, 191–203.

Matsunaga, T., Ishii, T., Matsumoto, S., Higuchi, M., Darvill, A., Albersheim, P., and O'Neill, M. A. (2004). Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes: implications for the evolution of vascular plants. *Plant Physiol.* 134, 339–351.

Mitchell, R. A., Dupree, P., and Shewry, P. R. (2007). A novel bioinformatics approach identifies candidate genes for the synthesis and feruloylation of arabinoxylan. *Plant Physiol.* 144, 43–53.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Curr. Opin. Plant. Biol.* 11, 266–277.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A., and Willats, W. G. T. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Mouille, G., Robin, S., Lecomte, M., Pagant, S., and Höfte, H. (2003). Classification and identification of Arabidopsis cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *Plant J.* 35, 393–404.

Niklas, K. J. (2004). The cell walls that bind the tree of life. *BioScience* 54, 831–841.

Niklas, K. J., and Kutschera, U. (2010). The evolution of the land plant life cycle. *New Phytol.* 185, 27–41.

Parre, E., and Geitmann, A. (2005). Pectin and the role of the physical properties of the cell-wall in pollen tube growth of *Solanum chacoense*. *Planta* 220, 582–592.

Park, S., Szumlanski, A. L., Gu, F., Guo, F., and Nielsen, E. (2011). A role for CSLD3 during cell-wall synthesis in apical plasma membranes of tip-growing root-hair cells. *Nat. Cell. Biol.* 13, 973– 980.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Pauly, M., and Keegstra, K. (2010). Plant cell wall polymers as precursors for biofuels. *Curr. Opin. Plant Biol.* 13, 305–312.

Popper, Z. A. (2008). Evolution and diversity of green plant cell walls. *Curr. Opin. Plant Biol.* 11, 286–292.

Popper, Z. A., and Tuohy, M. G. (2010). Beyond the green: understanding the evolutionary puzzle of plant and algal cell walls. *Plant Physiol.* 153, 373–383.

Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G., Tuohy, M. G., Kloareg, B., and Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant. Biol.* 62, 567–590.

Qu, Y., Egelund, J., Gilson, P. R., Houghton, F., Gleeson, P. A., Schultz, C. J., and Bacic, A. (2004). Identification of a novel group of putative *Arabidopsis thaliana* β-(1,3)-galactosyltransferases. *Plant Mol. Biol.* 68, 43–59.

Roberts, A. W., Roberts, E. M., and Delmer, D. P. (2002). Cellulose Synthase (CesA) Genes in the green alga *Mesotaenium caldariorum*. *Eukaryot. Cell* 1, 847–855.

Sarkar, P., Bosneaga, E., and Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *J. Exp. Bot.* 60, 3615–3635.

Scheible, W. R., and Pauly, M. (2004). Glycosyltransferases and cell wall biosynthesis: novel players and insights. *Curr. Opin. Plant. Biol.* 7, 285–295.

Scheller, H. V., and Ulvskov, P. (2010.) Hemicelluloses. *Annu. Rev. Plant. Biol.* 61, 263–289.

Scheller, H. V., Ulvskov, P., and Harholt, J. (2010). Stress-tolerant plants expressing mannosylglycerate-producing enzymes. US National Stage Patent Application No. 13/318,661. WIPO Patent WO/2010/129574A1. Available at: http://www.sumobrain.com/patents/wipo/Stress-tolerant-plants-expressing-mannosylglycerate/WO2010129574A1.pdf

Scherp, P., Grotha, R., and Kutschera, U. (2001). Occurrence and phylogenetic significance of cytokinesis-related callose in green algae, bryophytes, ferns and seed plants. *Plant Cell Reports* 20, 143–149.

Sørensen, I., Domozych, D., and Willats, W. G. (2010). How have plant cell walls evolved? *Plant Physiol.* 153, 366–372.

Sørensen, I., Pedersen, H. L., and Willats, W. G. (2009). An array of

possibilities for pectin. *Carbohydr. Res.* 344, 1872–1878.

Sørensen, I., Pettolino, F. A, Bacic, A., Ralph, J., Lu, F., O'Neill, M. A, Fei, Z., Rose, J. K. C., Domozych, D. S., and Willats, W. G. T. (2011). The charophycean green algae provide insights into the early origins of plant cell walls. *Plant J.* 68, 201–211.

Spatz, H.-C., Rowe, N., Speck, T., and Daviero, V. (1998). Biomechanics of hollow stemmed Sphenopsids: II. Calamites – to have or not to have secondary xylem. *Rev. Palaeobot. Palynol.* 102, 63–77.

Timme, R. E., and Delwiche, C. F. (2010). Uncovring the evolutionary origin of plant molecular processes: comparison of *Coleochaete* (Coleochaetales) and *Spirogyra* (Zygnematales) transcriptomes. *BMC Plant Biol.* 10, 96. doi: 10.1186/1471-2229-10-96

Timme, R. E., Bachvaroff, T. R., and Delwiche, C. F. (2012). Broad phylogenomic sampling and the sister lineage of land plants. *PLoS ONE* 7, e29696. doi:10.1371/journal.pone.0029696

Tsekos, I. (1999). The sites of cellulose synthesis in algae: diversity and evolution of cellulose-synthesizing enzyme complexes. *J. Phycol.* 35, 635–655.

Velasquez, S. M., Ricardi, M. M., Dorosz, J. G., Fernandez, P. V., Nadra, A. D., Pol-Fachin, L., Egelund, J., Gille, S., Harholt, J., Ciancia, M., Verli, H., Pauly, M., Bacic, A., Olsen, C. E., Ulvskov, P., Petersen, B. L, Somerville, C., Iusem, N. D., and Estevez, J. M. (2011). *O*-Glycosylated cell wall proteins are essential in root hair growth. *Science* 332, 1401–1403.

Verhertbruggen, Y., Marcus, S. E., Haeger, A., Verhoef, R., Schols, H. A., McCleary, B. V., McKee, L., Gilbert, H. J., and Knox, J. P. (2009). Developmental complexity of arabinan polysaccharides and their processing in plant cell walls. *Plant J.* 59, 413–425.

Vogel, J. P., Garvin, D. F., Mockler, T. C., Schmutz, J., Rokhsar, D., Bevan, M. W., Barry, K., Lucas, S., Harmon-Smith, M., Lail, K., Tice, H., Schmutz, J., Grimwood, J., McKenzie, N., Bevan, M. W., Huo, N., Gu, Y. Q., Lazo, G. R., Anderson, O. D., Vogel, J. P., You, F. M., Luo, M. C., Dvorak, J., Wright, J., Febrer, M., Bevan, M. W., Idziak, D., Hasterok, R., Garvin, D. F., Lindquist, E., Wang, M., Fox, S. E., Priest, H. D., Filichkin, S. A., Givan, S. A., Bryant, D. W., Chang, J. H., Mockler, T. C., Wu, H., Wu, W., Hsia, A. P., Schnable, P. S., Kalyanaraman, A., Barbazuk, B., Michael, T. P., Hazen, S. P., Bragg, J. N., Laudencia-Chingcuanco, D., Vogel, J. P., Garvin, D. F., Weng, Y., McKenzie, N., Bevan, M. W., Haberer, G., Spannagl, M., Mayer, K., Rattei, T., Mitros, T., Rokhsar, D., Lee, S. J., Rose, J. K., Mueller, L. A., York, T. L., Wicker, T., Buchmann, J. P., Tanskanen, J., Schulman, A. H., Gundlach, H., Wright, J., Bevan, M., de Oliveira, A. C., Maia Lda, C., Belknap, W., Gu, Y. Q., Jiang, N., Lai. J., Zhu, L., Ma, J., Sun, C., Pritham, E., Salse, J., Murat, F., Abrouk, M., Haberer. G., Spannagl. M., Mayer, K., Bruggmann, R., Messing, J., You, F. M., Luo, M. C., Dvorak, J., Fahlgren, N., Fox, S. E., Sullivan, C. M., Mockler, T. C., Carrington, J. C., Chapman, E. J., May, G. D., Zhai, J., Ganssmann, M., Gurazada, S. G., German, M., Meyers, B. C., Green, P. J., Bragg, J. N., Tyler, L., Wu, J., Gu, Y. Q., Lazo, G. R., Laudencia-Chingcuanco, D., Thomson, J., Vogel, J. P., Hazen, S. P., Chen, S., Scheller, H. V., Harholt, J., Ulvskov, P., Fox, S. E., Filichkin, S. A., Fahlgren, N., Kimbrel, J. A., Chang, J. H., Sullivan, C. M., Chapman, E. J., Carrington, J. C., Mockler, T. C., Bartley, L. E., Cao, P., Jung, K. H., Sharma, M. K., Vega-Sanchez, M., Ronald, P., Dardick, C. D., De Bodt, S., Verelst, W., Inzé, D., Heese, M., Schnittger, A., Yang, X., Kalluri, U. C., Tuskan, G. A., Hua, Z., Vierstra, R. D., Garvin, D. F., Cui, Y., Ouyang, S., Sun, Q., Liu, Z., Yilmaz, A., Grotewold, E., Sibout, R., Hematy, K., Mouille, G., Höfte, H., Michael, T., Pelloux, J., O'Connor, D., Schnable, J., Rowe, S., Harmon, F., Cass, C. L., Sedbrook, J. C., Byrne, M. E., Walsh, S., Higgins, J., Bevan, M., Li, P., Brutnell, T., Unver, T., Budak, H., Belcram, H., Charles, M., Chalhoub, B., and Baxter, I. (2010). Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463, 763–768.

Vreeland, V., Slomich, M., and Laetsch, W. M. (1984). Monoclonal antibodies as molecular probes for cell wall antigens of the brown alga *Fucus*. *Planta* 163, 506–517.

Waters, E. R. (2003). Molecular adaptation and the origin of land plants. *Mol. Phylogenet. Evol.* 29, 456–463.

Weng, J.-K., Li, X., Stout, J., and Chapple, C. (2008). Independent origins of syringyl lignin in vascular plants. *Proc. Nat. Acad. Sci. U.S.A.* 105, 7887–7892.

Willats, W. G. T., Knox, J. P., and Mikkelsen, J. D. (2006). Pectin: new insights into an old polymer are starting to gel. *Trends Food Sci. Technol.* 17, 97–104.

Willats, W. G. T., Orfila, C., Limberg, G., Buchholt, H. C., van Alebeek, G.-J. W. M., Voragen, A. G. J., Marcus, S. E., Christensen, T. M. I. E., Mikkelsen, J. D., Murray, B. S., and Knox, J. P. (2001). Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls: implications for pectin methyl esterase action, matrix properties and cell adhesion. *J. Biol. Chem.* 276, 19404–19413.

Wodniok, S., Brinkmann, H., Glöckner, G., Heidel, A. J., Philippe, H., Melkonian, M., and Becker, B. (2011). Origin of land plants: do conjugating green algae hold the key? *BMC Evol. Biol.* 11, 104. doi: 10.1186/1471-2148-11-104

Wolf, S., Mouille, G., and Pelloux, J. (2009). Homogalacturonan methyl-esterification and plant development. *Mol. Plant* 2, 851–860.

Xue, X., and Fry, S. C. (2012). Evolution of mixed-linkage (1→3, 1→4)-β-D-glucan (MLG) and xyloglucan in Equisetum (horsetails) and other monilophytes. *Ann. Bot.* 109, 873–886.

Yin, Y., Chen, H., Hahn, M. G., Mohnen, D., and Xu, Y. (2010). Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *Plant Physiol.* 153, 1729–1746.

Yin, Y., Huang, J., and Xu, Y. (2009). The cellulose synthase superfamily in fully sequenced plants and algae. *BMC Plant Biol.* 9, 99. doi: 10.1186/1471-2229-9-99

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Moss cell walls: structure and biosynthesis

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The genome sequence of the moss *Physcomitrella patens* has stimulated new research examining the cell wall polysaccharides of mosses and the glycosyl transferases that synthesize them as a means to understand fundamental processes of cell wall biosynthesis and plant cell wall evolution. The cell walls of mosses and vascular plants are composed of the same classes of polysaccharides, but with differences in side chain composition and structure. Similarly, the genomes of *P. patens* and angiosperms encode the same families of cell wall glycosyl transferases, yet, in many cases these families have diversified independently in each lineage. Our understanding of land plant evolution could be enhanced by more complete knowledge of the relationships among glycosyl transferase functional diversification, cell wall structural and biochemical specialization, and the roles of cell walls in plant adaptation. As a foundation for these studies, we review the features of *P. patens* as an experimental system, analyses of cell wall composition in various moss species, recent studies that elucidate the structure and biosynthesis of cell wall polysaccharides in *P. patens*, and phylogenetic analysis of *P. patens* genes potentially involved in cell wall biosynthesis.

Keywords: cell wall, polysaccharide, cellulose, cellulose synthesis complex, glycosyl transferase, moss, *Physcomitrella patens*

MOSS BIOLOGY AND EVOLUTION

The common ancestor of land plants is believed to have resembled extant mosses in having a biphasic life cycle with a dominant haploid gametophyte and rudimentary adaptations for tolerating the aerial environment. Because they have retained these characteristics, mosses are often referred to as "lower" plants (Mishler and Oliver, 2009). Despite this designation, mosses are highly successful, comprising more than 10,000 species adapted to diverse habitats ranging from submerged aquatic to desert (Buck and Goffinet, 2000). Mosses differ from vascular plants in the strategy they employ to survive in the dry aerial environment. Vascular plants are homeohydric with a thick cuticle to reduce dehydration, roots to extract water from the soil, and vascular tissue to distribute water internally. In contrast, mosses are poikilohydric, depending on a surface film of free water to maintain hydration. Although some mosses are confined to aquatic habitats, many are dehydration tolerant and some are desiccation tolerant (Mishler and Oliver, 2009).

The life cycle, morphology, and biochemistry of mosses have been influenced by selective pressure associated with poikilohydry. Because cellular water status is controlled by surface absorption and diffusion, moss organs are small and thin, and lignin is not required to provide support against gravity or the negative pressures generated during transpiration (Mishler and Oliver, 2009). In most mosses, haploid spores produce protonemal filaments that extend by apical division and tip growth (**Figure 1**). The filaments produce buds that develop into leafy gametophores, which enlarge by diffuse growth. Haploid spores are produced by diploid sporophytes, which develop from eggs fertilized by swimming sperm at the gametophore apex (Schumaker and Dietrich, 1998).

Although structurally simple, moss gametophytes contain different cell types. The protonema is differentiated into chloroplast-rich chloronemal cells and caulonemal cells, which elongate three times faster (Menand et al., 2007). In response to dehydration stress, protonemal cells resume division and differentiate into thick-walled, dehydration-tolerant brachycytes, and subtending tmema cells, which undergo programmed cell death (Decker et al., 2006). Gametophores include a stem, leaves, axillary hairs, and rhizoids. The stem typically consists of small, thick-walled epidermal and subepidermal cells, thin-walled parenchyma cells, and conducting cells. The conducting cells include hydroids and leptoids, which are functionally analogous to xylem and phloem (Buck and Goffinet, 2000). Similar to tracheary elements, hydroids are dead at maturity and connected by perforations, but they lack thick lignified secondary cell walls (Hebant, 1977). Leaves are typically one cell layer thick except for the midribs and margins, which may consist of multiple layers of differentiated cells. Leaf cells of *Sphagnum* species include photosynthetic chlorocytes and hyalocytes with elaborate cell wall thickenings. Other leaf cell specializations include papillae and various surface elaborations. Additional differentiated cell types form the gametangia, gametes, and sterile paraphyses. The sporophyte stalk, sporangium, and spores also consist of specialized cell types (Buck and Goffinet, 2000), including stomata (Sack and Paolillo, 1983).

