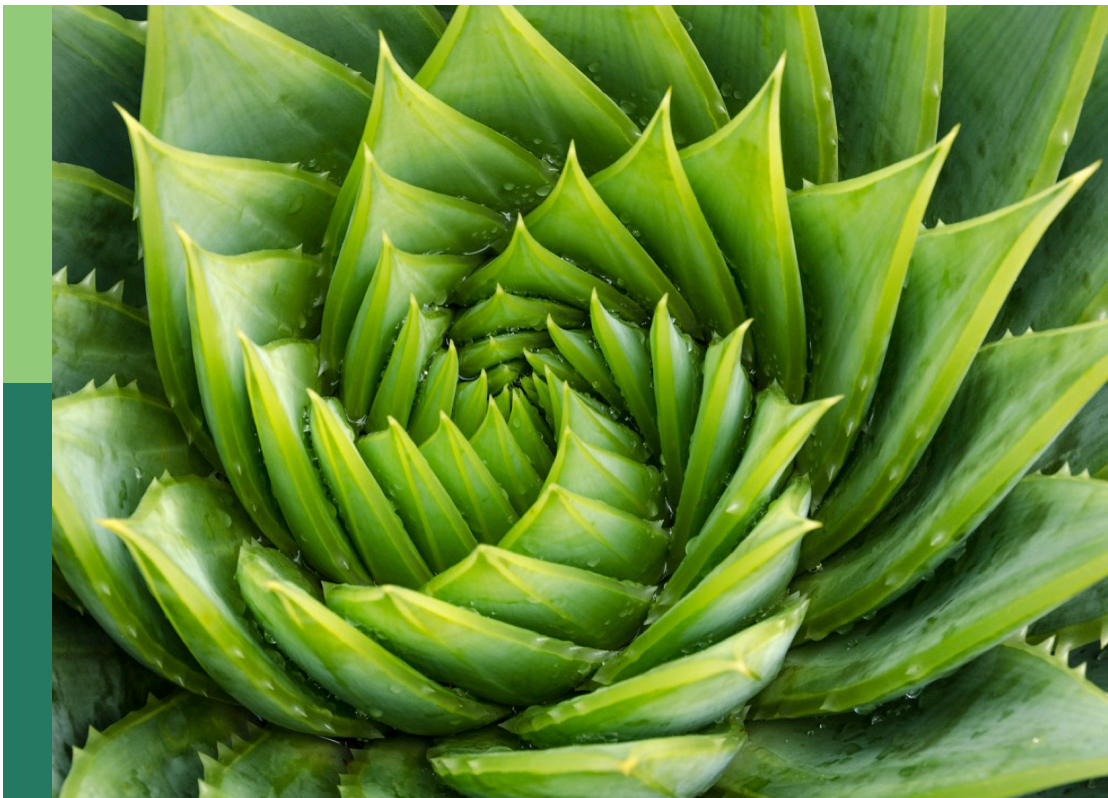
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Targeted genome editing for crop improvement

Edited by
Kejian Wang, Shuangxia Jin and Maria Raffaella Ercolano

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Targeted genome editing for crop improvement

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Table of contents

05 Editorial: Targeted genome editing for crop improvement
Maria Raffaella Ercolano and Kejian Wang

08 Impairment of Tomato *WAT1* Enhances Resistance to Vascular Wilt Fungi Despite Severe Growth Defects
Katharina Hanika, Danny Schipper, Shravya Chinnappa, Marian Oortwijn, Henk J. Schouten, Bart P. H. J. Thomma and Yuling Bai

22 Concurrent Disruption of Genetic Interference and Increase of Genetic Recombination Frequency in Hybrid Rice Using CRISPR/Cas9
Chaolei Liu, Yiwei Cao, Yufeng Hua, Guijie Du, Qing Liu, Xin Wei, Tingting Sun, Jianrong Lin, Mingguo Wu, Zhukuan Cheng and Kejian Wang

32 Multiplex Genome-Editing Technologies for Revolutionizing Plant Biology and Crop Improvement
Mohamed Abdelrahman, Zheng Wei, Jai S. Rohila and Kaijun Zhao

47 Employing CRISPR/Cas Technology for the Improvement of Potato and Other Tuber Crops
Tussipkan Dilnur and Shuga A. Manabayeva

63 C-to-G Base Editing Enhances Oleic Acid Production by Generating Novel Alleles of *FATTY ACID DESATURASE 2* in Plants
Mid-Eum Park, Jae-Young Yun and Hyun Uk Kim

74 An Efficient Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Protein 9 Mutagenesis System for Oil Palm (*Elaeis guineensis*)
Wan-Chin Yeap, Norkhairunnisa Che Mohd Khan, Norfadzilah Jamalludin, Muhammad Rashdan Muad, David Ross Appleton and Harikrishna Kulaveerasingam

87 The CRISPR/Cas9-Mediated Modulation of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* in Alfalfa Leads to Distinct Phenotypic Outcomes
Stacy D. Singer, Kimberley Burton Hughes, Udaya Subedi, Gaganpreet Kaur Dhariwal, Kazi Kader, Surya Acharya, Guanqun Chen and Abdelali Hannoufa

104 Generating Novel Male Sterile Tomatoes by Editing Respiratory Burst Oxidase Homolog Genes
Xiaojuan Dai, Huanan Han, Wei Huang, Lianghui Zhao, Minglei Song, Xuesong Cao, Chenglan Liu, Xiaomu Niu, Zhaobo Lang, Changle Ma and Hongtao Xie

113 Highly Efficient Generation of Canker-Resistant Sweet Orange Enabled by an Improved CRISPR/Cas9 System
Xiaoen Huang, Yuanchun Wang and Nian Wang

125 Efficient Genome Editing in *Setaria italica* Using CRISPR/Cas9 and Base Editors
Zhen Liang, Yuqing Wu, Lingling Ma, Yingjie Guo and Yidong Ran

135 An Efficient Hairy Root System for Validation of Plant Transformation Vector and CRISPR/Cas Construct Activities in Cucumber (*Cucumis sativus* L.)
Doai Van Nguyen, Trang Thi-Huyen Hoang, Ngoc Thu Le, Huyen Thi Tran, Cuong Xuan Nguyen, Yong-Hwan Moon, Ha Hoang Chu and Phat Tien Do

147 Modified Gene Editing Systems: Diverse Bioengineering Tools and Crop Improvement
Guoning Zhu and Hongliang Zhu

161 Functional Differentiation of *BnVTE4* Gene Homologous Copies in α-Tocopherol Biosynthesis Revealed by CRISPR/Cas9 Editing
Haiyan Zhang, Yuqin Shi, Mengdan Sun, Xuezhi Hu, Mengyu Hao, Yu Shu, Xue-Rong Zhou, Qiong Hu, Chao Li and Desheng Mei