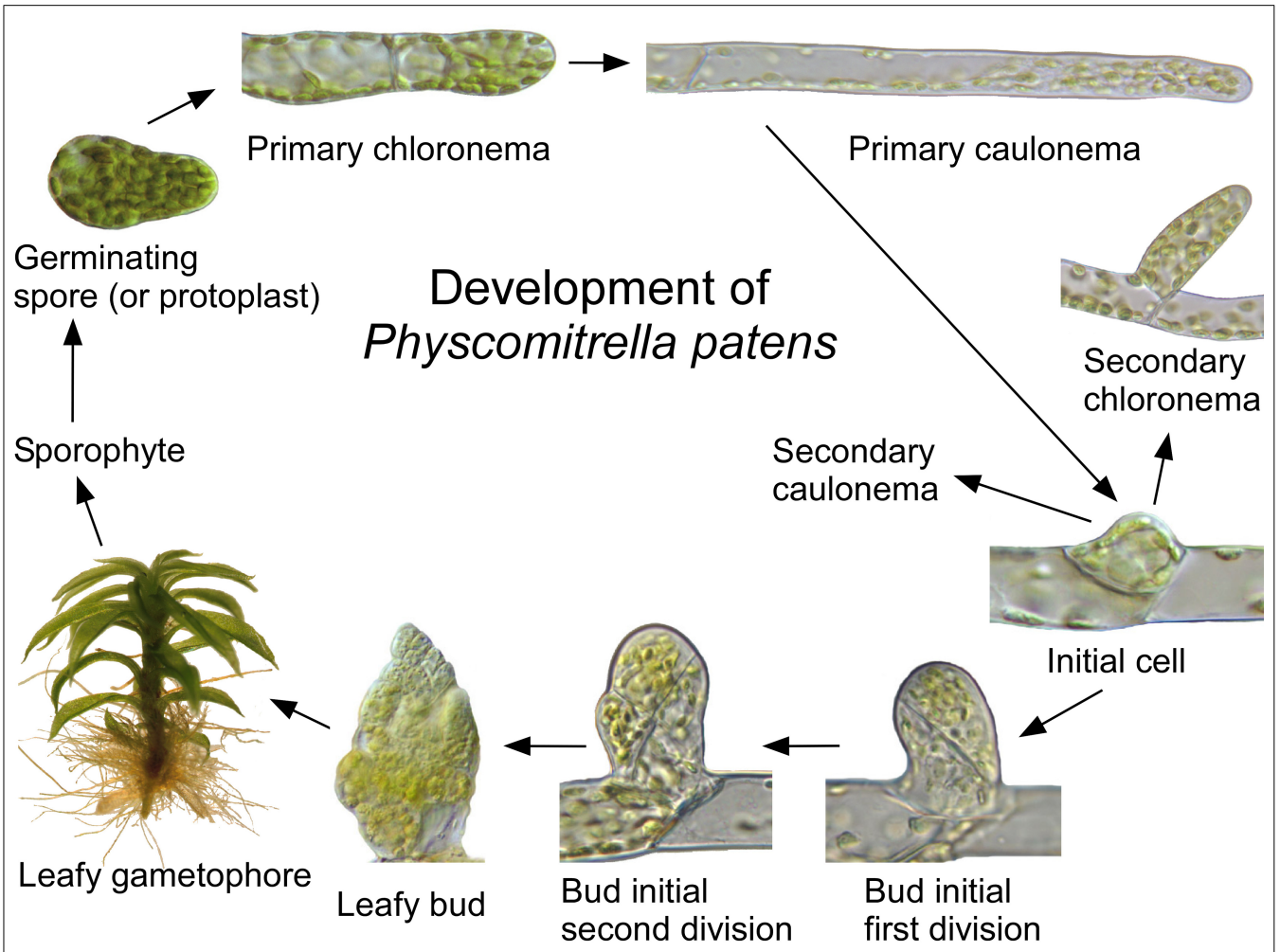


FIGURE 1 | Development of *Physcomitrella patens*. A haploid spore or protoplast germinates to form a primary chloronema with numerous chloroplasts and transverse cross walls, which subsequently differentiates into a more rapidly growing primary caulonema with fewer chloroplasts and oblique cross walls. The caulonemal subapical cells divide to produce initials cells, which develop into lateral secondary chloronemata, secondary caulonemata (not shown), or buds. Buds develop into leafy gametophores, which produce apical gametangia. Fertilization of an egg by swimming sperm at the gametophore apex produces a zygote, which develops into a diploid sporophyte consisting of a stalk and sporangium (not shown). Meiotic divisions within the sporangium generate haploid spores.

Although mosses share a poikilohydric ecological strategy and common body plan, their diversification and colonization of different habitats have been accompanied by the evolution of specialized morphological and biochemical adaptations that must be considered when inferring evolutionary trends from comparative studies of mosses and vascular plants. The mosses diverged from the land plant lineage between the liverworts and the hornworts, which most recent phylogenies place as sister to the vascular plants. The moss lineage includes the "true mosses" and three early divergent and ecologically specialized lineages, the aquatic Sphagnales, the desiccation-tolerant rock-dwelling Andreales, and the morphologically diverse Polytricales (Mishler and Oliver, 2009). Whereas mosses have retained primitive aspects of cell wall structure and composition due to poikilohydry, they have also evolved special cell wall adaptations that enabled them to colonize diverse habitats.

PHYSCOMITRELLA PATENS, THE MODEL MOSS SPECIES

As a member of the Funariales, *Physcomitrella patens* occupies a phylogenetic position at the base of the true mosses. As an inhabitant of moist soils that tolerates dehydration, but not desiccation, it represents a "primitive moss ecology" (Mishler and Oliver, 2009). This lack of specialization for extreme conditions combined with abundant genomic resources (Rensing et al., 2008), efficient production of transgenic genotypes, and ease of culture and experimental manipulation (Cove, 2005) provides an opportunity to relate the diversification of gene families to innovations in cell wall composition, structure, and development that accompanied the adaptation of plants to life on land. Other advantages of *P. patens* include the ability to produce large amounts of tissue consisting of a single cell type (chloronemal filaments) and rapid cell wall regeneration in protoplasts (Lee et al., 2005a; Lawton and Saidasan, 2011; Roberts et al., 2011).

CELL WALL ANALYSIS

Cell wall polysaccharide composition has been investigated in several moss species, including *P. patens*. As a complement to biochemical methods, immunological and affinity approaches employing antibodies and carbohydrate binding modules that recognize a variety of cell wall polysaccharides (Knox, 2008) have been used to examine the distribution of polysaccharides and with the microarray method known as comprehensive microarray polymer profiling (CoMPP; Moller et al., 2007).

CELLULOSE

Cellulose exists in the cell walls of all mosses that have been examined. It was detected in *P. patens* by CoMPP, sugar linkage analysis, and staining with Tinopal and CBM3A, a probe specific for crystalline cellulose (Kremer et al., 2004; Lee et al., 2005b, 2011; Moller et al., 2007; Nothnagel and Nothnagel, 2007; Goss et al., 2012). As is typical for cellulose, 5–20 nm wide microfibrils are visible in extracted and shadowed cell walls (**Figure 2A**). Microfibril impressions also occur in freeze-fractured plasma membranes (**Figure 2B**). Fibrils detected by atomic force microscopy on the surface of air-dried protonemal filaments were 250 nm in diameter (Wyatt et al., 2008), which is consistent with cellulose aggregation upon drying.

The *P. patens* genome includes seven *Cellulose Synthase* genes (*PpCESA3,-4,-5,-6,-7,-8*, and *-10* = *-11*) and three *CESA* pseudogenes (*PpCESA1,-2*, and *-9*; Roberts and Bushoven, 2007; Yin et al., 2009; Wise et al., 2011). Whereas seed plant CESAs are specialized for primary and secondary cell wall deposition, the CESAs of *P. patens* may be specialized for tip and diffuse growth. *PpCESA5* is required for leafy gametophore morphogenesis and is upregulated by cytokinin, which also induces gametophore development (Goss et al., 2012). Based on over-representation in EST libraries from cytokinin-treated cultures, *PpCesA4* and *-10* may also be involved in gametophore development. In contrast, *PpCESA6* is expressed in tip-growing protonemal filaments, rhizoids, and axillary hairs (Wise et al., 2011). Knockout mutants of *PpCESA6* and *-7*, which differ by three amino acids, have no morphological phenotype. Shorter gametophores were observed in one *PpCESA6/7* double mutant line (Wise et al., 2011). Analysis of additional *PpCESA* knockout mutants will be required to fully understand CESA diversification and functional specialization in *P. patens*.

Mosses including *Funaria hygrometrica* (Reiss et al., 1984; Rudolph and Schnepf, 1988; Rudolph et al., 1989) and *P. patens* have rosette-type cellulose synthesis complexes (CSCs). CSCs are abundant near apical cell tips, some apparently emerging from secretory vesicles (**Figures 2C,D**), and adjacent to forming cell plates (**Figure 2E**). Rosette-type CSCs of seed plants contain three CESA isoforms, and it has been suggested that *CESA* diversification was a prerequisite for rosette CSC evolution (Doblin et al., 2002). However, phylogenetic analyses indicate that the *PpCESA*s are not orthologs of the functionally specialized seed plant *CESA*s and that the common ancestor of mosses and seed plants had a single *CESA*. Thus, the hetero-oligomeric CSCs of seed plants evolved from homo-oligomeric rosettes, which may still exist in *P. patens* (Roberts and Bushoven, 2007). Yet, *P. patens* could have hetero-oligomeric CSCs if they evolved independently in mosses and seed plants. Published CESA phylogenies indicate that the divergence that produced primary and secondary cell wall CESAs preceded the diversification that resulted in hetero-oligomeric CSCs (Tanaka et al., 2003; Djerbi et al., 2005; Nairn and Haselkorn, 2005; Ranik and Myburg, 2006; Roberts and Bushoven, 2007; Kumar et al., 2009; Carroll and Specht, 2011). This implies that hetero-oligomeric CSCs evolved independently from homo-oligomeric primary and secondary CSCs. Although this scenario seems unparsimonious, the theory of constructive neutral evolution recently demonstrated for the V-ATPase complex in yeast (Doolittle, 2012; Finnigan et al., 2012) postulates that multisubunit complexes, such as CSCs, are driven towards a hetero-oligomeric state. Like CSCs, the transmembrane ring of yeast V-ATPase consists of three paralogous, but non-interchangeable, protein subunits. By reconstructing the common ancestor of two of these subunits and reintroducing historical mutations, Finnigan et al. (2012) showed that a gene duplication followed by complementary loss of specific interfaces involved in protein–protein interactions was responsible for the evolution of subunits that differ only in the

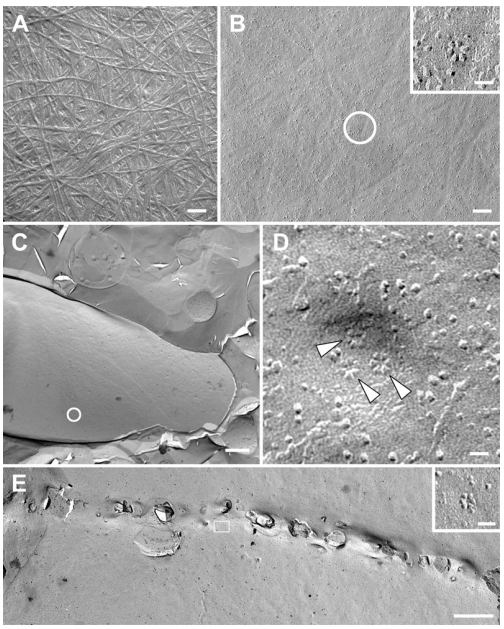


FIGURE 2 | Transmission electron micrographs of extracted and shadowed cell walls and platinum–carbon freeze fracture replicas from *Physcomitrella patens* protonemal filaments. (A) Cell wall extracted with 1 N NaOH and shadowed with platinum–carbon, showing microfibrils (bar = 100 nm). **(B)** Plasma membrane fracture with microfibril impressions and rosette-type cellulose synthesis complexes (circle and inset, bars = 100 nm for **(B)**, 20 nm for inset). **(C)** Plasma membrane fracture of a protonemal tip (bar = 2 μm). **(D)** Higher magnification view of the circled area in **D**, showing a presumed secretory vesicle containing at least one rosette-type cellulose synthesis complex (top arrowhead). The other two rosette cellulose synthesis complexes (bottom arrowheads) may have been in the plasma membrane beforehand or in the midst of secretion from the same vesicle when the cell was frozen (bar = 20 nm). **(E)** Fusion of a cell plate with the plasma membrane in a dividing protonemal tip cell with associated rosette cellulose synthesis complexes (box and inset, bars = 1 μm for **(E)**, 20 nm for inset).

positions that they occupy within the complex. In this process, a high-probability loss-of-function (i.e., the inability to interact with like subunits) is initially independent of selection, but the hetero-oligomeric condition becomes locked-in by selection as mutations accumulate. This driving of multimeric protein complexes toward increased complexity explains how the hetero-oligomeric state could have evolved independently in primary and secondary cell walls CSCs in seed plants and, possibly, in *P. patens*.

CROSS-LINKING GLYCANS

Xyloglucan has been detected in various moss species by the presence of isoprimeverose in driselase digests (Popper and Fry, 2003) and in *P. patens* by CoMPP using antibodies directed against non-fucosylated xyloglucan (Moller et al., 2007). Detailed structural analysis confirmed the absence of fucopyranosyl residues and revealed that *P. patens* xyloglucan has an XXGGG branching pattern and novel branched side chains containing galactosyluronic acid and arabinopyranosyl residues (Peña et al., 2008). Based on immunolabeling the leaves and stems are enriched in xyloglucan (Kulkarni et al., 2012). The five members of the *P. patens CSLC* family of putative xyloglucan synthases (Cocuron et al., 2007) form a clade separate from seed plant *CSLC*s (Roberts and Bushoven, 2007). The *P. patens* genome also encodes putative homologs of the *Arabidopsis* xyloglucan xylosyl transferases XXT1 and XXT2, and galactosyl transferases MUR3 and GT18 (Peña et al., 2008). Because xylopyranosyl residues of *P. patens* xyloglucan are substituted with galactosyluronic acid or arabinopyranosyl residues instead of galactopyranosyl residues, it was suggested that the *P. patens* homologs differ from MUR3/GT18 in substrate specificity (Peña et al., 2008). Although the *P. patens* genome encodes several members of GT37, which includes the *Arabidopsis* xyloglucan fucosyl transferase FUT1, sequence similarity is low (Peña et al., 2008) and the *P. patens* sequences are not in the FUT1 subclade (Del Bem and Vincentz, 2010), consistent with the lack of xyloglucan fucosylation. Differences in side chain frequency in xyloglucans extracted from protonemal and gametophore cell walls may be related to the roles of xyloglucan in tip and diffuse growth (Peña et al., 2008).

Mannans are present in walls of various bryophytes based on chemical analysis (Geddes and Wilkie, 1971; Popper and Fry, 2003) and in *P. patens* based on CoMPP, chemical analysis, and immunolabeling (Liepman et al., 2007; Moller et al., 2007; Nothnagel and Nothnagel, 2007; Lee et al., 2011). The *P. patens* genome includes three *CSLA* genes (Roberts and Bushoven, 2007), at least two of which have mannan/glucomannan synthase activity when expressed in insect cells (Liepman et al., 2007).

A recent immunolabeling study failed to detect xylan-specific epitopes in eight moss species, including *F. hygrometrica* (Carafa et al., 2005). However, xylan-specific epitopes were detected in *P. patens* by CoMPP, and sugar linkage analysis confirmed the presence of β-1,4-linked xylan (Moller et al., 2007). A detailed structural analysis revealed glucuronoxylan with a 1,4-linked β-D-xylan backbone and α-D-glucosyluronic acid side chains, but no 4-*O*-methyl-α-D-glucosyluronic acid side chains, indicating that *O*-methylation of glucosyluronic acid evolved after divergence of mosses and vascular plants. The *P. patens* xylan is also unusual in having pairs of side chains separated by a single xylosyl residue and possibly an unidentified pentosyl residue. Xylans from both seedless vascular plants and *P. patens* lack the reducing-end sequence characteristic of seed plant xylans (Kulkarni et al., 2012). Xylan has been immunolocalized in leaf cells and axillary hairs in *P. patens*, but little or none was detected in the stems and protonemal filaments (Kulkarni et al., 2012). Xylan backbone synthesis in *Arabidopsis* involves members of GT43 (IRX-9, IRX-9L, IRX-14, IRX-14L) and GT47 (IRX-10, IRX-10L). The major clades containing these proteins and their putative seed plant orthologs include *P. patens* sequences (Kulkarni et al., 2012). However, the *P. patens* members of the IRX-9/IRX-9L clade may not be their direct orthologs. Biosynthesis of the xylan reducing-end sequence in *Arabidopsis* involves members of GT8 (IRX-8/GAUT-12, PARVUS/GATL-1) and GT47 (IRX7). The *P. patens* genome encodes three basal members of the subclade containing GAUT-12 and three other AtGAUTs, five basal members of the clade containing GATL1 (Yin et al., 2010), and three basal members of the subclade containing IRX7 and other *Arabidopsis* sequences (Kulkarni et al., 2012). The *P. patens* genome also has orthologs of the *GUX* genes, which encode xylan glucoronosyl transferases (Harholt et al., 2012).