170 Development of a Genome-Edited Tomato With High Ascorbate Content During Later Stage of Fruit Ripening Through Mutation of *SlAPX4*
Ju Hui Do, Seo Young Park, Se Hee Park, Hyun Min Kim, Sang Hoon Ma, Thanh Dat Mai, Jae Sung Shim and Young Hee Joung

181 Expanding the Editing Window of Cytidine Base Editors With the Rad51 DNA-Binding Domain in Rice
Chunjie Wei, Hao Liu, Wenwen Wang, Pengyu Luo, Qiuling Chen, Rou Li, Chong Wang, José Ramón Botella and Hui Zhang

190 Advance of Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 System and Its Application in Crop Improvement
Yuchun Rao, Xi Yang, Chenyang Pan, Chun Wang and Kejian Wang

207 CRISRP/Cas9-Mediated Targeted Mutagenesis of Tomato Polygalacturonase Gene (*SlPG*) Delays Fruit Softening
Hongmei Nie, Yu Shi, Xueqing Geng and Guoming Xing

217 Molecular and Computational Strategies to Increase the Efficiency of CRISPR-Based Techniques
Lucia Mattiello, Mark Rütgers, Maria Fernanda Sua-Rojas, Rafael Tavares, José Sérgio Soares, Kevin Begcy and Marcelo Menossi

235 Increased sulfur-containing amino acid content and altered conformational characteristics of soybean proteins by rebalancing 11S and 7S compositions
Biao Wang, Da Teng, Cunhao Yu, Luming Yao, Xiaohong Ma and Tianlong Wu

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Editorial: Targeted genome editing for crop improvement

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KEYWORDS

CRISPR/Cas methodology, base editor (BE), prime editor, multiplex genome editing, agronomic traits

Editorial on the Research Topic:
Targeted genome editing for crop improvement

Introduction

The genome editing approach is revolutionizing both agricultural and plant research. Conventional breeding strategies are often time-consuming and laborious, and may not be able to maintain the rate of progress requested with the increasing food demand (Scheben et al., 2017). There is a pressing need of new breeding techniques for developing agriculture products in sustainable way. In recent years, the targeted genome editing technologies showed that the precise modification of a trait is feasibly (Anzalone et al., 2020). Important technological developments, including optimization of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) protocols in popular and neglected crops, and innovative methodologies approaches have been provided. In addition, several examples of traits improvement obtained through this methodology have already been made available (Zhu et al., 2020; Gao, 2021; Huang et al., 2021). This research topic presents the most recent advances in 19 publications, including 5 reviews, one method article and 13 research articles, contributed by 119 authors. The aim of this topic is to survey the major technological advances and application of genome editing in crops. Here we summarize these papers according the classification of contributions, mainly including the optimization of CRISPR/Cas systems in different crops and its applications in crops.

Advancements in genome editing technology

The CRISPR/Cas9 system represents the third generation of targeted genome editing technology (Gaj et al., 2013; Gupta et al., 2019). The Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) has provided answers to basic questions related to plant biology as well as to compelling breeding needs. Although, the simplicity, the high efficiency and specificity of CRISPR/Cas9 allowed its rapid and widespread diffusion in plant science (Huang et al., 2022). This system has become increasingly mature and has been widely used in gene knockout, knock-in, and regulation, as well as the improvement of yield, quality, and biological resistance of important crops (Rao et al.). Several attempts for enlarging the opportunities offered by CRISPR/Cas9 system have

been conducted. Engineering of Cas9, Cas12a, Cas12b, and Cas12f proteins was able to improve its efficiency (Mattiello et al.). The multiplex genome-editing (MGE) technologies allowed to enhanced mutations at multiple loci (Abdelrahman et al.). Recently, base editors (BEs) methodology displayed to be a powerful tool for altering desired trait in crops. Although the editing efficiency, and editing window are still not optimal, base substitution of target sequences by BEs can be accurately achieved. Moreover, prime editors (PEs) permitted to replace or insert sequences in crucial sites (Zhu and Zhu). In rice, although the fusion of a rice codon-optimized human Rad51 DNA-Binding Domain (DBD) protein between Cas9 nickase and the deaminase did not increase editing efficiency, the editing window of base editors was expanded. Similarly, the use of a specific rice Rad51 DBD homolog also expanded the editing window effectively (Wei et al.). Cytosyne and Adenine base editor (CBE and ABE) systems were successfully used to target the *SiALS* and *SiACC* genes in foxtail millet (*Setaria italica*). By utilizing CBE to target the *SiALS* gene, a homozygous herbicide-tolerant mutant was created (Liang et al.).

CRISPR/Cas systems targeting cell organelle genomes or RNA have also been explored and modified gene-editing systems made transgene-free plants more readily available. In various plant species, the hairy root induction system showed to be an effective method to study gene expression and function due to its fast-growing and high genetic stability (Jacobs and Martin, 2016; Gutierrez-Valdes et al.; Le et al.). An example hairy root induction mediated by *Rhizobium rhizogenes* for performing CRISPR/Cas9 editing was described in cucumber (*Cucumis sativus* L.). This system displayed to offer a wide range of possible applications to solve different challenges in cucumber as well as other cucumis plants (Van Nguyen et al.). The improvement of CRISPR-based technologies efficiency is enabling its implementation in a variety of crop plants, fostering the progress in both basic research and molecular breeding. Furthermore, the application of MGE and modified gene-editing technologies accelerated its use in crop-improvement programs.

Breeding achievements

In less than a decade afterward, CRISPR/Cas-based technology has been successfully used as a powerful and efficient tool for genome editing due to its simplicity, efficiency, and versatility. In this research topics, the application of the CRISPR/Cas system involved many crops, such as rice, cucumber, oilseed rape, soybean, tomato, sweet orange, oil palm, alfalfa and tuber crops (Tussipkan and Manabayeva). The target engineered genes were found to be associated with nutrient–antinutrient content, post-harvest factors, abiotic-biotic resistance, self-incompatibility and recombination traits.

Nutritional and post harvest traits

An interesting "proof-of-concept" approach was proposed in *Arabidopsis*, using ABE and CBE to obtain *FATTY ACID DESATURASE 2* (*FAD2*) alleles, whose functional alterations can reduce the unsaturation levels of fatty acids with acceptable plant growth defects. The authors claim that equivalent alleles may be generated in vegetable oil crops *via* precision genome editing for practical cultivation. Interestingly, all higher oleic-acid alleles turned out to arise within the g5 gRNA targeting region, which represents the cytosolic stretch of ER-membrane-bound FAD2 protein (Park et al.). An "trade-off" concept was presented in soybean using RNA interference to silence *CG-β-1* expression, which sharply raised the accumulation of 11S glycinin at the expense of reducing the content of 7S globulin (Wang et al.).

Vitamin E deficiency have a profound impact on human health. A daily supplement of vitamin E *via* high-quality rapeseed oil is the safest and most effective way to keep the nutritional requirement for the human body. Zhang et al. used CRISPR/Cas9 to perform targeted mutagenesis of *BnVTE4* homologs, the editing of which led to a significant change of the α-tocopherol content and the ratio between α- and γ-tocopherol, providing a theoretical basis for breeding high α-tocopherol content oilseed rape. Ascorbate is also an essential antioxidant substance for humans. The tomato (*Solanum lycopersicum*) gene *ASCORBATE PEROXIDASE 4* (*SlAPX4*), specifically induced during fruit ripening, is involved in the decrease of ascorbate. *SlAPX4* mutants, obtained by the CRISPR/Cas9 system, increased ascorbate content in ripened tomato fruits, but not in leaves (Do et al.). Those strategies supply novel formulas for food products.

In tomato, CRISPR/Cas9 system was also used to induce the targeted mutagenesis of the Polygalacturonase (PG) *SlPG* gene to delay the softening of tomato fruit. Mutated plants exhibited late fruit softening under natural conditions and lower water loss (Nie et al.).

Developmental and environmental defense traits

Alfalfa mutated genotypes in *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* (*MsSPL8)* genes using CRISPR/Cas9 technology displayed consistent morphological alterations, including reduced leaf size and early flowering. Plants with the highest number of mutated *MsSPL8* alleles exhibited significant decreases in internode length, plant height, shoot and root biomass, root length and drought tolerance (Singer et al.). In oil palm, the development of a transient protoplast assay and the generation of stable transformants allowed the CRISPR/Cas9 cleavage of *phytoene desaturase* (*EgPDS*) with good efficiency. The CRISPR/Cas9 system was further used to target the *brassinosteroid-insensitive 1* (*EgBRI1*) gene, which resulted in premature necrosis shoots and stunted phenotype mutants (Yeap et al.). Tomato mutant lines carrying targeted deletions of *Walls Are Thin 1* (*WAT1*) gene *SlWAT1* showed enhanced resistance to *Verticillium dahlia*, *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*), but severe growth defects (Hanika et al.). A significant improvement to sweet orange genome editing was developed by choosing superior promoters [*Cestrum yellow leaf curling virus* (CmYLCV) or *Citrus sinensis* ubiquitin (CsUbi) promoter] to drive Cas9 and optimizing culture temperature. The author generated canker-resistant sweet orange by mutating the effector binding element (EBE) of canker susceptibility gene *CsLOB1*, which is required for *Xanthomonas citri* subsp. *citri* (*Xcc*) infection (Huang et al.).

Reactive oxygen species (ROS), which act as key regulators of anther development, are mediated by *Respiratory Burst Oxidase Homolog* (*RBOH*) genes. Knockout mutations by CRISPR/Cas9 of both *LeRBOH* and *LeRBOHE*, two tomato anther-expressed genes, resulted in complete male sterility. Further analysis of mutants provided helpful information for understanding how *RBOH* genes regulate tomato reproduction process (Dai et al.). Additionally, manipulation of the distribution and frequency of meiotic recombination events to increase genetic diversity and disrupting genetic interference is a hot-topic in crop breeding. Null mutants of the *ZEP1* gene, which encodes the central component of the meiotic synaptonemal complex (SC), produced male sterile mutants. Genetic recombination frequency was greatly increased and genetic interference was completely eliminated by crossing the *zep1* mutants with a male fertile variety. The remained female fertility of the *zep1* mutants makes it possible to break linkage drag. This study provides a potential approach to increase genetic diversity and fully eliminate genetic interference in rice breeding (Liu et al.).

Perspectives

The Research Topic on *Targeted Genome Editing for Crop Improvement* collected innovative contributions on recent advances made in the field of plant GENOME EDITING. With the rapid development of genome editing technologies and functional genomics, it is foreseeable that many new optimized gene editing systems will emerge. Because of the limitation inherent in time constraints, this research topic did not cover the development, optimization, and application of gene editing systems for as many crops as possible. We hope that more original research and critical review papers on novel genome editing technologies such as CRISPR gene editing, MGE, base editing, and primer editing will be published in coming topics of Frontiers in Plant Science, helping to better guide the future research.

Author contributions

ME and KW have made a substantial, direct, and intellectual contribution to the work, and approved it for publication in Frontiers in Plant Science.

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ORIGINAL RESEARCH
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Impairment of Tomato *WAT1* Enhances Resistance to Vascular Wilt Fungi Despite Severe Growth Defects

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Verticillium dahliae is a particularly notorious vascular wilt pathogen of tomato and poses a reoccurring challenge to crop protection as limited qualitative resistance is available. Therefore, alternative approaches for crop protection are pursued. One such strategy is the impairment of disease susceptibility (*S*) genes, which are plant genes targeted by pathogens to promote disease development. In Arabidopsis and cotton, the *Walls Are Thin 1* (*WAT1*) gene has shown to be a *S* gene for *V. dahliae*. In this study, we identified the tomato *WAT1* homolog Solyc04g080940 (*SlWAT1*). Transient and stable silencing of *SlWAT1*, based on virus-induced gene silencing (VIGS) and RNAi, respectively, did not consistently lead to reduced *V. dahliae* susceptibility in tomato. However, CRISPR-Cas9 tomato mutant lines carrying targeted deletions in *SlWAT1* showed significantly enhanced resistance to *V. dahliae,* and furthermore also to *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*). Thus, disabling the tomato *WAT1* gene resulted in broad-spectrum resistance to various vascular pathogens in tomato. Unfortunately these tomato CRISPR mutant lines suffered from severe growth defects. In order to overcome the pleiotropic effect caused by the impairment of the tomato *WAT1* gene, future efforts should be devoted to identifying tomato *SlWAT1* mutant alleles that do not negatively impact tomato growth and development.

Keywords: *Verticillium*, *Fusarium*, susceptibility gene, resistance breeding, pleiotropic effect

INTRODUCTION

Vascular wilt pathogens cause diseases in many annual and perennial crops (Yadeta and Thomma, 2013). Vascular pathogens of tomato include fungi such as *Fusarium* (Michielse and Rep, 2009) and *Verticillium* (Fradin and Thomma, 2006), as well as bacteria such as *Clavibacter* (Nandi et al., 2018), *Ralstonia* (Peeters et al., 2013), and *Xanthomonas* (Potnis et al., 2015). Vascular pathogens are hard to combat once they invaded a plant host (Yadeta and Thomma, 2013). The soil-borne fungus *Verticillium dahliae* is particularly hard to control due to its wild host range that comprises hundreds of hosts and its persisting resting structures in the soil (Fradin and Thomma, 2006). Crop protection therefore relies on the use of resistant plant varieties. For *V. dahliae* only

one monogenic resistance gene, the *Ve1* tomato gene, has been cloned so far (Fradin et al., 2009). This resistance is based on the recognition of the *V. dahliae* avirulence protein Ave1 by the resistance (R) protein encoded by the *Ve1* gene (de Jonge et al., 2012). However, this resistance has been overcome by *V. dahliae* strains that have purged the *Ave1* gene, posing a reoccurring challenge for tomato cultivation (Grogan, 1979; Dobinson et al., 1996; de Jonge et al., 2012; Usami et al., 2017).