Mixed-linkage β-glucans (MLGs) are apparently absent from bryophyte cell walls (Popper and Fry, 2003). The *P. patens* genome lacks members of the *CSLF* and *CSLH* families (Roberts and Bushoven, 2007), which are involved in MLG biosynthesis in cereals (Burton et al., 2006; Doblin et al., 2009; Nemeth et al., 2010; Vega-Sanchez et al., 2012), but absent in dicots. While MLGs have been detected in seedless plants, including *Equisetum* (Sørensen et al., 2008) and *Selaginella* (Harholt et al., 2012), there is no evidence that these polymers are synthesized by CSLF and CSLH proteins.

The *P. patens* genome also lacks members of the *CSLB*, *CSLE*, and *CSLG* families found in seed plants. Although the biosynthetic functions of the encoded proteins are unproven, they may be involved in cell wall polysaccharide biosynthesis (Richmond and Somerville, 2000). The *P. patens* genome includes eight *CSLD*s, which have been linked to the biosynthesis of β-1,4 glucan (Park et al., 2011) and mannan (Yin et al., 2011) in angiosperms. The use of *P. patens* as a relatively simple model organism may help to clarify CSLD function.

PECTINS

Pectin epitopes, including homogalacturonan, β-1,4-galactan, and α-1,5-arabinan were detected by CoMPP and sugar linkage analysis in *P. patens* cell walls (Moller et al., 2007). Desterified pectin and RG-I were also detected by immunofluorescence in leaves and stems of *P. patens* (Kulkarni et al., 2012). Water conducting cells of several species and hyaline cells from *Sphagnum* label preferentially with antibodies directed at the arabinosylated β-1,4-galactan epitope of RG-I (Ligrone et al., 2002; Kremer et al., 2004). The cell walls of some moss species may contain an RG-II-like polysaccharide based on the presence of 2-methyl-fucose and 2-methyl-xylose (apiose and aceric acid were not detected) and release of cross-linked borate by driselase (Matsunaga et al., 2004). However, the

polymer was not isolated in sufficient quantities for structural characterization. The *P. patens* genome encodes putative homologs of pectin biosynthesis enzymes AtGAUT1 and the RGXTs, but not AtGAUT7, QUA1, or ARAD1 (Harholt et al., 2012). Putative homologs of CMP-Kdo-synthase have also been identified, further supporting the presence of an RG-II-like polysaccharide in mosses (Matsunaga et al., 2004).

GLYCOPROTEINS

The presence and functional significance of arabinogalactan proteins (AGPs) in the cell walls of *P. patens* is supported by cross-reactivity with Yariv reagent and monoclonal antibodies, as well as growth inhibition by Yariv reagent and in AGP knockouts (Lee et al., 2005a,b; Moller et al., 2007). *Physcomitrella* AGP glycans contain unusual terminal 3-*O*-methyl-L-rhamnosyl residues in addition to the (1,3,6)-linked galactopyranosyl, terminal arabinofuranosyl and (1,4)-linked glucuronopyranosyl residues typical of angiosperm AGPs (Fu et al., 2007). Genes encoding a classical AGP, three AG peptides, and two fasciculin-like AGPs were identified in a *P. patens* EST database (Lee et al., 2005b) and an AGP was among the proteins identified in a proteomic analysis of dehydration response (Cui et al., 2012). Extensin was weakly detected in *P. patens* by CoMPP. Genome searches identified homologs of GT77 proteins implicated in extensin glycosylation (Harholt et al., 2012), but not extensin itself (Lawton and Saidasan, 2011). However, a comprehensive analysis of cell wall protein genes in *P. patens* has not been reported.

CALLOSE

Callose has been detected in mosses, including *P. patens*, by aniline blue cytochemistry (Scherp et al., 2001; Tang, 2007; Schuette et al., 2009) and CoMPP (Moller et al., 2007). As in other plants and algae, callose is involved in normal developmental processes, including cytokinesis (Scherp et al., 2001) and spore formation (Schuette et al., 2009), and it also forms in response to wounding (Abel et al., 1989; Scherp et al., 2001; Tang, 2007). The association of callose with different developmental stages and stimuli is not unexpected since the *P. patens* genome contains 12 putative *Callose Synthase* (*CalS*) genes that cluster in three clades with *Arabidopsis CalS* genes (Schuette et al., 2009).

LIGNIN

Most reports of lignin in mosses have not withstood further scrutiny (Weng and Chapple, 2010; Espiñeira et al., 2011). However, the presence of lignin-like compounds in mosses is consistent with identification of *P. patens* homologs of genes encoding lignin biosynthesis enzymes (Xu et al., 2009).

CUTICLE

Although the protonemal filaments of mosses apparently lack cuticles, a hydrophobic cuticle-like layer has been reported in some moss gametophores and sporophytes (Cook and Graham, 1998; Budke et al., 2011). Although some authors have indicated that *P. patens* gametophores lack a cuticle (Liénard et al., 2008), other histochemical studies have suggested that a cuticle is present (Wyatt et al., 2008) in this species. No analysis of *P. patens* genes potentially involved in cuticle biosynthesis has been reported.

PROSPECTS

While detailed structures of *P. patens* cell wall polysaccharides are now being revealed, few of the *P. patens* glycosyl transferases have been characterized functionally. Further studies in *P. patens* along with comparative studies that seek to identify cell wall characteristics that correlate with adaptation to diverse habitats can enhance our understanding of cell wall biosynthesis and land plant evolution by elucidating the relationships among glycosyl transferase functional diversification, cell wall structural and biochemical specialization, and the roles of cell wall properties in plant adaptation.

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REFERENCES

Abel, W. O., Knebel, W., Koop, H.-U., Marienfeld, J. R., Quader, H., Reski, R., Schnepf, E., and Spörlein, B. (1989). A cytokinin-sensitive mutant of the moss, *Physcomitrella patens*, defective in chloroplast division. *Protoplasma* 152, 1–13.

Buck, W. R., and Goffinet, B. (2000). "Morphology and classification of mosses," in *Bryophyte Biology*, eds A. J. Shaw, and B. Goffinet (Cambridge: Cambridge University Press), 71–149.

Budke, J. M., Goffinet, B., and Jones, C. S. (2011). A hundred-year-old question: is the moss calyptra covered by a cuticle? A case study of *Funaria hygrometrica*. *Ann. Bot.* 107, 1279–1286.

Burton, R. A., Wilson, S. M., Hrmova, M., Harvey, A. J., Shirley, N. J., Medhurst, A., Stone, B. A., Newbigin, E. J., Bacic, A., and Fincher, G. B. (2006). Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1,3;1,4)- β-D-glucans. *Science* 311, 1940–1942.

Carafa, A., Duckett, J. G., Knox, J. P., and Ligrone, R. (2005). Distribution of cell-wall xylans in bryophytes and tracheophytes: new insights into basal interrelationships of land plants. *New Phytol.* 168, 231–240.

Carroll, A., and Specht, C. D. (2011). Understanding plant cellulose synthases through a comprehensive investigation of the cellulose synthase family sequences. *Front. Plant Sci.* 2:5. doi: 10.3389/fpls.2011.00005

Cocuron, J.-C., Lerouxel, O., Drakakaki, G., Alonso, A. P., Liepman, A. H., Keegstra, K., Raikhel, N., and Wilkerson, C. G. (2007). A gene from the cellulose synthase-like C family encodes a β-1,4 glucan synthase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8550–8555.

Cook, M. E., and Graham, L. E. (1998). Structural similarities between surface layers of selected charophycean algae and bryophytes and the cuticles of vascular plants. *Int. J. Plant Sci.* 159, 780–787.

Cove, D. (2005). The moss *Physcomitrella patens*. *Annu. Rev. Genet.* 39, 339–358.

Cui, S., Hu, J., Guo, S., Wang, J., Cheng, Y., Dang, X., Wu, L., and He, Y. (2012). Proteome analysis of *Physcomitrella patens* exposed to progressive dehydration and rehydration. *J. Exp. Bot.* 63, 711–726.

Decker, E. L., Frank, W., Sarnighausen, E., and Reski, R. (2006). Moss systems biology en route: phytohormones in *Physcomitrella* development. *Plant Biol.* 8, 397–405.

Del Bem, L. E., and Vincentz, M. G. (2010). Evolution of xyloglucan-related genes in green plants. *BMC Evol. Biol.* 10, 341. doi: 10.1186/1471-2148-10-341

Djerbi, S., Lindskog, M., Arvestad, L., Sterky, F., and Teeri, T. T. (2005). The genome sequence of black cottonwood (*Populus trichocarpa*) reveals 18 conserved cellulose synthase (*CesA*) genes. *Planta* 221, 739–746.

Doblin, M. S., Kurek, I., Jacob-Wilk, D., and Delmer, D. P. (2002). Cellulose

biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol.* 43, 1407–1420.

Doblin, M. S., Pettolino, F. A., Wilson, S. M., Campbell, R., Burton, R. A., Fincher, G. B., Newbigin, E., and Bacic, A. (2009). A barley cellulose synthase-like *CSLH* gene mediates (1,3;1,4)- β-D-glucan synthesis in transgenic *Arabidopsis. Proc. Natl. Acad. Sci. U.S.A.* 106, 5996–6001.

Doolittle, W. F. (2012). Evolutionary biology: a ratchet for protein complexity. *Nature* 481, 270–271.

Espiñeira, J. M., Novo Uzal, E., Gómez Ros, L. V., Carrión, J. S., Merino, F., Ros Barceló, A., and Pomar, F. (2011). Distribution of lignin monomers and the evolution of lignification among lower plants. *Plant Biol.* 13, 59–68.

Finnigan, G. C., Hanson-Smith, V., Stevens, T. H., and Thornton, J. W. (2012). Evolution of increased complexity in a molecular machine. *Nature* 481, 360–364.

Fu, H., Yadav, M. P., and Nothnagel, E. A. (2007). *Physcomitrella patens* arabinogalactan proteins contain abundant terminal 3-*O*-methyl-L-rhamnosyl residues not found in angiosperms. *Planta* 226, 1511–1524.

Geddes, D. S., and Wilkie, K. C. B. (1971). Hemicelluloses from the stem tissues of the aquatic moss *Fontinalis antipyretica. Carbohydr. Res.* 18, 333–335.

Goss, C. A., Brockmann, D. J., Bushoven, J. T., and Roberts, A. W. (2012). A *cellulose synthase* (*CESA*) gene essential for gametophore morphogenesis in the moss *Physcomitrella patens. Planta* 235, 1355–1367.

Harholt, J., Sørensen, I., Fangel, J., Roberts, A., Willats, W. G. T., Scheller, H. V., Petersen, B. L., Banks, J. A., and Ulvskov, P. (2012). The glycosyltransferase repertoire of the spikemoss *Selaginella moellendorffii* and a comparative study of its cell wall. *PLoS ONE* 7, e35846. doi: 10.1371/journal.pone.0035846

Hebant, C. (1977). *The Conducting Tissues of Bryophytes.* Vaduz: J. Cramer.

Knox, J. P. (2008). Revealing the structural and functional diversity of plant cell walls. *Curr. Opin. Plant Biol.* 11, 308–313.

Kremer, C., Pettolino, F., Bacic, A., and Drinnan, A. (2004). Distribution of cell wall components in *Sphagnum* hyaline cells and in liverwort and hornwort elaters. *Planta* 219, 1023–1035.

Kulkarni, A. R., Peña, M. J., Avci, U., Mazumder, K., Urbanowicz, B. R., Pattathil, S., Yin, Y., O'Neill, M. A., Roberts, A. W., Hahn, M. G., Xu, Y., Darvill, A. G., and York, W. S. (2012). The ability of land plants to synthesize glucuronoxylans predates the evolution of tracheophytes. *Glycobiology* 22, 439–451.

Kumar, M., Thammannagowda, S., Bulone, V., Chiang, V., Han, K.-H., Joshi, C. P., Mansfield, S. D., Mellerowicz, E., Sundberg, B., Teeri, T., and Ellis, B. E. (2009). An update on the nomenclature for the cellulose synthase genes in *Populus. Trends Plant Sci.* 14, 248–254.

Lawton, M. A., and Saidasan, H. (2011). "Cell wall genomics in the recombinogenic moss *Physcomitrella patens*," in *Routes to Cellulosic Ethanol*, eds M. S. Buckeridge, and G. H. Goldman (Berlin: Springer), 241–261.

Lee, K. J. D., Knight, C. D., and Knox, J. P. (2005a). *Physcomitrella patens*: a moss system for the study of plant cell walls. *Plant Biosyst.* 139, 16–19.

Lee, K. J. D., Sakata, Y., Mau, S.-L., Pettolino, F., Bacic, A., Quatrano, R. S., Knight, C. D., and Knox, J. P. (2005b). Arabinogalactan proteins are required for apical cell extension in the moss *Physcomitrella patens. Plant Cell* 17, 3051–3065.

Lee, K. J. D., Marcus, S. E., and Knox, J. P. (2011). Cell wall biology: perspectives from cell wall imaging. *Mol. Plant* 4, 212–219.

Liénard, D., Durambur, G., Kiefer-Meyer, M.-C., Nogué, F., Menu-Bouaouiche, L., Charlot, F., Gomord, V., and Lassalles, J.-P. (2008). Water transport by aquaporins in the extant plant *Physcomitrella patens. Plant Physiol.* 146, 1207–1218

Liepman, A. H., Nairn, C. J., Willats, W. G. T., Sørensen, I., Roberts, A. W., and Keegstra, K. (2007). Functional genomic analysis supports conservation of function among *Cellulose synthase-like A* gene family members and suggests diverse roles of mannans in plants. *Plant Physiol.* 143, 1881–1893.

Ligrone, R., Vaughn, K. C., Renzaglia, K. S., Knox, J. P., and Duckett, J. G. (2002). Diversity in the distribution of polysaccharide and glycoprotein epitopes in the cell walls of bryophytes: new evidence for the multiple evolution of water-conducting cells. *New Phytol.* 156, 491–508.

Matsunaga, T., Ishii, T., Matsumoto, S., Higuchi, M., Darvill, A., Albersheim, P., and O'Neill, M. A. (2004). Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. Implications for the evolution of vascular plants. *Plant Physiol.* 134, 339–351.

Menand, B., Calder, G., and Dolan, L. (2007). Both chloronemal and caulonemal cells expand by tip growth in the moss *Physcomitrella patens. J. Exp. Bot.* 58, 1843–1849.

Mishler, B. D., and Oliver, M. J. (2009). "Putting *Physcomitrella patens* on the tree of life: the evolution and ecology of mosses," in *The Moss Physcomitrella patens*, eds C. D. Knight, P. F. Perroud, and D. Cove (Chichester: Blackwell), 1–15.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A., and Willats, W. G. T. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *Plant J.* 50, 1118–1128.

Nairn, C. J., and Haselkorn, T. (2005). Three loblolly pine *CesA* genes expressed in developing xylem are orthologous to secondary cell wall *CesA* genes of angiosperms. *New Phytol.* 166, 907–915.

Nemeth, C., Freeman, J., Jones, H. D., Sparks, C., Pellny, T. K., Wilkinson, M. D., Dunwell, J., Andersson, A. A. M., Åman, P., Guillon, F., Saulnier, L., Mitchell, R. A. C., and Shewry, P. R. (2010). Down-regulation of the *CSLF6* gene results in decreased (1,3;1,4)- β-D-glucan in endosperm of wheat. *Plant Physiol.* 152, 1209–1218.

Nothnagel, A. L., and Nothnagel, E. A. (2007). Primary cell wall structure in the evolution of land plants. *J. Integr. Plant Biol.* 49, 1271–1278.

Park, S., Szumlanski, A. L., Gu, F., Guo, F., and Nielsen, E. (2011). A role for CSLD3 during cell-wall synthesis in apical plasma membranes of tip-growing root-hair cells. *Nat. Cell Biol.* 13, 973–980.

Peña, M. J., Darvill, A. G., Eberhard, S., York, W. S., and O'Neill, M. A. (2008). Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants. *Glycobiology* 18, 891–904.

Popper, Z. A., and Fry, S. C. (2003). Primary cell wall composition of bryophytes and charophytes. *Ann. Bot.* 91, 1–12.

Ranik, M., and Myburg, A. A. (2006). Six new cellulose synthase genes from *Eucalyptus* are associated with primary and secondary cell wall biosynthesis. *Tree Physiol.* 26, 545–556.