To address the recurrent problem of the breakdown of *R* gene-mediated resistance, alternative approaches can be pursued, such as the impairment of disease susceptibility (*S*) genes (Pavan et al., 2010; Gawehns et al., 2013). *S* genes are host genes that play an important role in disease establishment by the pathogen. *S* genes can function in a multitude of ways, including early recognition of the pathogen, negative regulation of immune responses, or pathogen sustenance (van Schie and Takken, 2014). Nevertheless, *S* genes also have functions for the host. *S* gene-mediated resistance, or rather loss-of-susceptibility, is achieved by circumventing the manipulation of these gene products by the pathogen, preferably whilst keeping the intrinsic function for the host intact. In wild germplasm such impaired *S* gene alleles can occur naturally, for example as loss-of-function mutations or as promoter mutations leading to impaired expression (Chu et al., 2006; Bai et al., 2008; Gao et al., 2015). Alternatively, these impairments can be introduced by random mutagenesis or by targeted genome editing, for example using CRISPR-Cas9 (Zaidi et al., 2018; Dong and Ronald, 2019). Impairment of *S* genes can be associated with severe pleiotropic effect as a consequence of not only impairment of its function for the pathogen, but also impairment of its intrinsic role for the host. For instance for the *defense no death 1* (*dnd1*) mutant, loss-of-susceptibility to *Pseudomonas syringae* is accompanied by dwarfism in Arabidopsis and tomato, and spontaneous lesion formation in potato (Sun et al., 2016). An important benefit, however, is that impairment of *S* genes can lead to non-race specific resistance to different strains of a given pathogen (Jørgensen, 1992), or even to broad-spectrum resistance to multiple pathogens (Wang et al., 2018). This illustrates the potential of *S*-gene mediated resistance in crop protection.

An example of broad-spectrum resistance to different vascular pathogens is provided by the *walls are thin 1* (*wat1*) mutant (Denancé et al., 2013). This mutant was identified in an Arabidopsis screen for cell wall mutants (Ranocha et al., 2010), and displays resistance to the bacteria *Ralstonia solanacearum* and *Xanthomonas campestris*, and the fungi *V. dahliae, Verticillium albo-atrum*, and *Plectoshaerella cucumerina* (Denancé et al., 2013). *WAT1* encodes a tonoplast-localized auxin transporter (Ranocha et al., 2013), but its exact role in so-called "vascular immunity" is not yet understood. *WAT1* orthologs have been characterized in several plant species (Ranocha et al., 2010), and recently its role as susceptibility factor in cotton was investigated, demonstrating that simultaneous transient silencing of three *WAT1* homologs resulted in increased resistance to *V. dahliae* (Tang et al., 2019). In this study, we aimed to identify the tomato *WAT1* ortholog and examine its role as a susceptibility factor in tomato for vascular pathogens such as *Verticillium* spp. and *Fusarium oxysporum*.

MATERIALS AND MATHODS

Plant and Fungi Growth Conditions

All tomato genotypes were grown in the greenhouse (Unifarm, Wageningen University & Research, Netherlands) at 21/19°C (day/night) at 60% relative humidity and a minimal light intensity of 100 W/m^2 in potting soil (Potgrond 4, Horticoop, Katwijk, Netherlands). *V. dahliae* (strain JR2, race 1), *V. albo-atrum* (strain CBS385.91, race 1) and *F. oxysporum* f. sp. *lycopersici* (stain Bt.01, race 1) were maintained on potato dextrose agar (PDA) at room temperature in the dark.

Virus-Induced Gene Silencing

Virus-induced gene silencing (VIGS) was carried out as described previously using tobacco rattle virus (TRV) (Liu et al., 2002; Fradin et al., 2009; Verlaan et al., 2013). Briefly, a gene-specific 150–300 bp fragment was amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs, Bioké, Leiden, Netherlands) with primers mentioned in **Supplementary Table 1**. The obtained fragment was cloned into the tobacco rattle virus 2 (TRV2) vector (Liu et al., 2002) using Gateway cloning and subsequently transformed into *Agrobacterium tumefaciens* strain GV3103. As negative control a TRV2 vector containing a fragment of the β-*Glucuronidase* (*GUS*) gene was used (Wu et al., 2011; Senthil-Kumar and Mysore, 2014). Moreover, a TRV2 vector carrying a fragment of the tomato *phytoene desaturase* (*PDS*) gene was used as a positive control as it triggers photobleaching upon effective silencing.

Generation of CRISPR-Cas9 and RNAi Lines

To design single guide RNAs (sgRNAs) the "CCTop – CRISPR/Cas9 target online predictor"[1] (Stemmer et al., 2015) was used and for target site evaluation the tomato genome (*Solanum lycopersicum* Solyc2.5) was used as reference. Only sgRNAs with a maximum of one exonic off-target site were selected. All sgRNAs were verified to contain a GC-content[2] between 30 and 80% and presence of required secondary structures was evaluated[3] (Zuker, 2003) according to Liang et al., 2016. Different scoring tools[4,5,6] (Wong et al., 2015; Chari et al., 2017; Sanson et al., 2018) were used to select the best sgRNAs which met most of the criteria. In total, four sgRNAs were designed (**Supplementary Table 1**).

Golden Gate Cloning (Engler et al., 2008) was used to clone the constructs, and plasmids were obtained from Addgene[7]: pICH86966 (level 0 plasmid for amplification), pICSL01009 (level 0 plasmid containing AtU6), pICH47751 (level 1 sgRNA1), pICH47761 (level 1 sgRNA2), pICH47772 (level 1 sgRNA3), pICH47781 (level 1 sgRNA4), pICH47732 (level 1 containing

[1]https://crispr.cos.uni-heidelberg.de/

[2]http://www.endmemo.com/bio/gc.php

[3]http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form

[4]https://sgrnascorer.cancer.gov/

[5]https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

[6]http://crispr.wustl.edu/

[7]https://www.addgene.org/

NPTII), pICH47742 (level 1 containing Cas9), pICH41822 (linker), and pAGM4723 (level 2 binary vector) (Weber et al., 2011). Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Bleiswijk, Netherlands) was used to amplify sgRNAs, and PCR products were purified with QIAquick PCR Purification Kit (Qiagen Benelux B.V., Venlo, Netherlands). Level 1 plasmids were digested using *Bsa*I/*Eco*31I and ligated using T4 DNA ligase (Thermo Scientific, Bleiswijk, Netherlands) and cloned into *Escherichia coli* strain DH5α (Thermo Scientific, Bleiswijk, Netherlands). Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen Benelux B.V., Venlo, Netherlands). Level 2 plasmids were digested using *Bpi*I/*Bps*I and ligated using T4 DNA ligase (Thermo Scientific, Bleiswijk, Netherlands), cloned into *E. coli* strain DH5α, purified and sequenced. All plasmids were cloned into *A. tumefaciens* strain AGL1+virG. Transformation of tomato cultivar Moneymaker (MM) was carried out as described previously (Huibers et al., 2013).

To generate the *WAT1* RNAi construct, the same fragment that was used for VIGS was cloned from the TRV2 vector into the pHellsgate8 vector (Helliwell and Waterhouse, 2003) using Gateway cloning (Katzen, 2007). Subsequently the construct was transformed into *A. tumefaciens* strain AGL1+virG. Tomato transformation of cultivar MM was carried out as described previously (Huibers et al., 2013).

Pathogen Inoculations, Phenotyping, and Fungal Biomass Quantification

Verticillium dahliae, V. albo-atrum, and *F. oxysporum* (*Fol*) inoculations were carried out with root dipping as described previously (Fradin et al., 2009; Boshoven, 2017). For phenotyping, stunting (%) between inoculated and mock-inoculated plants was calculated based on plant canopy area at 21 days post inoculation (dpi) using ImageJ (Abramoff et al., 2004) as follows:

$$stunting\ (\%) = \left(1 - \frac{canopy\ area\ of\ V.dahliae\ inoculated\ plants}{average\ canopy\ area\ of\ mock\ treated\ plants}\right) \times 100\%$$

To quantify fungal biomass, stems sections (∼ 2 cm around the cotyledons) were harvested at 21 dpi and freeze-dried for 48 h. Subsequently, material was ground, and DNA was isolated using CTAB buffer (200 mM Tris–HCl pH 7.5, 50 mM EDTA pH 8.0, 2 M NaCl, 2% CTAB). Fungal biomass was determined on genomic DNA targeting the *ITS* gene (*V. dahliae, V. albo-atrum*, and *F. oxysporum*) relative to the reference gene *SlRUB* (**Supplementary Table 1**) with the CFX96 Real-time System (Bio-Rad, Veenendaal, Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, Netherlands). Data were normalized to MM with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

DNA Isolation and Genotyping

To genotype RNAi and CRISPR plants, DNA was isolated from young leaves using CTAB buffer (1 M Tris–HCl pH 7.5, 0.5 M EDTA pH 8.0, 5 M NaCl, 2% CTAB). PCR was performed with DreamTaq DNA polymerase (Thermo Scientific, Bleiswijk, Netherlands) and corresponding primers (**Supplementary Table 1**). PCR products were sequenced by Marcrogen Europe (Amsterdam, Netherlands).

RNA Isolation and Quantitative Reverse Transcription PCR

To quantify silencing levels in RNAi plants, root material was harvested at 21 dpi and snap-frozen in liquid nitrogen. Total RNA was isolated with the MagMAX-96 Total RNA Isolation Kit (Invitrogen, Bleiswijk, Netherlands) using a KingFisher Flex System (Thermo Scientific, Bleiswijk, Netherlands) and synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, Netherlands) according to the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was carried out with the CFX96 Real-time System (Bio-Rad, Veenendaal, Netherlands) and SYBR Green Master Mix (Bio-Rad, Veenendaal, Netherlands) according to the manufacturer's instructions. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) relative to the *tomato elongation factor 1 α* (*SlEF1α*) (**Supplementary Table 2**). Data were normalized to transformants devoid of the silencing construct or, when not available, to MM plants.

RESULTS

Transient Silencing of *WAT1* in Tomato Might Reduce Susceptibility to *V. dahliae*

To identify tomato *WAT1* orthologs, the amino acid sequence of the Arabidopsis *WAT1* gene (At1g75500) was obtained from TAIR[8] and used as query in a blastP search against the Sol genomics database (ITAG release 4.0[9]). Two close homologs were identified and phylogenetic trees were constructed using Phylogeny.fr (Dereeper et al., 2008). Results showed that the tomato gene Solyc04g080940 (hereafter *SlWAT1*) has the highest homology of 74.7% to *AtWAT1* (**Figure 1A**). To functionally test the *SlWAT1* gene for a role as *S* gene in tomato, *A. tumefaciens*-mediated VIGS was used for transient silencing of *SlWAT1*. One TRV construct (TRV::SlWAT1) was made (**Figure 1B**), which resulted in a significant reduction in relative expression of *SlWAT1* to approximately 49.6% in plants treated with TRV::SlWAT1 when compared with TRV::GUS-treated plants (**Figure 1C**). To screen for reduced susceptibility to *V. dahliae* resulted from silencing *SlWAT1,* stunting based on canopy area was calculated between mock- and *V. dahliae*-inoculated plants at 21 dpi. Compared with *V. dahliae*-inoculated TRV::GUS plants, *V. dahliae*-inoculated plants treated with the TRV::SlWAT1 construct showed significantly less stunting in three out of the eight performed experiments (**Figure 1D**, panels 1, 3, and 8).