Reiss, H. D., Schnepf, E., and Herth, W. (1984). The plasma membrane of the *Funaria* caulonema tip cell: morphology and distribution of particle rosettes, and the kinetics of cellulose synthesis. *Planta* 160, 428–435.

Rensing, S. A., Lang, D., Zimmer, A. D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P. F., Lindquist, E. A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W. B., Barker, E., Bennetzen, J. L., Blankenship, R., Cho, S. H., Dutcher, S. K., Estelle, M., Fawcett, J. A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K. A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D. R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P. J., Sanderfoot, A., Schween, G., Shiu, S.-H., Stueber, K., Theodoulou, F. L., Tu, H., Van De Peer, Y., Verrier, P. J., Waters, E., Wood, A., Yang, L., Cove, D., Cuming, A. C., Hasebe, M., Lucas, S., Mishler, B. D., Reski, R., Grigoriev, I. V., Quatrano, R. S., and Boore, J. L. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319, 64–69.

Richmond, T. A., and Somerville, C. R. (2000). The cellulose synthase superfamily. *Plant Physiol.* 124, 495–498.

Roberts, A. W., and Bushoven, J. T. (2007). The cellulose synthase (*CESA*) gene superfamily of the moss *Physcomitrella patens. Plant Mol. Biol.* 63, 207–219.

Roberts, A. W., Dimos, C., Budziszek, M. J., Goss, C. A., and Lai, V. (2011). "Knocking out the wall: protocols for gene targeting in *Physcomitrella patens*," in *The Plant Cell Wall: Methods and Protocols, Methods in Molecular Biology*, ed. Z. A. Popper (Berlin: Springer), 273–290.

Rudolph, U., Gross, H., and Schnepf, E. (1989). Investigations of the turnover of the putative cellulose-synthesizing particle "rosettes" within the plasma membrane of *Funaria hygrometrica* protonema cells. II. Rosette structure and the effects of cycloheximide, actinomycin D, 2,6-dichlorobenzonitrile, biofluor, heat shock, and plasmolysis. *Protoplasma* 148, 57–69.

Rudolph, U., and Schnepf, E. (1988). Investigations of the turnover of the putative cellulose-synthesizing particle "rosettes" within the plasma membrane of *Funaria hygrometrica* protonema cells. I. Effects of monensin and cytochalasin B. *Protoplasma* 143, 63–73.

Sack, F., and Paolillo, D. J. Jr. (1983). Structure and development of walls in *Funaria* stomata. *Am. J. Bot.* 70, 1019–1030.

Scherp, P., Grotha, R., and Kutschera, U. (2001). Occurrence and phylogenetic significance of cytokinesis-related callose in green algae, bryophytes, ferns and seed plants. *Plant Cell Rep.* 20, 143–149.

Schuette, S., Wood, A. J., Geisler, M., Geisler-Lee, J., Ligrone, R., and Renzaglia, K. S. (2009). Novel localization of callose in the spores of *Physcomitrella patens* and phylogenomics of the callose synthase gene family. *Ann. Bot.* 103, 749–756.

Schumaker, K. S., and Dietrich, M. A. (1998). Hormone-induced signaling during moss development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 501–523.

Sørensen, I., Pettolino, F. A., Wilson, S. M., Doblin, M. S., Johansen, B., Bacic, A., and Willats, W. G. T. (2008). Mixed-linkage (1→3),(1→4)- β-D-glucan is not unique to the Poales and is an abundant component of *Equisetum arvense* cell walls. *Plant J.* 54, 510–521.

Tanaka, K., Murata, K., Yamazaki, M., Onosato, K., Miyao, A., and Hirochika, H. (2003). Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. *Plant Physiol.* 133, 73–83.

Tang, C.-T. C. (2007). *The Wound Response in Arabidopsis thaliana and Physcomitrella patens.* Ph.D. dissertation, Rutgers University.

Vega-Sanchez, M. E., Verhertbruggen, Y., Christensen, U., Chen, X., Sharma, V., Varanasi, P., Jobling, S. A., Talbot, M., White, R. G., Joo, M., Singh, S., Auer, M., Scheller, H. V., and Ronald, P. C. (2012). Loss of cellulose synthase-like F6 function affects mixed-linkage glucan deposition, cell wall mechanical properties and defense responses in vegetative tissues of rice. *Plant Physiol.* 159, 56–69.

Weng, J.-K., and Chapple, C. (2010). The origin and evolution of lignin biosynthesis. *New Phytol.* 187, 273–285.

Wise, H. Z., Saxena, I. M., and Brown, R. M. Jr. (2011). Isolation and characterization of the cellulose synthase genes *PpCesA6* and *PpCesA7* in *Physcomitrella patens. Cellulose* 18, 371–384.

Wyatt, H. D. M., Ashton, N. W., and Dahms, T. E. S. (2008). Cell wall architecture of *Physcomitrella patens* is revealed by atomic force microscopy. *Botany* 86, 385–397.

Xu, Z., Zhang, D., Hu, J., Zhou, X., Ye, X., Reichel, K. L., Stewart, N. R., Syrenne, R. D., Yang, X., Gao, P., Shi, W., Doeppke, C., Sykes, R. W., Burris, J. N., Bozell, J. J., Cheng, Z.-M., Hayes, D. G., Labbe, N., Davis, M., Stewart, C. N. Jr., and Yuan, J. S. (2009). Comparative genome analysis of lignin biosynthesis gene families across the plant kingdom. *BMC Bioinformatics* 10(Suppl. 11), S3. doi: 10.1186/1471-2105-10-S11-S3

Yin, L., Verhertbruggen, Y., Oikawa, A., Manisseri, C., Knierim, B., Prak, L., Jensen, J. K., Knox, J. P., Auer, M., Willats, W. G. T., and Scheller, H. V. (2011). The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal *Arabidopsis* development. *Mol. Plant* 4, 1024–1037.

Yin, Y., Chen, H., Hahn, M. G., Mohnen, D., and Xu, Y. (2010). Evolution and function of the plant cell wall synthesis-related glycosyltransferase family 8. *Plant Physiol.* 153, 1729–1746.

Yin, Y., Huang, J., and Xu, Y. (2009). The cellulose synthase superfamily in fully sequenced plants and algae. *BMC Plant Biol.* 9, 99. doi: 10.1186/1471-2229-9-99

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We are good to grow: dynamic integration of cell wall architecture with the machinery of growth

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Despite differences in cell wall composition between the type I cell walls of dicots and most monocots and the type II walls of commelinid monocots, all flowering plants respond to the same classes of growth regulators in the same tissue-specific way and exhibit the same growth physics. Substantial progress has been made in defining gene families and identifying mutants in cell wall-related genes, but our understanding of the biochemical basis of wall extensibility during growth is still rudimentary. In this review, we highlight insights into the physiological control of cell expansion emerging from genetic functional analyses, mostly in Arabidopsis and other dicots, and a few examples of genes of potential orthologous function in grass species. We discuss examples of cell wall architectural features that impact growth independent of composition, and progress in identifying proteins involved in transduction of growth signals and integrating their outputs in the molecular machinery of wall expansion.

Keywords: cell wall, cellulose, dicots, extensibility, grasses, growth, pectin, signaling

INTRODUCTION

Cell expansion integrates "loosening" of existing architecture with synthesis and deposition of new wall components. Golgi-associated syntheses of matrix polysaccharides and their subsequent secretion is coordinated closely with cellulose synthesis at the plasma membrane, which is in turn coordinated with dynamic assembly and rearrangement during wall extension (Cosgrove, 2005). Multiple signal pathways regulate wall biophysics to permit cell expansion by controlling expression of cell wall-related genes. For example, the brassinosteroid-activated transcription factor BES1 binds to promoter elements of most cellulose synthase (*CesA*) genes (Xie et al., 2011), and NAC and MYB domain-containing transcriptional factors bind to AC-elements of promoters of genes involved in polysaccharide and monolignol syntheses (Zhao and Dixon, 2011).

In elongating cells, cellulose microfibrils are laid down in helical patterns transverse to the long axis of the cell, and are separated and reoriented by tangential forces generated by turgor. Walls of grasses and those of other flowering plants interlace the microfibrils with different matrix constituents (Carpita and Gibeaut, 1993). All dicots and about one-half of all monocots have type I cell walls: a framework of cellulose microfibrils cross-linked primarily by xyloglucans (XyGs) and embedded in a complex matrix of pectic polysaccharides (McCann and Roberts, 1991; Carpita and Gibeaut, 1993); in type II cell walls of the grasses and other commelinid monocots, cellulose microfibrils are cross-linked primarily with glucuronoarabinoxylans (GAXs). Pectins are a small proportion of the matrix polymers, with GAXs providing most of the negatively charged matrix of the type II cell wall (Carpita, 1996). Structural proteins comprise up to 10% of mass of type I walls, whereas networks of phenylpropanoids are deposited in the type II wall. In contrast to other commelinids, the grasses (Poales) contain a mixed-linkage $(1 \rightarrow 3),(1 \rightarrow 4)$-β-D-glucan that is synthesized during cell expansion of grasses and hydrolyzed when growth ceases (Buckeridge et al., 2004).

Cell elongation results in more subtle changes in cell wall composition and architecture than can be revealed by chemical analyses of whole plant organs. Immuno-labeling experiments using a panel of monoclonal antibodies reveals that unique combinations of epitopes are present in highly nuanced patterns along the Arabidopsis root (Pattathil et al., 2010). Infrared spectra of cell walls of maize coleoptiles show that distinct compositional changes occur at 1.5-day intervals (McCann et al., 2007). This dynamic, cellular heterogeneity may be required for wall-modifying activities to alter biophysical properties at precise developmental stages. Tobacco leaves expressing an inducible cucumber α-expansin (*CsEXP1*) promotes leaf growth maximally at the mid-stage of leaf growth (Sloan et al., 2009).

ARCHITECTURAL CHANGES IN CELL WALLS IMPACT ANISOTROPIC GROWTH

When the structure of the cell wall is severely compromised, the consequence is a strong inhibition of organ elongation. Mutants of primary wall cellulose synthases *CesA1*, *radial swelling1* (*rsw1*), and *CesA6*, *procuste1 (prc1)*, have reduced anisotropic growth in roots and hypocotyls (Arioli et al., 1998; Fagard et al., 2000). In *prc1*, cellulose content is only reduced by about one-third, but the mutation results in a fourfold inhibition of growth (Fagard et al., 2000). Anisotropic distributions of microfibril angles deposited during the initial growth phase can account for the growth inhibition if the wall is modeled as a composite material (MacKinnon et al., 2006). Reorientation from transverse to longitudinal in *procuste* hypocotyls also results in failure to fill gaps in cellulose deposition

in some regions, possibly contributing to a tendency for cells to rupture (Anderson et al., 2010).

Mutations in genes whose functions are associated with delivery or activation of cellulose synthases, and other plasma membrane-resident or membrane-associated proteins, also result in cellulose deficiency and inhibition of elongation growth. *CELLULOSE SYNTHASE INTERACTIVE1* (*CSI1*) is a microtubule-associated protein that bridges CESA complexes and cortical microtubules, mutation of which affects movement of CESA complexes in the plasma membrane (Gu et al., 2010; Li et al., 2012). Site-directed mutations of the phosphorylation sites of CESA1 to mimic always-on or always-off states provide evidence that CESAs are direct targets of signal pathways impacting elongation (Chen et al., 2010). Mutations in a plasma membrane-associated endo-(1 → 4)-β-D-glucanase, *korrigan* in Arabidopsis (Nicol et al., 1998) and rice (Zhou et al., 2006) or in *COBRA* genes, encoding glycosylphosphatidyl inositol-anchored proteins, are cellulose-deficient and compromised in organ elongation in Arabidopsis (Schindelman et al., 2001; Roudier et al., 2005) and rice (Dai et al., 2011).

Loss of cell wall strength to resist turgor pressure is expected with cellulose deficiencies, but similar swelling phenotypes can occur in mutants unaffected in cellulose synthesis. Mutations in two UDP-sugar interconversion pathway genes encoding UDP-glucose dehydrogenases cause significant reduction of substitutions to the XyG backbone, arabinan side chains of rhamnogalacturonan (RG) I, and the apiose-containing side chains A and B of RG II (Reboul et al., 2011), with phenotypes of swollen and misshapen roots and cotyledons, and shorter hypocotyls and reproductive organs.

Xyloglucan endo-β-transglucosylases/hydrolases (XET/XTHs) catalyze the molecular grafting and/or hydrolysis of XyGs in the primary type I cell wall (Rose et al., 2002; Eklof and Brumer, 2010). Galactose-deficient *mur3* XyGs bind to cellulose *in vivo* and *in vitro* as do wild-type XyGs, but are exceptionally poor substrates for XET – a feature that correlates with cell swelling at the end of growth (Peña et al., 2004). When XyGs of different molecular sizes are fed to pea stems, large polymers of XyG cross-link cellulose microfibrils and slow growth by action of endogenous XET activity, whereas XyG oligosaccharides promote cell elongation (Takeda et al., 2002). RNAi lines with reduced levels of AtXTH18 show decreased primary root growth compared to that of wild-type (Osato et al., 2006), and AtXTH14 and AtXTH26 reduced the extension of heat-inactivated isolated cell walls under constant-load extension (Maris et al., 2009). Also, when growing roots were exposed to either recombinant XTH protein, cell elongation is reduced in a concentration-dependent manner and abnormal root hairs are formed, suggesting a role for XET activity in stiffening of the side-walls of root hairs and cells of the elongation zone.

While XET activities may be associated with maintenance of tensile strength by religating XyGs during growth, the actual stress relaxation of the walls required for growth is induced by expansins (Cosgrove, 2005). The expansin superfamily falls into two major groups, called α- and β-expansin, in both dicots and grasses, but with many more β-expansins in the grasses (Sampedro and Cosgrove, 2005). The α-expansins disrupt hydrogen bonds between polysaccharides (Cosgrove, 2000), including cellulose microfibrils in filter paper (McQueen-Mason and Cosgrove, 1994). The β-expansin clade also contains maize group-1 pollen allergens, which, unlike α-expansins, solubilize homogalacturonans (HGs) and highly arabinose-substituted GAXs from the middle lamellae of maize silks during pollen growth (Tabuchi et al., 2011).

However, modifications to pectins also impact wall mechanical properties in dicots. The *mur1* mutant of Arabidopsis is deficient in GDP-mannose dehydratase activity, resulting in the absence of fucose residues in cell wall polymers of the shoot (Bonin et al., 1997). A slight dwarfism and greatly reduced tensile strength of the floral stem suggested that the fucose-containing side-group of XyG might be important for cross-linking during growth. However, O'Neill et al. (2001) showed that the reduced stem growth and tensile strength of the *mur1* mutant is rescued to near wild-type levels by spraying plants with excess boron, thus promoting the dimerization of fucose-deficient side-chains of RG II. Ryden et al. (2003) showed that the tensile strength of *mur1* etiolated hypocotyls was about half that of wild-type but could be similarly rescued. Rescue of the *mur1* phenotype with boron alone shows that RG II dimers are load-bearing and important for cell and organ growth.

Coordinated cell growth in the context of an organ is far more complex than changing biomechanical properties of individual cell walls, and for which the biophysics of cell layers and volumes control organ form (Green, 1996). Osmotic manipulation of wall tension shows regions of "stiffening" interpreted to provide the mechanical determinants for cell patterning (Kierzkowski et al., 2012). The smooth surface of a meristem gives rise to defined undulations by asymmetric cell expansion in a few cells, and these asymmetries pre-stage the pattern of phyllotaxis (Green et al., 1996). A physical undulation induced by asymmetric application of expansin or induction of its expression is sufficient to induce an entire developmental program of organ development, changing phyllotaxis in the apical meristem or leaf shape (Fleming et al., 1997; Pien et al., 2001).

Pectins also appear to be involved in transduction of biophysical signals. HG is synthesized as a heavily methyl-esterified polymer that are de-esterified by pectin methyl esterases (PMEs) to variable extents during cell elongation. Like expansins, modification of methyl esterification of cell wall pectins is linked to organ initiation and control of the normal pattern of phyllotaxis in the apical meristem (Peaucelle et al., 2008, 2011a). Arabidopsis *PME5* is regulated by the homeodomain transcription factor BELLRINGER; in the *bellringer* mutant, PME5 activity is enhanced in the meristem (Peaucelle et al., 2011b). In contrast, *PME5* expression is down-regulated in the mutant, which results in reduced internode elongation. These data suggest a dual function for *BELLRINGER* – a repressor of *PME* in the meristem dome and an activator of *PME* in the elongating stem.

Hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) become cross-linked in walls at the cessation of growth. However, recent work demonstrates that failure to glycosylate these proteins results in defects early in cell growth. An extensin is required for proper cell plate formation during cytokinesis (Cannon et al., 2008). The Arabidopsis prolyl 4-hydroxylase (AtP4H) hydroxylates prolines of glycoproteins, such as extensins, which are *O*-glycosylated with arabinosyl and galactosyl residues by ER- and Golgi-resident glycosyltransferases (Shpak et al., 1999;

Gille et al., 2009), and then cross-linked upon delivery to the cell wall (Held et al., 2004). Further complexity in structure is revealed by expression of "glycomodules" of synthetic extensins, where variations in the extent of hydroxylation and glycosylation are observed in a cell and tissue-specific manner (Estévez et al., 2006). Blocking *O*-glycosylation, either chemically by inhibition of P4H with ethyl-3,4-dihydroxybenzoate or α,α-dipyridyl, or genetically by insertional mutagenesis, result in aberrancies in root hair elongation in Arabidopsis (Velasquez et al., 2011).

STRUCTURE/FUNCTION RELATIONSHIPS MAY BE DIFFICULT TO UNCOVER BECAUSE OF FEEDBACK/COMPENSATION MECHANISMS IN MUTANT GENOTYPES

While severe defects in wall architecture affect anisotropic growth, there is an enormous plasticity in composition and architecture that may compensate, at least in part, for architectural deficiencies. The cellulose synthesis inhibitor dichlorobenzonitrile induces tomato and tobacco culture cells to synthesize a modified type I wall of more highly cross-linked pectin and protein to replace a cellulose–xyloglucan network, whereas barley cells cross-link GAXs with a more extensive polyphenolic network to make a cellulose-free type II wall (Shedletzky et al., 1992). Arabidopsis cells habituated to grow in the cellulose synthesis inhibitor isoxaben alter their cell walls to compensate for the loss of this structural scaffold, and this response is accompanied by strong up-regulation of a glycine-rich cell-wall protein, the *CELLULOSE SYNTHASE-LIKE D5*, a trichome birefringence-like glycosyltransferase (Bischoff et al., 2010), and a putative glycosyltransferase of unknown function (Manfield et al., 2004). The *mur10* mutation in a secondary wall *CESA7* results in alterations to pectin side-group composition in primary walls in cells surrounding the vasculature of Arabidopsis (Bosca et al., 2006).

In contrast, primary wall cellulose deficiencies resulting from either mutated *CesA3* or *CesA1* result in ectopic lignification (Caño-Delgado et al., 2000, 2003). In both Arabidopsis and rice, mutations in other genes associated with cellulose synthesis, such as *korrigan* and *cobra*, can result in increased pectin content and/or ectopic lignification (Nicol et al., 1998; Sato et al., 2001; Zhou et al., 2006). In the temperature-sensitive mutant allele of *korrigan*, *altered cell wall1*, an increase in pectin content of 62% is observed when cellulose content is reduced by 60% at the restrictive temperature (Sato et al., 2001). In rice, mutation in a *COBRA*-like gene, *Brittle Culm-Like4*, causes a dwarf phenotype with fewer tillers than the wild-type (Dai et al., 2011), and an increase in levels of pectin: several *CesA* and *CslF* genes are up-regulated in the mutant compared to wild-type despite reduced cellulose content, suggesting that interference with cellulose deposition elicits a positive feedback mechanism. Two cellulose-deficient dwarf mutant alleles of *kobito1* (*kob1*), a cell wall-localized protein (Lertpiriyapong and Sung, 2003), have increased pectin content, and an increase in the ectopic deposition of both callose and lignin in dark-grown seedlings (Pagant et al., 2002).

Mutations in a XyG-specific fucosyltransferase (*mur2*) and galactosyltransferase (*mur3*) alter or eliminate the α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 2)-side group, yet the mutant plants are indistinguishable from wild-type (Vanzin et al., 2002; Madson et al., 2003). These mutants compensate for these mutations by enhancing activity of a second galactosyl transferase that adds galactose to the middle xylosyl residue (Peña et al., 2004). Although acid-growth and wall extensibility is reduced, expansins still induce extension growth in the XyG-less *xxt1/xxt2* mutant, even though they are missing the primary target of their activity (Park and Cosgrove, 2012a). While the shoots and floral stem are indistinguishable from wild-type in the *mur3* mutant, tensile strength is reduced in etiolated hypocotyls (Ryden et al., 2003; Peña et al., 2004). The reduced tensile strength was traced to a near absence of galactosyl side-groups on hypocotyl XyG (Peña et al., 2004). Cell growth is similar to wild-type, but the *mur3* hypocotyls present an abnormal swelling and bulging along with an increased diameter of both epidermal and underlying cortical cells.

We have depicted cross-bridging of cellulose microfibrils with XyGs and GAXs as the principal load-bearing interaction in our cell wall models (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). However, double mutations eliminating function of two GT34 xylosyltransferases, *XXT1* and *XXT2*, produce plants with no detectable XyG (Cavalier et al., 2008). Plants grow more slowly, are slightly dwarfed, and form short root hairs with bulging bases, but are otherwise surprisingly healthy. The reduction in XyG content slightly reduces stiffness and tensile strength of *xxt2* and *xxt1xxt2* mutant hypocotyls but does not significantly impact extensibility or organ growth. A third xylosyltransferase mutant, *xxt5*, has a phenotype similar to those observed in *xxt1xxt2* double mutants, and reduced XyG content and xylosylation of the glucan backbone (Zabotina et al., 2008). Extensibility is enhanced several-fold in the *xxt1/xxt2* mutant by treatment of tissues with endoxylanase, polygalacturonase, and other treatments that disrupt matrix polysaccharides other than XyGs, indicating that GAX and HG may functionally replace XyGs (Park and Cosgrove, 2012a). These authors conclude that the inherently high extensibility of the mutant over wild-type indicates a reinforcing role for XyGs as well as being the optimal extensibility determinant for which GAX and HG cannot completely substitute. Taken together, these observations reveal a curious paradox – loss of galactosylation of XyG impacts wall tensile strength in organ failure tests, but complete loss of XyG does not. Park and Cosgrove (2012b) also showed that creep can be induced in wild-type, but not in *xxt1/xxt2* mutant plants, by enzymes active against both cellulose and XyG; a cocktail of XyG-specific and cellulose-specific enzyme activities is not effective. These results suggest that the load-bearing connection between microfibrils and XyGs is in a relatively inaccessible region of interaction rather than the extended regions of XyG that span between microfibrils (Park and Cosgrove, 2012b).

MEDIATORS OF SIGNAL TRANSDUCTION PATHWAYS AND CELL WALL STATUS HAVE BEEN IDENTIFIED

The *Catharanthus roseus* (Cr) receptor-like kinase (RLK1) family contains 17 Arabidopsis members, of which four, *FERONIA* (*FER*), *THESEUS1* (*THE1*), *HERCULES1* (*HERK1*), and *HERK2*, are implicated in regulation of cell wall deposition and extensibility during growth (Hématy and Höfte, 2008; Steinwand and Kieber, 2010). The *the1* mutant was identified as a suppressor of the hypocotyl elongation defect observed in the CESA-defective *procuste* (Hématy et al., 2007). No visible change in growth or

development is seen in *the1* alone, but combining *the1* mutation with *herk1* or *herk2* results in decreases in petiole length and shoot growth (Guo et al., 2009a,b). The *the1:herk1* double mutant produces a severe dwarf phenotype in the loss-of-function *BRASSINOSTEROID RECEPTOR1* (*bri1*) mutant but partially suppresses the excessive cell elongation phenotype of the gain-of-function mutant of the transcription factor involved in *brassinosteroid response1*, *bes1-D* (Guo et al., 2009b). Mutations in two other Arabidopsis leucine-rich repeat (LRR)-RLKs, *FEI1* and *FEI2*, cause swollen-root phenotypes (Xu et al., 2008).

Matrix polymers other than cellulose are also involved in signal pathways. Extracellular domains of *WALL-ASSOCIATED KINASES* (*WAK*s) directly bind to pectin (Seifert and Blaukopf, 2010; Kohorn and Kohorn, 2012). Mutation of WAK2 or expression of antisense *WAK2* or *WAK4* to reduce levels of WAK proteins results in reduction in cell elongation (Lally et al., 2001; Wagner and Kohorn, 2001; Kohorn et al., 2006). Expression of genes associated with cell wall biogenesis and pathogen response is WAK2-dependent, suggesting a role in relaying pectin-based signals from the cell wall (Kohorn et al., 2009). Arabinogalactan proteins (AGPs) are highly glycosylated extracellular proteins anchored to the plasma membrane without kinase activities. Exogenous AGP induces somatic embryogenesis in carrot cells (van Hengel et al., 2001), and apical cell elongation in moss depends on a functional AGP (Lee et al., 2005). The Arabidopsis *fasciclin-like arabinogalactan1* (*fla*1) mutant reduces shoot formation in callus regeneration (Johnson et al., 2011), and mutation of an *AtAGP19* results in reduction of hypocotyl elongation, but, unlike mutations in structural elements, the reduced growth does not result from changes in numbers of cells, cell width, numbers of layers or hypocotyl diameter (Yang et al., 2007).

PROSPECTS

Despite differences in the structural components of plant cell walls of dicots and grasses, the architectural principles of their construction are similar, giving rise to biophysical properties that underpin common mechanisms of growth. Perturbations to wall architecture, by altering cellulose synthesis and orientation, cross-linking glycan substitution or methyl esterification of the pectin matrix, reveal sensing mechanisms that result in feedback to other biosynthetic pathways. While some candidate components of the sensing mechanisms are receptor kinases or arabinogalactan proteins, a major challenge will be untangling the direct responses of cells to signal transduction mechanisms from the many indirect effects of a life-long deficiency in mutant genotypes. Establishing systems for inducible timing of wall perturbations, introduced by interference with wall synthesis or signal pathways, is a promising approach to unravel the direct integration of growth signals, wall architecture, and biophysical mechanisms of growth.

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REFERENCES

Anderson, C. T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. *Plant Physiol.* 152, 787–796.

Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998). Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science* 279, 717–720.

Bischoff, V., Nita, S., Neumetzler, L., Schindelasch, D., Urbain, A., Eshed, R., Persson, S., Delmer, D., and Scheible, W. R. (2010). *TRICHOME BIREFRINGENCE* and its homolog At5g01360 encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis*. *Plant Physiol.* 153, 590–602.

Bonin, C. P., Potter, I., Vanzin, G. F., and Reiter, W. D. (1997). The *MUR1* gene of *Arabidopsis thaliana* encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de novo synthesis of GDP-L-fucose. *Proc. Natl. Acad. Sci. U.S.A.* 94, 2085–2090.

Bosca, S., Barton, C. J., Taylor, N. G., Ryden, P., Neumetzler, L., Pauly, M., Roberts, K., and Seifert, G. J. (2006). Interactions between MUR10/CesA7-dependent secondary cellulose biosynthesis and primary cell wall structure. *Plant Physiol.* 142, 1353–1363.

Buckeridge, M. S., Rayon, C., Urbanowicz, B., Tine, M. A. S., and Carpita, N. C. (2004). Mixed linkage (1 → 3),(1 → 4)-β-D-glucans of grasses. *Cereal Chem.* 81, 115–127.

Cannon, M. C., Terneus, K., Hall, Q., Tan, L., Wang, Y., Wegenhart, B. L., Chen, L., Lamport, D. T., Chen, Y., and Kieliszewski, M. J. (2008). Self-assembly of the plant cell wall requires an extensin scaffold. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2226–2231.

Caño-Delgado, A. I., Metzlaff, K., and Bevan, M. W. (2000). The *eli1* mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. *Development* 127, 3395–3405.

Caño-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J.* 34, 351–362.

Carpita, N. C. (1996). Structure and biogenesis of the cell walls of grasses. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 445–476.

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Chen, S., Ehrhardt, D. W., and Somerville, C. R. (2010). Mutations of cellulose synthase (CESA1) phosphorylation sites modulate anisotropic cell expansion and bidirectional mobility of cellulose synthase. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17188–17193.

Cosgrove, D. J. (2000). Loosening of plant cell walls by expansins. *Nature* 407, 321–326.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nature Rev. Mol. Cell Biol.* 6, 850–861.

Dai, X., You, C., Chen, G., Li, X., Zhang, Q., and Wu, C. (2011). *OsBC1L4* encodes a COBRA-like protein that affects cellulose synthesis in rice. *Plant Mol. Biol.* 75, 333–345.

Eklof, J. M., and Brumer, H. (2010). The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* 153, 456–466.

Estévez, J. M., Kieliszewski, M. J., Khitrov, N., and Somerville, C. (2006). Characterization of synthetic hydroxyproline-rich proteoglycans with arabinogalactan protein and extensin motifs in *Arabidopsis*. *Plant Physiol.* 142, 458–470.

Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., McCann, M., Rayon, C., Vernhettes, S., and Höfte, H. (2000). *PROCUSTE1* encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. *Plant Cell* 12, 2409–2423.

Fleming, A. J., McQueen-Mason, S., Mandel, T., and Kuhlemeier, C. (1997). Induction of leaf primordia

by the cell wall protein expansin. *Science* 276, 1415–1418.

Gille, S., Haensel, U., Ziemann, M., and Pauly, M. (2009). Identification of plant cell wall mutants by means of a forward chemical genetic approach using hydrolases. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14699–14704.

Green, P. B. (1996). Expression of form and pattern in plants – A role for biophysical fields. *Sem. Cell Dev. Biol.* 7, 903–911.

Green, P. B., Steele, C. S., and Rennich, S. C. (1996). Phyllotactic patterns: a biophysical mechanism for their origin. *Ann. Bot.* 77, 515–527.

Gu, Y., Kaplinsky, N., Bringmann, M., Cobb, A., Carroll, A., Sampathkumar, A., Baskin, T. I., Persson, S., and Somerville, C. R. (2010). Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12866–12871.

Guo, H., Li, L., Ye, H., Yu, X., Algreen, A., and Yin, Y. (2009a). Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 7648–7653.

Guo, H., Ye, H., Li, L., and Yin, Y. (2009b). A family of receptor-like kinases are regulated by BES1 and involved in plant growth in *Arabidopsis thaliana*. *Plant Signal. Behav.* 4, 784–786.

Held, M. A., Tan, L., Kamyab, A., Hare, M., Shpak, E., and Kieliszewski, M. J. (2004). Di-isodityrosine is the intermolecular cross-link of isodityrosine-rich extensin analogs cross-linked in vitro. *J. Biol. Chem.* 279, 55474–55482.

Hématy, K., and Höfte, H. (2008). Novel receptor kinases involved in growth regulation. *Curr. Opin. Plant Biol.* 11, 321–328.

Hématy, K., Sado, P. E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S., Renou, J. P., and Höfte, H. (2007). A receptor-like kinase mediates the response of *Arabidopsis* cells to the inhibition of cellulose synthesis. *Curr. Biol.* 17, 922–931.

Johnson, K. L., Kibble, N. A. J., Bacic, A., and Schultz, C. J. (2011). A fasciclin-like arabinogalactan-protein (FLA) mutant of *Arabidopsis thaliana*, *fla1*, shows defects in shoot regeneration. *PLoS ONE* 6, e25154. doi: 10.1371/journal.pone.0025154

Kierzkowski, D., Nakayama, N., Routier-Kierzkowski, A.-L., Weber, A., Bayer, E., Schorderet, M., Reinhardt, D., Kuhlemeier, C., and Smith, R. C. (2012). Elastic domains regulate growth and organogenesis in the plant shoot apical meristem. *Science* 335, 1096–1099.

Kohorn, B. D., and Kohorn, S. L. (2012). The cell wall-associated kinases, WAKS, as pectin receptors. *Front. Plant Sci.* 3:88. doi: 10.3389/fpls.2012.00088

Kohorn, B. D., Johansen, S., Shishido, A., Todorova, T., Martinez, R., Defeo, E., and Obregon, P. (2009). Pectin activation of MAP kinase and gene expression is WAK2 dependent. *Plant J.* 60, 974–982.

Kohorn, B. D., Kobayashi, M., Johansen, S., Riese, J., Huang, L. F., Koch, K., Fu, S., Dotson, A., and Byers, N. (2006). An *Arabidopsis* cell wall-associated kinase required for invertase activity and cell growth. *Plant J.* 46, 307–316.