[8]https://www.arabidopsis.org/

[9]https://solgenomics.net/tools/blast/

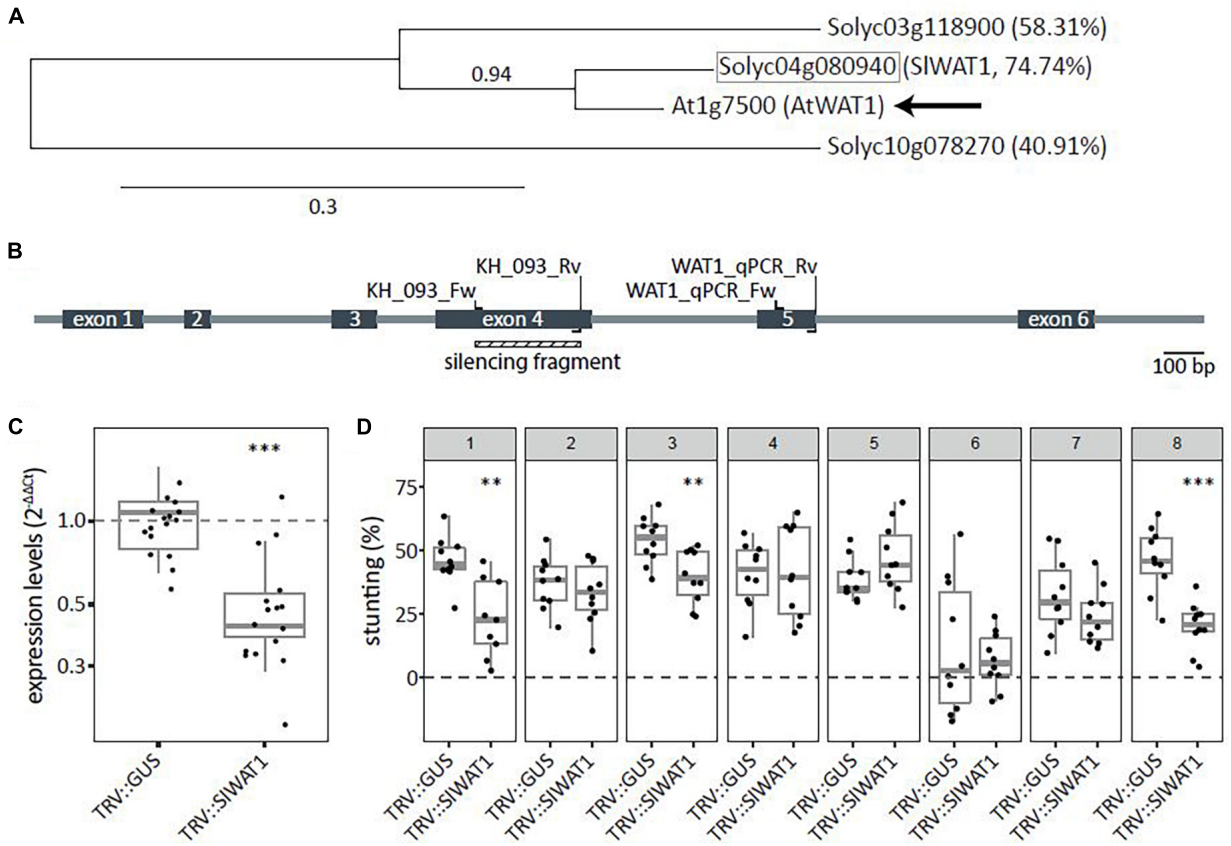


FIGURE 1 | Transient silencing of *WAT1* in tomato resulted in reduced susceptibility to *Verticillium dahliae*. **(A)** Phylogenetic tree based on amino acid sequences for *WAT1* including potential ortholog from tomato (highlighted in gray square). Percentages indicate protein sequence similarity to AtWAT1 marked with an arrow. Number above node indicate branch support values. **(B)** Schematic overview of *SlWAT1* (Solyc04g080940) with intron/exon locations as well as primers for cloning the VIGS fragment and for monitoring the *SlWAT1* expression. **(C)** Expression levels ($2^{-\Delta\Delta Ct}$) of plants treated with TRV::SlWAT1 in stems normalized to TRV::GUS 2 weeks after *Agrobacterium tumefaciens* treatment on a log10 scale. Data of two independent experiments with $n \geq 6$ per experiment per genotype (*t*-test when compared with TRV::GUS with $^{***}p < 0.001$). **(D)** Stunting (%) in *Verticillium dahliae*-inoculated plants when compared with the average of mock-inoculated plants at 21 dpi after transient silencing of *SlWAT* in eight independent experiments with $n \geq 8$ per experiment per genotype ($n \geq 8$, *t*-test when compared with TRV::GUS with $^{**}p < 0.01$ and $^{***}p < 0.001$).

RNAi Knock-Down of *SlWAT1* Did Not Confirm Loss of Susceptibility to *V. dahliae*

Since the silencing effect *via* VIGS was transient and patchy, an RNAi approach was taken to further verify the role of *SlWAT1* in *V. dahliae* susceptibility. An RNAi construct was made with the VIGS fragment (**Figure 1A**) and used to transform the tomato cultivar MM. Several primary transformants (T1) were evaluated by testing for stable integration of the silencing construct and by determining residual *WAT1* expression levels. In the T1 transformants relative *WAT1* expression varied greatly, from 11 to 270%, when compared with the expression levels found in leaves of control plants (**Supplementary Table 2**). Five T1 transformants with reduced *SlWAT1* expression were transferred to the greenhouse for T2 seed production. However, seeds were only obtained from three transformants, TV181034, TV181036, and TV181037, which were used for further study (**Supplementary Table 2**).

T2 plants derived from the three transformants, TV181034, TV181036, and TV181037, were tested for presence of the silencing construct with a NPTII- and 35S specific-PCR (**Supplementary Table 1**). This revealed that 7 out of 45 plants (15.5%), 17 out of 89 plants (19.1%), and 9 out of 90 plants (10.0%) of the plants of the T2 families TV181034, TV181036, and TV181037, respectively, did not carry the silencing construct (**Figure 2A**). When compared with plants lacking the construct (− NPTII/35S) of all families, the expression of *SlWAT1* in the roots of plants carrying the silencing construct (+ NPTII/35S) was significantly reduced, with the residual expression of on average 20.2, 54.4, and 65.5% for the family TV181036, TV181034, and TV181037, respectively (**Figure 2B**). We also determined whether the presence of the silencing construct affected plant growth in the absence of *V. dahliae* inoculation. No significant difference in canopy area of mock-inoculated plants was found for any of the T2 families compared with mock-inoculated MM plants (**Figure 2C**).

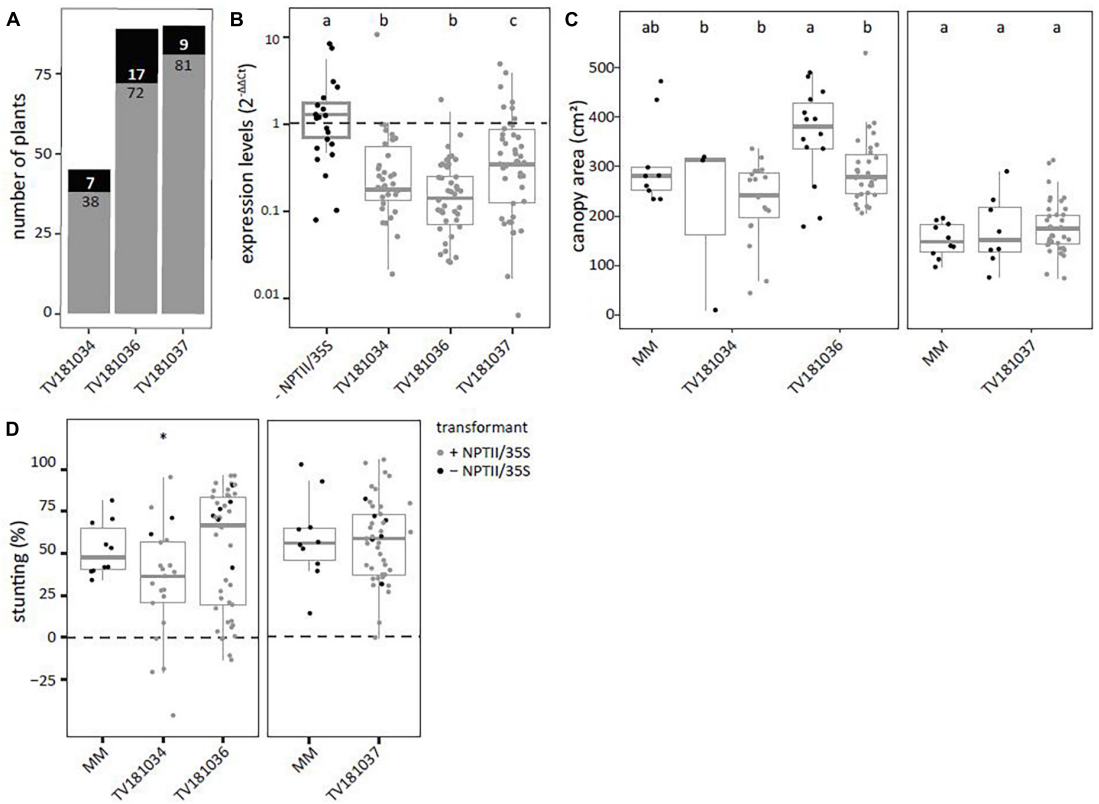


FIGURE 2 | Reduced susceptibility to *V. dahliae* was found in *SlWAT1* T2 RNAi family TV181034, but not for TV181036 and TV181037. **(A)** Total number of plants with (gray) and without (black) the silencing construct for each of the obtained T2 RNAi families. **(B)** Expression levels of *SlWAT1* in roots collected at 21 dpi of plants with the silencing construct (+ NPTII/35S, gray dots) relatively to plants without the silencing construct (– NPTII/35S, black dots) for all families. Data were normalized using $2^{-\Delta\Delta Ct}$ to *SlEF1*α with $n \geq 4$ per family (ANOVA with Fisher's unprotected LSD with $p = 0.05$ on ΔCt values). **(C)** Canopy area (cm^2) of mock-inoculated plants at 21 dpi for T2 RNAi families with (gray) and without (black) the silencing construct ($n \geq 3$, ANOVA with Fisher's unprotected LSD, $p = 0.05$). **(D)** Stunting of *V. dahliae*-inoculated plants when compared with the average stunting of mock-inoculated plants per genotype at 21 dpi ($n \geq 5$, *t*-test with $^*p < 0.05$). a–c: Genotypes having average values with a letter in common are not statistically significant different (at $P = 0.05$).

To test for loss of susceptibility to *V. dahliae*, plants from all three T2 families were challenged with *V. dahliae*. To this end, stunting based on canopy area was calculated between mock- and *V. dahliae*-inoculated plants for each of the genotypes at 21 dpi and compared with *V. dahliae*-induced stunting in MM plants (**Figure 2D**). Of the three tested families, a significant reduction in stunting of *V. dahliae*-inoculated plants was found only in the family TV181034 with a value of on average 32.2 vs 52.6% in *V. dahliae*-inoculated MM plants.

To confirm the results of the T2 generation, two plants per T2 family were kept for T3 seed production. T3 plants with and without the RNAi construct were identified (**Figure 3A**). In the T3 families derived from T2 plants of TV181034-46 and –53 as well as TV181036-54 and –59, a significant reduction of *SlWAT1* expression to 12.0, 41.4, 35.2 and 30.6%, respectively, were found in the roots of plants carrying the RNAi constructs (**Figure 3B**). Unfortunately, *SlWAT1* was not silenced at all in plants carrying the RNAi construct for families TV181037-73 and –74, as *SlWAT1* expression was on average 39.4 and 36.5% higher than in control plants lacking the RNAi construct, respectively (**Figure 3B**). No statistically significant difference in canopy area was found for plants of any of the T3 RNAi families when compared with the canopy area of MM plants (**Figure 3C**). This is in agreement with the results obtained in the T2 families (**Figure 2C**), indicating that the presence of the silencing construct did not significantly affect plant growth at this developmental stage.

The plants of the six T3 RNAi families were challenged with *V. dahliae*, and similar levels of stunting of *V. dahliae*-inoculated plants were found for most plants of the T3 families when compared with *V. dahliae*-inoculated MM plants (**Figure 2D**). Only in one T3 family, TV181034-46, stunting of *V. dahliae*-inoculated plants was significantly reduced to (on average) 39.5% when compared with *V. dahliae*-inoculated MM plants that displayed an average stunting of 56.6%.

To quantify *V. dahliae* colonization in the T3 RNAi families, fungal biomass was quantified on genomic DNA by targeting the *ITS* gene of *V. dahliae* relative to the tomato reference gene *SlRUB* (**Supplementary Table 1**) in stems of *V. dahliae*-inoculated plants at 21 dpi for each genotype. No significant reduction in fungal biomass in plants of all six T3 RNAi families was found when compared with *V. dahliae*-inoculated MM plants (**Figure 3E**).