Lally, D., Ingmire, P., Tong, H. Y., and He, Z. H. (2001). Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. *Plant Cell* 13, 1317–1331.

Lee, K. J. D., Sakata, Y., Mau, S.-L., Pettolino, F., Bacic, A., Quatrano, R. S., Knight, C. D., and Knox, J. P. (2005). Arabinogalactan proteins are required for apical cell extension in the moss *Physcomitrella patens*. *Plant Cell* 17, 3051–3065.

Lertpiriyapong, K., and Sung, Z. R. (2003). The *elongation defective1* mutant of *Arabidopsis* is impaired in the gene encoding a serine-rich secreted protein. *Plant Mol. Biol.* 53, 581–595.

Li, S., Lei, L., Somerville, C. R., and Gu, Y. (2012). Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 185–190.

MacKinnon, I. M., Sturcova, A., Sugimoto-Shirasu, K., His, I., McCann, M. C., and Jarvis, M. C. (2006). Cell-wall structure and anisotropy in *procuste*, a cellulose synthase mutant of *Arabidopsis thaliana*. *Planta* 224, 438–448.

Madson, M., Dunand, C., Li, X., Verma, R., Vanzin, G. F., Caplan, J., Shoue, D. A., Carpita, N. C., and Reiter, W. D. (2003). The *MUR3* gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. *Plant Cell* 15, 1662–1670.

Manfield, I. W., Orfila, C., McCartney, L., Harholt, J., Bernal, A. J., Scheller, H. V., Gilmartin, P. M., Mikkelsen, J. D., Knox, J. P., and Willats, W. G. T. (2004). Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *Plant J.* 40, 260–275.

Maris, A., Suslov, D., Fry, S. C., Verbelen, J. P., and Vissenberg, K. (2009). Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis* and their effect on root growth and cell wall extension. *J. Exp. Bot.* 60, 3959–3972.

McCann, M. C., Defernez, M., Urbanowicz, B. R., Tewari, J. C., Langewisch, T., Olek, A., Wells, B., Wilson, R. H., and Carpita, N. C. (2007). Neural network analyses of infrared spectra for classifying cell wall architectures. *Plant Physiol.* 143, 1314–1326.

McCann, M. C., and Roberts, K. (1991). "Architecture of the primary cell wall," in *The Cytoskeletal Basis of Plant Growth and Form*, ed. C. W. Lloyd. (London: Academic Press), 109–129.

McQueen-Mason, S., and Cosgrove, D. J. (1994). Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6574–6578.

Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H., and Höfte, H. (1998). A plasma membrane-bound putative endo-1,4-β-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J.* 17, 5563–5576.

O'Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001). Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 294, 846–849.

Osato, Y., Yokoyama, R., and Nishitani, K. (2006). A principal role for AtXTH18 in *Arabidopsis thaliana* root growth: a functional analysis using RNAi plants. *J. Plant Res.* 119, 153–162.

Pagant, S., Bichet, A., Sugimoto, K., Lerouxel, O., Desprez, T., McCann, M., Lerouge, P., Vernhettes, S., and Höfte, H. (2002). KOBITO1 encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in Arabidopsis. *Plant Cell* 14, 2001–2013.

Park, Y. B., and Cosgrove, D. J. (2012a). Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1/xxt2* mutant of *Arabidopsis*. *Plant Physiol.* 158, 465–475.

Park, Y. B., and Cosgrove, D. J. (2012b). A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. *Plant Physiol.* 158, 1933–1943.

Pattathil, S., Avci, U., Baldwin, D., Swennes, A. G., McGill, J. A., Popper, Z., Bootten, T., Albert, A., Davis, R. H., Chennareddy, C., Dong, R., O'Shea, B., Rossi, R., Leoff, C., Freshour, G., Narra, R., O'Neil, M., York, W. S., and Hahn, M. G. (2010). A comprehensive toolkit of plant cell wall glycan-directed monoclonal antibodies. *Plant Physiol.* 153, 514–525.

Peaucelle, A., Louvet, R., Johansen, J. N., Hoefte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). *Arabidopsis* phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. *Curr. Biol.* 18, 1943–1948.

Peaucelle, A., Louvet, R., Johansen, J. N., Salsac, F., Morin, H., Fournet, F., Belcram, K., Gillet, F., Höfte, H., Laufs, P., Mouille, G., and Pelloux, J. (2011a). The transcription factor BELLRINGER modulates phyllotaxis by regulating the expression of a pectin methylesterase in Arabidopsis. *Development* 138, 4733–4741.

Peaucelle, A., Braybrook, S. A., Le Guillou, L., Bron, E., Kuhlemeier, C., and Höfte, H. (2011b). Pectin-induced changes in cell wall mechanics underlie organ initiation in Arabidopsis. *Curr. Biol.* 21, 1720–1726.

Peña, M. J., Ryden, P., Madson, M., Smith, A. C., and Carpita, N. C. (2004). The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in Arabidopsis during growth. *Plant Physiol.* 134, 443–451.

Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C., and Fleming, A. (2001). Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11812–11817.

Reboul, R., Geserick, C., Pabst, M., Frey, B., Wittmann, D., Luetz-Meindl, U., Leonard, R., and Tenhaken, R. (2011). Down-regulation of UDP-glucuronic acid biosynthesis leads to swollen plant cell walls and severe developmental defects associated with changes in pectic polysaccharides. *J. Biol. Chem.* 286, 39982–39992.

Rose, J. K. C., 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.

Roudier, F., Fernandez, A. G., Fujita, M., Himmelspach, R., Borner, G.

H., Schindelman, G., Song, S., Baskin, T. I., Dupree, P., Wasteneys, G. O., and Benfey, P. N. (2005). COBRA, an *Arabidopsis* extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. *Plant Cell* 17, 1749–1763.

Ryden, P., Sugimoto-Shirasu, K., Smith, A. C., Findlay, K., Reiter, W. D., and McCann, M. C. (2003). Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. *Plant Physiol.* 132, 1033–1040.

Sampedro, J., and Cosgrove, D. J. (2005). The expansin superfamily. *Genome Biol.* 6, 242–250.

Sato, S., Kato, T., Kakegawa, K., Ishii, T., Liu, Y. G., Awano, T., Takabe, K., Nishiyama, Y., Kuga, S., Sato, S., Nakamura, Y., Tabata, S., and Shibata, D. (2001). Role of the putative membrane-bound endo-1,4-β-glucanase KORRIGAN in cell elongation and cellulose synthesis in *Arabidopsis thaliana*. *Plant Cell Physiol.* 42, 251–263.

Schindelman, G., Morikami, A., Jung, J., Baskin, T. I., Carpita, N. C., Derbyshire, P., McCann, M. C., and Benfey, P. N. (2001). *COBRA* encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. *Genes Dev.* 15, 1115–1127.

Seifert, G. J., and Blaukopf, C. (2010). Irritable walls: the plant extracellular matrix and signaling. *Plant Physiol.* 153, 467–478.

Seifert, G. J., and Blaukopf, C. (2010). Irritable walls: the plant extracellular matrix and signaling. *Plant Physiol.* 153, 467–478.

Shedletzky, E., Shmuel, M., Trainin, T., Kalman, S., and Delmer, D. (1992). Cell-wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-dichlorobenzonitrile – A comparison between two dicotyledonous plants and a gramineous monocot. *Plant Physiol.* 100, 120–130.

Shpak, E., Leykam, J. F., and Kieliszewski, M. J. (1999). Synthetic genes for glycoprotein design and the elucidation of hydroxyproline-*O*-glycosylation codes. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14736–14741.

Sloan, J., Backhaus, A., Malinowski, R., McQueen-Mason, S., and Fleming, A. J. (2009). Phased control of expansin activity during leaf development identifies a sensitivity window for expansin-mediated induction of leaf growth. *Plant Physiol.* 151, 1844–1854.

Steinwand, B. J., and Kieber, J. J. (2010). The role of receptor-like kinases in regulating cell wall function. *Plant Physiol.* 153, 479–484.

Tabuchi, A., Li, L. C., and Cosgrove, D. J. (2011). Matrix solubilization and cell wall weakening by β-expansin (group-1 allergen) from maize pollen. *Plant J.* 68, 546–559.

Takeda, T., Furuta, Y., Awano, T., Mizuno, K., Mitsuishi, Y., and Hayashi, T. (2002). Suppression and acceleration of cell elongation by integration of xyloglucans in pea stem segments. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9055–9060.

van Hengel, A. J., Tadesse, Z., Immerzeel, P., Schols, H., van Kammen, A., and de Vries, S. C. (2001). *N*-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. Plant Physiol. 125, 1880–1890.

Vanzin, G. F., Madson, M., Carpita, N. C., Raikhel, N. V., Keegstra, K., and Reiter, W. D. (2002). The *mur2* mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3340–3345.

Velasquez, S. M., Ricardi, M. M., Dorosz, J. G., Fernandez, P. V., Nadra, A. D., Pol-Fachin, L., Egelund, J., Gille, S., Harholt, J., Ciancia, M., Verli, H., Pauly, M., Bacic, A., Olsen, C. E., Ulvskov, P., Petersen, B. L., Somerville, C., Iusem, N. D., and Estevez, J. M. (2011). *O*-Glycosylated cell wall proteins are essential in root hair growth. *Science* 332, 1401–1403.

Wagner, T. A., and Kohorn, B. D. (2001). Wall-associated kinases are expressed throughout plant development and are required for cell expansion. *Plant Cell* 13, 303–318.

Xie, L., Yang, C., and Wang, X. (2011). Brassinosteroids can regulate cellulose biosynthesis by controlling the expression of *CESA* genes in *Arabidopsis*. *J. Exp. Bot.* 62, 4495–4506.

Xu, S. L., Rahman, A., Baskin, T. I., and Kieber, J. J. (2008). Two leucine-rich repeat receptor kinases mediate signaling, linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. *Plant Cell* 20, 3065–3079.

Yang, J., Sardar, H. S., McGovern, K. R., Zhang, Y. Z., and Showalter, A. M. (2007). A lysine-rich arabinogalactan protein in *Arabidopsis* is essential for plant growth and development, including cell division and expansion. *Plant J.* 49, 629–640.

Zabotina, O. A., van de Ven, W. T. G., Freshour, G., Drakakaki, G., Cavalier, D., Mouille, G., Hahn, M. G., Keegstra, K., and Raikhel, N. V. (2008). Arabidopsis *XXT5* gene encodes a putative α-1,6-xylosyltransferase that is involved in xyloglucan biosynthesis. *Plant J.* 56, 101–115.

Zhao, Q., and Dixon, R. A. (2011). Transcriptional networks for lignin biosynthesis: more complex than we thought? *Trends Plant Sci.* 16, 227–233.

Zhou, H. L., He, S. J., Cao, Y. R., Chen, T., Du, B. X., Chu, C. C., Zhang, J. S., and Chen, S. Y. (2006). OsGLU1, a putative membrane-bound endo-1,4-β-D-glucanase from rice, affects plant internode elongation. *Plant Mol. Biol.* 60, 137–151.

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Comparative structure and biomechanics of plant primary and secondary cell walls

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Recent insights into the physical biology of plant cell walls are reviewed, summarizing the essential differences between primary and secondary cell walls and identifying crucial gaps in our knowledge of their structure and biomechanics. Unexpected parallels are identified between the mechanism of expansion of primary cell walls during growth and the mechanisms by which hydrated wood deforms under external tension. There is a particular need to revise current "cartoons" of plant cell walls to be more consistent with data from diverse approaches and to go beyond summarizing limited aspects of cell walls, serving instead as guides for future experiments and for the application of new techniques.

Keywords: cellulose, creep, deformation, growth, primary cell walls, secondary cell walls, xyloglucan

INTRODUCTION

Primary and secondary cell walls are microfibril-based nanocomposites that differ in the arrangement, mobility and structure of matrix polymers, the higher-order organization of microfibrils into bundles and discrete lamellae, their rheological and mechanical properties, and their roles in the life of the plant. Wall structure has sometimes been compared with that of fiberglass – a plastic matrix reinforced by glass-fibers – but this analogy fails to account for the complex rheological behavior of plant walls. In this mini-review we summarize recent insights linking the molecular organization of cell walls with their mechanical and rheological properties.

Primary cell walls are synthesized during growth and typically are relatively thin, pliant, highly hydrated structures. The primary wall must be *strong* to withstand the tensile forces arising from turgor pressure, *extensible* to allow wall stress relaxation which motivates cell water uptake and physical enlargement of the cell (Hamant and Traas, 2010), and *incorporative*, meaning capable of linking newly deposited wall polymers into the load-bearing structure. These properties partly derive from the physical structure of the wall, but they also involve dynamic actions by expansin and xyloglucan endotransglycosylase/hydrolase (Cosgrove, 2005; Eklof and Brumer, 2010).

Secondary cell walls provide strength and rigidity in plant tissues that have ceased growing. Any tall terrestrial plant requires stems with bending strength and with water-conducting tissues that can withstand negative pressures (Koch et al., 2004; Speck and Burgert, 2011). Secondary walls therefore need compressive as well as tensile strength, but not extensibility. Nevertheless, under some conditions they can undergo various deformation processes that resemble, to some degree, primary cell wall growth.

STRUCTURE AND BIOMECHANICS OF GROWING WALLS

Primary walls are comprised of 15–40% cellulose, 30–50% pectic polysaccharides, and 20–30% xyloglucans and lesser amounts of arabinoxylans and structural proteins, on a dry weight basis, structured into one or more lamellae. The pectic polysaccharides are particularly important for wall hydration, which is essential for the slippage and separation of cellulose microfibrils during expansive growth. Notable deviations from this generic composition include the primary walls of:

- grasses, where arabinoxylans constitute most of the matrix; mixed-linkage glucans transiently comprise 10–20% of wall mass (Carpita et al., 2001; Gibeaut et al., 2005);
- celery and sugar beet parenchyma, which are rich in cellulose and pectin, but have little hemicellulose (Thimm et al., 2002; Zykwinska et al., 2007b).

Understanding how mechanical performance emerges from molecular structure – particularly the mechanism of cell wall expansion – has been a key *raison d'être* of primary cell wall models for decades (Keegstra et al., 1973; Preston, 1974; Cosgrove, 2005), yet despite significant progress we still have a long way to go to achieve the "consistency of molecular structure with the physical properties of the walls during growth" (Carpita and Gibeaut, 1993).

Studies of the viscoelastic properties of primary walls – summarized in numerous reviews (Preston, 1974; Cosgrove, 1993; Geitmann, 2010) – show that unaided viscoelastic extension of walls decays rapidly, amounting to a few % extension, whereas in the living plant it continues for hours or days and results in extensions of 50–100% or more. Normal cell wall expansion is not a simple result of the wall's constitutive properties but results from continuous action by the cell or by wall-associated proteins.

It follows that measures of wall viscoelasticity may correlate with wall structure at any given instant, but these physical measures generally do not encompass this active aspect of wall extensibility, which has sometimes been described as chemorheological creep, meaning polymer creep that depends on chemical or enzymatic modification (Ray and Ruesink, 1962). The gap between *in vitro* and *in vivo* extensibility is at least partly filled by the action of expansins, which induce stress relaxation and sustained creep of cell walls (Sampedro and Cosgrove, 2005). Although we know expansin structure in atomistic detail (Yennawar et al., 2006), the details of its loosening action on the wall remain enigmatic, in part because our understanding of the molecular architecture of the primary wall is incomplete.

CONVENTIONAL VIEW OF XYLOGLUCAN'S ROLE IN WALL MECHANICS

According to current depictions of primary walls, cellulose microfibrils are coated by xyloglucans and tethered by them to form a load-bearing network, with pectins functioning as a co-extensive, space-filling matrix that separates the microfibrils. This "tethered network model" (**Figure 1A**) might exhibit turgor-driven yield and creep resembling real primary cell walls if xyloglucan binding was dynamic and reversible (Veytsman and Cosgrove, 1998; Dyson and Jensen, 2010). However, if xyloglucan bound cellulose tightly and irreversibly, as appears to be true, this network would behave more like a viscoelastic solid, with retarded elasticity due to the time needed for unfolding of xyloglucan tethers and viscous movement of the cellulose microfibrils within the pectic matrix (Abasolo et al., 2009). On the other hand, there is some doubt that xyloglucan–cellulose binding has sufficient strength to withstand the tensile forces in the wall (Thompson, 2005). This issue seems an open question that might be addressed by molecular dynamics simulations (Bergenstrahle et al., 2009). A thorough analysis is complicated by microfibril structure which has distinct hydrophobic and hydrophilic surfaces as well as disordered regions, which likely differ in xyloglucan binding. Furthermore, xyloglucan may be entrapped within microfibrils or bundles of microfibrils, further complicating such analysis.

The elastic behavior of a multi-lamellate version of the tethered network has been modeled by a finite-element simulation, absent the pectin component (Kha et al., 2010). A key advantage of this approach is that the spatial distribution and range of tensile loads can be calculated for individual tethers. This may be important for extending the model to incorporate viscoelastic extension and creep behavior of walls in a realistic way.

Using another approach, Dyson et al. (2012) modeled the effects of stretch-dependent cross-link breakage on cell wall-yielding behavior, incorporating potential effects of expansin and xyloglucan endotransglycosylase/endohydrolase. The results resemble Lockhart's semi-empirical equation in which the rate of wall expansion is given by $\varphi\ (P - Y)$, that is, extensibility φ times turgor pressure P in excess of the yield threshold Y (Lockhart, 1965). Moreover, the model offers a molecular interpretation of φ as due to the pectin viscosity and Y as related to the density of xyloglucan tethers. These are testable predictions.

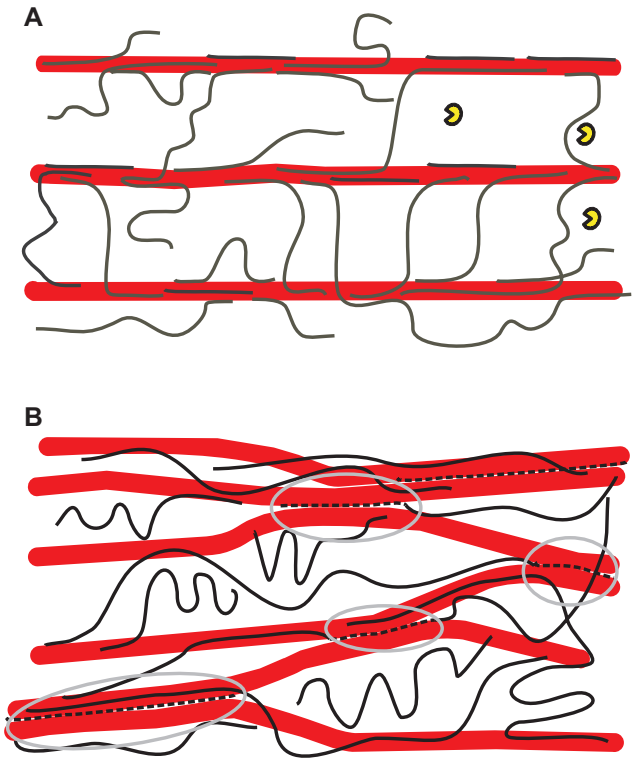


FIGURE 1 | Alternative hypothetical architectures of cellulose–xyloglucan networks in primary cell walls. (A) The tethered network model in which xyloglucans (black lines) fully coat the surfaces of cellulose microfibrils (larger red rods) and additionally span the 20–40 nm gap between adjacent cellulose microfibrils as load-bearing tethers. The distance between microfibrils is large enough to permit penetration of a xyloglucanase-specific endoglucanase (yellow "Pacman" symbols) of the type used by Park and Cosgrove (2012a). This model is based a variety of results (Scheller and Ulvskov, 2010; Hayashi and Kaida, 2011) but is still hypothetical and recent work is at odds with it. In this model, the primary means of cell wall expansion is by cutting the xyloglucan tethers or by destabilizing the xyloglucan–cellulose interaction, resulting in wall stress relaxation and yielding. The directionality of growth depends on the net orientation of cellulose microfibrils (Baskin, 2005; Kerstens et al., 2001). **(B)** A revised architecture based on the enzyme/biomechanics analysis of Park and Cosgrove (2012a), in which the load-bearing xyloglucan (broken black lines highlighted by gray ellipses) is a minor fraction of the total xyloglucan and is trapped between microfibrils, so it is not accessible to xyloglucan-specific endoglucanase. By this arrangement the xyloglucan glues microfibrils into a network of microfibril bundles which serve to protect it from lytic action by xyloglucan-specific endoglucanases as well as xyloglucan endotransglycosylase/hydrolase. The gray circles demark the limited region that bear the static tensile forces generated by turgor pressure. An arrangement as shown here could account for the lack of wall loosening caused by xyloglucan endotransglycosylase/hydrolases (Saladie et al., 2006; Maris et al., 2009; Miedes et al., 2011).

GROWING DOUBTS ABOUT XYLOGLUCAN'S ROLE

Despite the widespread acceptance of the tethered network model, recent results have led to some doubts about its correctness. As mentioned above, some walls contain little hemicellulose. Attempts to measure xyloglucan bound to cellulose by ^{13}C-NMR in intact *Arabidopsis* and mung bean walls concluded that $<10\%$ of the cellulose surface is coated with xyloglucan, contrary to expectations of the dominant model (Bootten et al., 2004; Dick-Perez

et al., 2011). Much of the xyloglucan interacted with pectins, which showed extensive interactions with cellulose. Neutral pectins bind to cellulose *in vitro*, although less avidly than does xyloglucan (Zykwinska et al., 2007a, 2008).

Another blow to the tethered network model came from analysis of mutants defective in xyloglucan synthesis which displayed only modest growth defects despite the complete absence of xyloglucan (Cavalier et al., 2008; Zabotina et al., 2012). Further analysis showed that pectins and xylans assumed greater biomechanical roles in the xyloglucan-deficient walls (Park and Cosgrove, 2012b) which were mechanically weaker but simultaneously less extensible because they lacked the major target of α-expansin. Xyloglucan may also affect microfibril structure or aggregation (Atalla et al., 1993), with indirect consequences for the effectiveness of wall-loosening agents. This idea draws some support from NMR analyses (Dick-Perez et al., 2011) and confocal microscopy (Anderson et al., 2010) which indicate that the xyloglucan-deficient walls contain larger cellulose microfibrils or microfibril bundles compared with wildtype walls.

A revised view of xyloglucan's role recently emerged from experiments in which substrate-specific endoglucanases were assessed for their ability to induce wall creep (Park and Cosgrove, 2012a). Although xyloglucan-specific endoglucanases removed much of the xyloglucan, they did not increase wall creep or compliance. Cellulose-specific endoglucanases were likewise inactive in biomechanical assays. Only enzymes able to hydrolyze both xyloglucan and cellulose were active in these assays, with large biomechanical effects associated with digestion of ∼0.3% of the total xyloglucan. These results argue against the tether model (**Figure 1A**), as such tethers would be digested by xyloglucan-specific enzymes. **Figure 1B** shows one possible scheme consistent with the new results, where the biomechanically significant xyloglucan is restricted to inaccessible junctions between microfibrils.

These studies de-emphasize the role of xyloglucan as the exclusive moderator of cell wall mechanics and indicate that other matrix polysaccharides may fill in for some xyloglucan functions. This idea is further reinforced by studies showing pectin de-esterification is linked with enhanced growth in the *Arabidopsis* hypocotyl (Pelletier et al., 2010) and in the outgrowth of leaf and flower primordia in the shoot apical meristem (Peaucelle et al., 2008, 2011). These effects of pectin methyl esterase are hard to understand in terms of wall structure because pectin de-esterification, by itself, should lead to enhanced ionic cross linking of pectins, thereby rigidifying the wall (Zhao et al., 2008). Indirect effects of pectin de-esterification may be linked to these growth enhancements (Wolf and Greiner, 2012). Moreover, pectin may play a greater role in specialized walls found in pollen tubes and in some algae (Fayant et al., 2010; Rojas et al., 2011; Palin and Geitmann, 2012).

BIOMECHANICS OF SECONDARY CELL WALLS

In most angiosperms the functions of water conduction and support are divided between vessels and interfascicular xylem cells, patterned through the influence of distinct transcription factors (Guo et al., 2009; Ohtani et al., 2011) and intercellular signaling networks (Fuchs et al., 2011). The requirement for tensile and compressive strength is satisfied by a high content of rather uniformly oriented cellulose, synthesized by a distinct set of cellulose synthases and normally lignified. In trees the result is wood (Mellerowicz and Sundberg, 2008). Xylem tissues broadly similar in structure and function, although often with less lignin or none (Carlquist and Schneider, 1998; Hepworth et al., 1998; Jung and Engels, 2002) are found in herbaceous plants, bamboos, and palms.

Like primary cell walls, secondary cell walls are composite materials based on 3-nm cellulose microfibrils, with lignin, xylans, and glucomannans replacing xyloglucans and pectins (Mellerowicz and Sundberg, 2008). Secondary cell walls are less hydrated than primary cell walls, containing only about 30% water at saturation. In coniferous wood at least, the cellulose microfibrils form loose bundles 10–20 nm across with direct lateral adhesion between adjacent microfibrils over part of their length (Fernandes et al., 2011). Most of the lignin and hemicelluloses are thought to lie outside these aggregates (Salmen, 2004; Fernandes et al., 2011), but hemicellulosic glucomannans are closely associated with cellulose (Salmen and Bergstrom, 2009; **Figure 2**). In contrast to the dispersed cellulose orientations in primary cell walls, the cellulose microfibrils are wound around wood cells in a helix whose pitch is defined by the microfibril angle, the angle that the microfibrils make with the cell axis (Barnett and Bonham, 2004). Tree saplings have high microfibril angles giving flexibility in response to wind and snow, while mature trees have low microfibril angles providing the stiffness to avoid buckling under the compressive loads imposed by their weight (Altaner and Jarvis, 2008). A tree's mechanical history is recorded in the variation of microfibril angle across the annual rings of the trunk.

Because of the importance of wood as a construction material its mechanical properties have been studied much more intensively than those of growing plant cells (Salmen, 2004). Models and tentative mechanisms have been suggested for three kinds of deformation: (1) viscoelastic (reversible) deformation; (2) an irreversible time-dependent form of deformation termed here "viscoplastic" (Flores et al., 2011); (3) mechano-sorptive deformation. Only (2) and (3) are irreversible and therefore directly analogous to the growth of primary cell walls, but it is not clear to what extent (1)–(3) are mechanistically related. All three may therefore suggest insights or potential experimental approaches that might help to understand the physical processes occurring when primary cell walls elongate.

VISCOELASTIC STRETCHING AND BENDING

Dry secondary cell walls with small microfibril angles show purely viscoelastic behavior, either in tensile or bending experiments at constant stress or by stress relaxation. The viscous component increases with the angle between the microfibrils and the stress (Bonfield et al., 1996; Gril et al., 2004) and has been modeled as due to viscous shear in the lignin–hemicellulose matrix between elastic microfibrils (Engelund and Svensson, 2011) or microfibril aggregates. The calculated free energy of activation was consistent with the breaking of four to six hydrogen bonds in each sliding event (Bonfield et al., 1996). Chemical delignification (Kohler and Spatz, 2002) or down regulation of lignin synthesis (Koehler and Telewski, 2006) generally reduced stiffness,

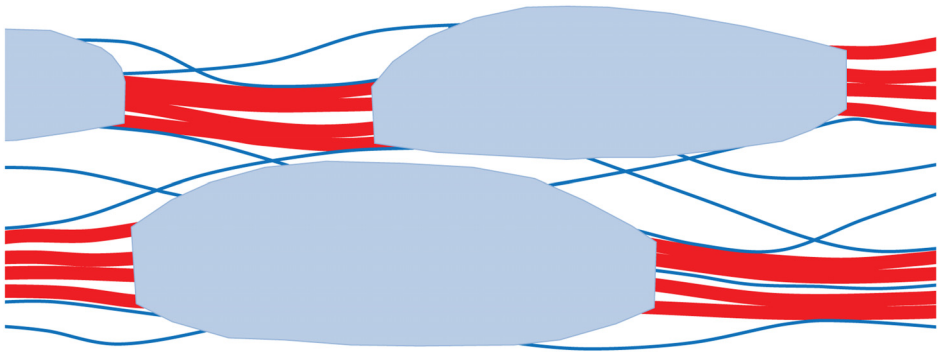


FIGURE 2 | Hypothetical architecture of polymer network in secondary cell walls of conifer wood. Loosely aggregated bundles of cellulose microfibrils (red) are coated with a disordered xylan–lignin complex (shaded light blue). Partially oriented glucomannan chains (blue lines) adhere by hydrogen bonding to the cellulose aggregates and acetylated segments of these glucomannans bridge between the aggregates. For clarity the structure is shown much more open than is the case: the free space, filled by water *in vivo*, is only about 40% of the total volume. This is much less than the free space in hydrated primary cell walls. Based on Terashima et al. (2009) and Fernandes et al. (2011).

demonstrating involvement of lignin. There was also less direct evidence for involvement of hemicelluloses (Assor et al., 2009; Bjurhager et al., 2010), but mechanisms remain unclear at the molecular level.

VISCOPLASTIC DEFORMATION ABOVE THE YIELD THRESHOLD

When fully hydrated as in the living tree, wood with high microfibril angle shows a two-phase load-deformation curve. Above a threshold stress it becomes less stiff. This second phase of elongation is irreversible but this does not result from overt damage, because when the stress is reduced below the yield threshold the elongated sample remains as stiff as before (Kohler and Spatz, 2002; Keckes et al., 2003). Mechanically, then, elongation of wood above the yield threshold resembles turgor-driven extension growth as described by the Lockhart equation. The term "molecular Velcro" was coined to describe this form of deformation in wood because it is simulated by hook-and-loop fasteners that can be pulled apart, moved and refastened without permanent loss of strength (Keckes et al., 2003). Molecular Velcro tensile behavior has been demonstrated for wet wood samples in which the cellulose microfibrils are oriented at 20–45° to the cell axis. If the wood is dry or the microfibril angle is low, the yield threshold rises until the sample breaks before reaching it (Kamiyama et al., 2005). Because of this technical problem it is common to use isolated single wood cells, which can be stretched to greater elongation than bulk wood samples without breaking (Keckes et al., 2003). The helical arrangement of the microfibrils tends to untwist on stretching, so single wood cells need to be rotationally restrained to match their behavior in wood, where each cell is locked to the next and cannot twist (Fratzl et al., 2004).

The molecular Velcro phenomenon requires that cellulose microfibrils should slide past one another and should also rotate closer to the line of stress (Flores et al., 2011): these two deformations must be balanced if the cell is not to twist (Fratzl et al., 2004). The yield threshold then corresponds to the abrupt onset of sliding between aggregates of microfibrils. Several mechanisms have been suggested to trigger this sliding process. The original suggestion was that hemicelluloses anchored to two adjacent microfibrils were entangled and that a threshold shear stress was required to break the entanglement, allowing the microfibrils to slide (Keckes et al., 2003). There is doubt about whether the orientation of hemicelluloses is consistent with entanglement interactions (Salmen and Bergstrom, 2009), and a more recent explanation of this mechanism (Speck and Burgert, 2011) substitutes lateral association of two hemicellulose chains, stabilized by hydrogen bonding over a number of residues. A further alternative mechanism involves the peeling of a hemicellulose chain off the microfibril surface by the component of the stress perpendicular to the microfibril aggregates, allowing them to slide parallel to one another until contact is restored (Altaner and Jarvis, 2008). All these triggering mechanisms focus on hemicelluloses and assume topologies rather like the "tethered network" model for primary walls, but there is evidence for the involvement of lignin as well (Kohler and Spatz, 2002) as for viscoelastic extension below the yield stress.

HYGROMECHANICAL DEFORMATION

Wood deforms progressively when maintained under a constant, often quite small, tensile or bending load through repeated cycles of drying and wetting (Bengtsson, 2001; Entwistle and Zadoroshnyj, 2008). The cumulative deformation is largely permanent unless the load is reversed. In that respect hygromechanical creep resembles viscoplastic deformation above the yield threshold, and it has been suggested to require breaking of hydrogen bonds between hemicelluloses and cellulose under hydrated conditions and their re-formation, in different locations, on drying (Entwistle and Zadoroshnyj, 2008).

All three of these forms of deformation of wood seem to be focused in the matrix region between microfibrils or microfibril aggregates, and increase simultaneously in magnitude with microfibril angle (Bengtsson, 2001; Fratzl et al., 2004; Gril et al., 2004), but it does not follow that they share exactly the same mechanism. There is an interesting parallel with the unexplained observation that stress relaxation parameters for the stretching of primary walls often correlate with growth (Nakamura et al., 2002).

CONCLUSION

Although primary walls of growing cells possess inherent viscoelastic–viscoplastic properties arising from the polymeric nature of their structure, cell wall expansion is primarily a dynamic process requiring the action of expansins or other wall-loosening agents. Deformation of wood is considered to be physical. Nevertheless there are similarities. Both processes are sensitive to microfibril orientation and both involve interactions of hydrated non-cellulosic polysaccharides with cellulose surfaces. The topology of these interactions in response to the local distribution of stresses – details best conveyed in cartoons like **Figure 1** – are only now starting to be understood. In secondary cell walls the key structural elements are not individual microfibrils but bundles of microfibrils, and the possibility that this is also true in primary cell walls deserves to be explored. The research communities working on primary and secondary cell walls are rather separate, but both could benefit from converging and exchanging ideas.

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REFERENCES

Abasolo, W., Eder, M., Yamauchi, K., Obel, N., Reinecke, A., Neumetzler, L., Dunlop, J. W., Mouille, G., Pauly, M., Hofte, H., and Burgert, I. (2009). Pectin may hinder the unfolding of xyloglucan chains during cell deformation: implications of the mechanical performance of Arabidopsis hypocotyls with pectin alterations. *Mol. Plant* 2, 990–999.

Altaner, C. M., and Jarvis, M. C. (2008). Modelling polymer interactions of the "molecular Velcro" type in wood under mechanical stress. *J. Theor. Biol.* 253, 434–445.

Anderson, C. T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. *Plant Physiol.* 152, 787–796.

Assor, C., Placet, V., Chabbert, B., Habrant, A., Lapierre, C., Pollet, B., and Perre, P. (2009). Concomitant changes in viscoelastic properties and amorphous polymers during the hydrothermal treatment of hardwood and softwood. *J. Agric. Food Chem.* 57, 6830–6837.

Atalla, R. H., Hackney, J. M., Uhlin, I., and Thompson, N. S. (1993). Hemicelluloses as structure regulators in the aggregation of native cellulose. *Int. J. Biol. Macromol.* 15, 109–112.

Barnett, J. R., and Bonham, V. A. (2004). Cellulose microfibril angle in the cell wall of wood fibres. *Biol. Rev.* 79, 461–472.

Baskin, T. I. (2005). Anisotropic expansion of the plant cell wall. *Annu. Rev. Cell Dev. Biol.* 21, 203–222.

Bengtsson, C. (2001). Mechano-sorptive bending creep of timber – influence of material parameters. *Holz Roh Werkst.* 59, 229–236.

Bergenstrahle, M., Thormann, E., Nordgren, N., and Berglund, L. A. (2009). Force pulling of single cellulose chains at the crystalline cellulose-liquid interface: a molecular dynamics study. *Langmuir* 25, 4635–4642.

Bjurhager, I., Olsson, A. M., Zhang, B., Gerber, L., Kumar, M., Berglund, L. A., Burgert, I., Sundberg, B., and Salmen, L. (2010). Ultrastructure and mechanical properties of *Populus* wood with reduced lignin content caused by transgenic down-regulation of cinnamate 4-hydroxylase. *Biomacromolecules* 11, 2359–2365.

Bonfield, P. W., Mundy, J., Robson, D. J., and Dinwoodie, J. M. (1996). The modelling of time-dependant deformation in wood using chemical kinetics. *Wood Sci. Technol.* 30, 105–115.

Bootten, T. J., Harris, P. J., Melton, L. D., and Newman, R. H. (2004). Solid-state 13C-NMR spectroscopy shows that the xyloglucans in the primary cell walls of mung bean (*Vigna radiata* L.) occur in different domains: a new model for xyloglucan–cellulose interactions in the cell wall. *J. Exp. Bot.* 55, 571–583.

Carlquist, S., and Schneider, E. L. (1998). Origin and nature of vessels in monocotyledons. 5. Araceae subfamily Colocasioideae. *Bot. J. Linn. Soc.* 128, 71–86.

Carpita, N. C., Defernez, M., Findlay, K., Wells, B., Shoue, D. A., Catchpole, G., Wilson, R. H., and McCann, M. C. (2001). Cell wall architecture of the elongating maize coleoptile. *Plant Physiol.* 127, 551–565.

Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30.

Cavalier, D. M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O. A., Hahn, M. G., Burgert, I., Pauly, M., Raikhel, N. V., and Keegstra, K. (2008). Disrupting two *Arabidopsis thaliana* xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* 20, 1519–1537.

Cosgrove, D. J. (1993). Wall extensibility: its nature, measurement, and relationship to plant cell growth. *New Phytol.* 124, 1–23.

Cosgrove, D. J. (2005). Growth of the plant cell wall. *Nat. Rev. Mol. Cell Biol.* 6, 850–861.

Dick-Perez, M., Zhang, Y., Hayes, J., Salazar, A., Zabotina, O. A., and Hong, M. (2011). Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. *Biochemistry* 50, 989–1000.

Dyson, R. J., Band, L. R., and Jensen, O. E. (2012). A model of crosslink kinetics in the expanding plant cell wall: yield stress and enzyme action. *J. Theor. Biol.* 307C, 125–136.

Dyson, R. J., and Jensen, O. E. (2010). A fibre-reinforced fluid model of anisotropic plant cell growth. *J. Fluid Mech.* 655, 472–503.

Eklof, J. M., and Brumer, H. (2010). The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. *Plant Physiol.* 153, 456–466.

Engelund, E. T., and Svensson, S. (2011). Modelling time-dependent mechanical behaviour of softwood using deformation kinetics. *Holzforschung* 65, 231–237.

Entwistle, K. M., and Zadoroshnyj, A. (2008). The recovery of mechano-sorptive creep strains. *J. Mater. Sci.* 43, 967–973.

Fayant, P., Girlanda, O., Chebli, Y., Aubin, C.-E., Villemure, I., and Geitmann, A. (2010). Finite element model of polar growth in pollen tubes. *Plant Cell* 22, 2579–2593.

Fernandes, A. N., Thomas, L. H., Altaner, C. M., Callow, P., Forsyth, V. T., Apperley, D. C., Kennedy, C. J., and Jarvis, M. C. (2011). Nanostructure of cellulose microfibrils in spruce wood. *Proc. Natl. Acad. Sci. U.S.A.* 108, E1195–E1203.

Flores, E. I. S., Neto, E. A. D., and Pearce, C. (2011). A large strain computational multi-scale model for the dissipative behaviour of wood cell-wall. *Comput. Mater. Sci.* 50, 1202–1211.

Fratzl, P., Burgert, I., and Keckes, J. (2004). Mechanical model for the deformation of the wood cell wall. *Z. Metallkd.* 95, 579–584.

Fuchs, M., Van Bel, A. J. E., and Ehlers, K. (2011). Do symplasmic networks in cambial zones correspond with secondary growth patterns? *Protoplasma* 248, 141–151.

Geitmann, A. (2010). Mechanical modeling and structural analysis of the primary plant cell wall. *Curr. Opin. Plant Biol.* 13, 693–699.

Gibeaut, D. M., Pauly, M., Bacic, A., and Fincher, G. B. (2005). Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. *Planta* 221, 729–738.

Gril, J., Hunt, D., and Thibaut, B. (2004). Using wood creep data to discuss the contribution of cell-wall reinforcing material. *C. R. Biol.* 327, 881–888.

Guo, Y., Qin, G. J., Gu, H. Y., and Qu, L. J. (2009). Dof5.6/HCA2, a Dof transcription factor gene, regulates interfascicular cambium formation and vascular tissue development in *Arabidopsis*. *Plant Cell* 21, 3518–3534.

Hamant, O., and Traas, J. (2010). The mechanics behind plant development. *New Phytol.* 185, 369–385.

Hayashi, T., and Kaida, R. (2011). Functions of xyloglucan in plant cells. *Mol. Plant* 4, 17–24.

Hepworth, D. G., Vincent, J. F. V., and Schuch, W. (1998). Using viscoelastic properties of the woody tissue from tobacco plants (*Nicotiana tabacum*) to comment on the molecular structure of cell walls. *Ann. Bot.* 81, 729–734.

Jung, H. G., and Engels, F. M. (2002). Alfalfa stem tissues: cell wall deposition, composition, and degradability. *Crop Sci.* 42, 524–534.

Kamiyama, T., Suzuki, H., and Sugiyama, J. (2005). Studies of the structural change during deformation in *Cryptomeria japonica* by time-resolved synchrotron small-angle X-ray scattering. *J. Struct. Biol.* 151, 1–11.

Keckes, J., Burgert, I., Fruhmann, K., Muller, M., Kolln, K., Hamilton, M., Burghammer, M., Roth, S. V., Stanzl-Tschegg, S., and Fratzl, P. (2003). Cell-wall recovery after irreversible

deformation of wood. *Nat. Mater.* 2, 810–814.
Keegstra, K., Talmadge, K. W., Bauer, W. D., and Albersheim, P. (1973). The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* 51, 188–196.
Kerstens, S., Decraemer, W. F., and Verbelen, J.-P. (2001). Cell walls at the plant surface behave mechanically like fiber-reinforced composite materials. *Plant Physiol.* 127, 381–385.
Kha, H., Tuble, S. C., Kalyanasundaram, S., and Williamson, R. E. (2010). WallGen, software to construct layered cellulose–hemicellulose networks and predict their small deformation mechanics. *Plant Physiol.* 152, 774–786.
Koch, G. W., Sillett, S. C., Jennings, G. M., and Davis, S. D. (2004). The limits to tree height. *Nature* 428, 851–854.
Koehler, L., and Telewski, F. W. (2006). Biomechanics and transgenic wood. *Am. J. Bot.* 93, 1433–1438.
Kohler, L., and Spatz, H. C. (2002). Micromechanics of plant tissues beyond the linear-elastic range. *Planta* 215, 33–40.
Lockhart, J. A. (1965). An analysis of irreversible plant cell elongation. *J. Theor. Biol.* 8, 264–275.
Maris, A., Suslov, D., Fry, S. C., Verbelen, J. P., and Vissenberg, K. (2009). Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of *Arabidopsis* and their effect on root growth and cell wall extension. *J. Exp. Bot.* 60, 3959–3972.
Mellerowicz, E. J., and Sundberg, B. (2008). Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties. *Curr. Opin. Plant Biol.* 11, 293–300.
Miedes, E., Zarra, I., Hoson, T., Herbers, K., Sonnewald, U., and Lorences, E. P. (2011). Xyloglucan endotransglucosylase and cell wall extensibility. *J. Plant Physiol.* 168, 196–203.
Nakamura, Y., Wakabayashi, K., Kamisaka, S., and Hoson, T. (2002). Effects of temperature on the cell wall and osmotic properties in dark-grown rice and azuki bean seedlings. *J. Plant Res.* 115, 455–461.
Ohtani, M., Nishikubo, N., Xu, B., Yamaguchi, M., Mitsuda, N., Goue, N., Shi, F., Ohme-Takagi, M., and Demura, T. (2011). A NAC domain protein family contributing to the regulation of wood formation in poplar. *Plant J.* 67, 499–512.
Palin, R., and Geitmann, A. (2012). The role of pectin in plant morphogenesis. *Biosystems.* doi: 10.1016/j.biosystems.2012.04.006 [Epub ahead of print].
Park, Y. B., and Cosgrove, D. J. (2012a). A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. *Plant Physiol.* 158, 1933–1943.
Park, Y. B., and Cosgrove, D. J. (2012b). Changes in cell wall biomechanical properties in the xyloglucan-deficient *xxt1/xxt2* mutant of *Arabidopsis. Plant Physiol.* 158, 465–475.
Peaucelle, A., Braybrook, S. A., Le Guillou, L., Bron, E., Kuhlemeier, C., and Hofte, H. (2011). Pectin-induced changes in cell wall mechanics underlie organ initiation in *Arabidopsis. Curr. Biol.* 21, 1720–1726.
Peaucelle, A., Louvet, R., Johansen, J. N., Hofte, H., Laufs, P., Pelloux, J., and Mouille, G. (2008). *Arabidopsis* phyllotaxis is controlled by the methyl-esterification status of cell-wall pectins. *Curr. Biol.* 18, 1943–1948.
Pelletier, S., Van Orden, J., Wolf, S., Vissenberg, K., Delacourt, J., Ndong, Y. A., Pelloux, J., Bischoff, V., Urbain, A., Mouille, G., Lemonnier, G., Renou, J. P., and Hofte, H. (2010). A role for pectin de-methylesterification in a developmentally regulated growth acceleration in dark-grown *Arabidopsis* hypocotyls. *New Phytol.* 188, 726–739.
Preston, R. D. (1974). *The Physical Biology of Plant Cell Walls.* London: Chapman & Hall.
Ray, P. M., and Ruesink, A. W. (1962). Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells. *Dev. Biol.* 4, 377–397.
Rojas, E. R., Hotton, S., and Dumais, J. (2011). Chemically mediated mechanical expansion of the pollen tube cell wall. *Biophys. J.* 101, 1844–1853.
Saladie, M., Rose, J. K., Cosgrove, D. J., and Catala, C. (2006). Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. *Plant J.* 47, 282–295.
Salmen, L. (2004). Micromechanical understanding of the cell-wall structure. *C. R. Biol.* 327, 873–880.
Salmen, L., and Bergstrom, E. (2009). Cellulose structural arrangement in relation to spectral changes in tensile loading FTIR. *Cellulose* 16, 975–982.
Sampedro, J., and Cosgrove, D. J. (2005). The expansin superfamily. *Genome Biol.* 6, 242.
Scheller, H. V., and Ulvskov, P. (2010). Hemicelluloses. *Annu. Rev. Plant Biol.* 61, 263–289.
Speck, T., and Burgert, I. (2011). Plant stems: functional design and mechanics. *Annu. Rev. Mater. Res.* 41, 169–193.
Terashima, N., Kitano, K., Kojima, M., Yoshida, M., Yamamoto, H., and Westermark, U. (2009). Nanostructural assembly of cellulose, hemicellulose, and lignin in the middle layer of secondary wall of ginkgo tracheid. *J. Wood Sci.* 55, 409–416.
Thimm, J. C., Burritt, D. J., Sims, I. M., Newman, R. H., Ducker, W. A., and Melton, L. D. (2002). Celery (*Apium graveolens*) parenchyma cell walls: cell walls with minimal xyloglucan. *Physiol. Plant.* 116, 164–171.
Thompson, D. S. (2005). How do cell walls regulate plant growth? *J. Exp. Bot.* 56, 2275–2285.
Veytsman, B. A., and Cosgrove, D. J. (1998). A model of cell wall expansion based on thermodynamics of polymer networks. *Biophys. J.* 75, 2240–2250.
Wolf, S., and Greiner, S. (2012). Growth control by cell wall pectins. *Protoplasma* 249(Suppl. 2), 169–175.
Yennawar, N. H., Li, L. C., Dudzinski, D. M., Tabuchi, A., and Cosgrove, D. J. (2006). Crystal structure and activities of EXPB1 (Zea m 1), a β-expansin and group-1 pollen allergen from maize. *Proc. Natl. Acad. Sci. U.S.A.* 103, 14664–14671.
Zabotina, O. A., Avci, U., Cavalier, D., Pattathil, S., Chou, Y. H., Eberhard, S., Danhof, L., Keegstra, K., and Hahn, M. G. (2012). Mutations in multiple *XXT* genes of *Arabidopsis* reveal the complexity of xyloglucan biosynthesis. *Plant Physiol.* 159, 1367–1348.
Zhao, Q., Yuan, S., Wang, X., Zhang, Y., Zhu, H., and Lu, C. (2008). Restoration of mature etiolated cucumber hypocotyl cell wall susceptibility to expansin by pretreatment with fungal pectinases and EGTA in vitro. *Plant Physiol.* 147, 1874–1885.
Zykwinska, A., Gaillard, C., Buleon, A., Pontoire, B., Garnier, C., Thibault, J. F., and Ralet, M. C. (2007a). Assessment of in vitro binding of isolated pectic domains to cellulose by adsorption isotherms, electron microscopy, and X-ray diffraction methods. *Biomacromolecules* 8, 223–232.
Zykwinska, A., Thibault, J. F., and Ralet, M. C. (2007b). Organization of pectic arabinan and galactan side chains in association with cellulose microfibrils in primary cell walls and related models envisaged. *J. Exp. Bot.* 58, 1795–1802.
Zykwinska, A., Thibault, J. F., and Ralet, M. C. (2008). Competitive binding of pectin and xyloglucan with primary cell wall cellulose. *Carbohydr. Polym.* 74, 957–961.

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