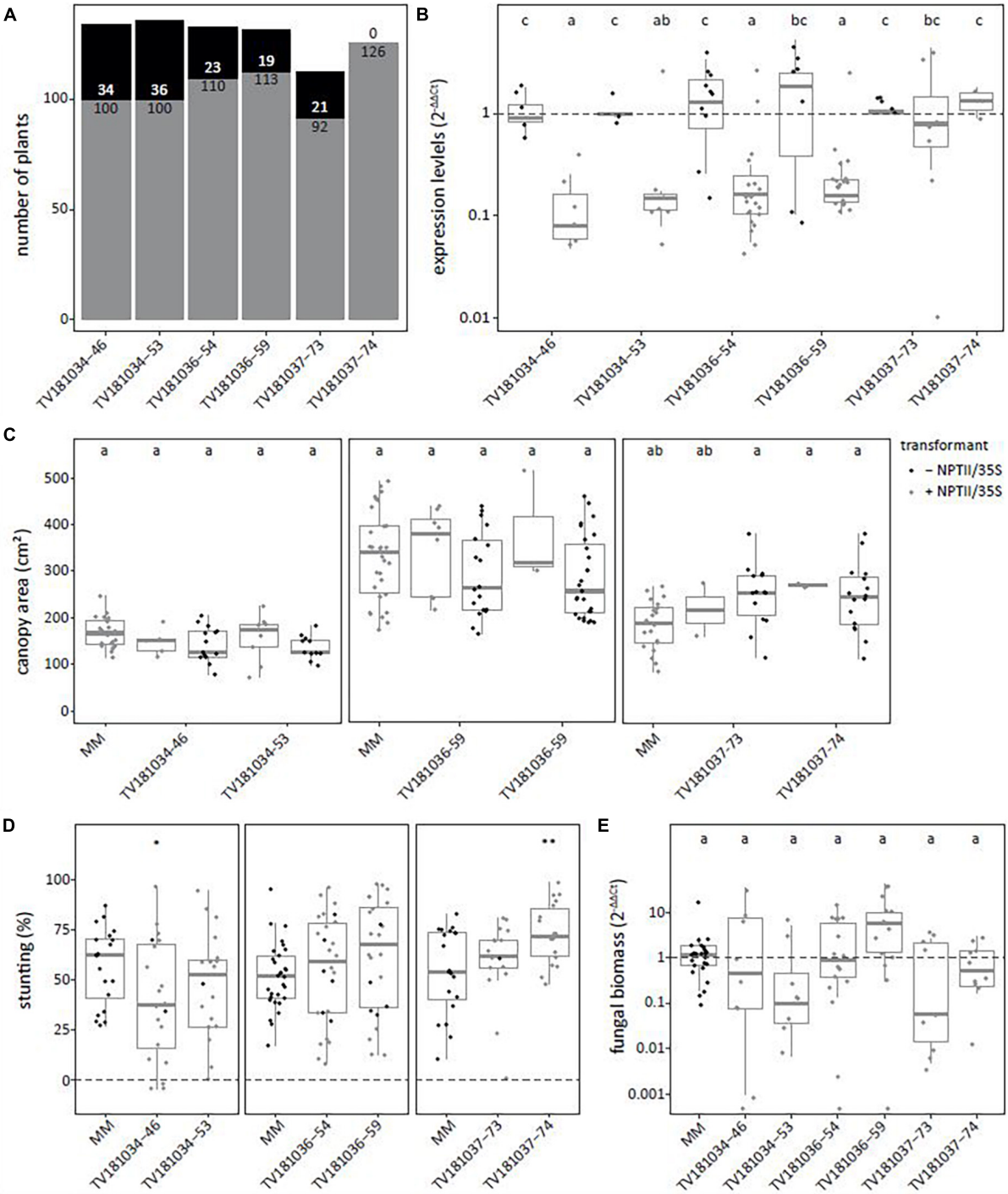


FIGURE 3 | Loss of susceptibility to *V. dahliae* was not confirmed in all *SlWAT1* T3 RNAi families. **(A)** Total number of plants with (gray) and without (black) the silencing construct for six T3 RNAi families which were obtained from three different T2 RNAi families. **(B)** Expression levels of T3 *SlWAT1* RNAi families relative to plants without the silencing construct (– NPTII/35S, gray) and normalized using $2^{-\Delta\Delta Ct}$ to *SlEF1*α with $n \geq 4$ per family with (gray) and without (black) the silencing construct (ANOVA with Fisher's unprotected LDS with $p = 0.05$ on ΔCt values). **(C)** Canopy area (cm^2) of mock-inoculated plants at 21 days post inoculation (dpi) for T3 *SlWAT1* RNAi families with (gray) and without (black) the silencing construct. Data from two independent experiments with $n \geq 2$ (ANOVA with Fisher's unprotected LSD, $p = 0.05$). **(D)** Stunting (%) of *V. dahliae*-inoculated plants when compared with the average stunting of mock-inoculated plants per genotype at 21 dpi. Box plots represent data with $n \geq 8$ plants per experimental repeat (*t*-test when compared with MM with $^{*}p < 0.05$ and $^{**}p < 0.01$). **(E)** Relative fungal biomass in the stems of RNAi plants at 21 dpi with *V. dahliae*. This was calculated as the ratio of the *V. dahliae* ITS gene amplification in comparison with the tomato *SlRUB* gene (**Supplementary Table 1**) and normalized the *V. dahliae*-inoculated MM plants using $2^{-\Delta\Delta Ct}$ on a log10 scale with $n \geq 7$ per family (ANOVA with Fisher's unprotected LSD, $p = 0.05$ on ΔCt). a–c: Genotypes having average values with a letter in common are not statistically significant different (at $P = 0.05$).

Targeted Deletion in *SlWAT1* Leads to Loss-of-Susceptibility to *V. dahliae* Despite Severe Growth Defects

In order to circumvent interference of residual *SlWAT1* expression as shown for the RNAi families, we explored approaches for targeted knock-out. To this end, stable transformants using CRISPR-Cas9 were generated. The CRISPR-Cas9 construct was designed with four sgRNAs that targeted sequences in exons 3, 4, and 5 of the *SlWAT1* gene (**Figure 4A**). The use of multiple sgRNAs increased the possibility of creating large deletions due to the occurrence of double stranded breaks at multiple sgRNAs locations simultaneously (Do et al., 2019). T1 transformants were evaluated for the occurrence of mutations in *SlWAT1* with a gene-specific PCR and gel electrophoresis to detect aberrantly sized PCR products (**Supplementary Table 1**). By focusing on large deletions, small deletions, small insertions, and single nucleotide polymorphisms might have been missed. Three transformants (#10, #19, and #28) showed a relatively large deletion, while for a fourth transformant (#23) an additional band appeared above the wild-type band (**Supplementary Figure 1**). However, T2 seeds from only one of these mutants, TV181046 (#19), were obtained as the other transformants were either not successfully transferred from *in vitro* conditions to soil, did not set fruits, or did not produce seeds.

First, plants from the T2 CRISPR family TV181046 were genotyped to confirm the presence of a mutation by sequencing. In fact, seedlings of TV181046 were found to carry bi-allelic mutations with either a smaller deletion (allele 1), or a larger deletion (allele 2), or heterozygous plants that carry both types of deletions (**Figures 4A,B**). The smaller 352 bp deletion (allele 1), located in exon 4, led to a 121 amino acid deletion, while the larger 1,291 bp deletion (allele 2), spanning exons 3, 4, and partly 5, resulted in a 197 amino acid deletion. As only one T2 CRISPR line was obtained, we propagated plants with the heterozygous deletions as well as homozygous plants for each mutant allele to obtain a larger panel of genotypes (T3) for testing. Seeds from three T3 CRISPR lines were obtained, TV181046-16, –18, and –23, genotyped and found to be heterozygous for the deletions (TV181046-16 and –23) and homozygous for allele 1 (TV181046-18) (**Figure 4B**).

As for neither of the two deletions (allele 1 and allele 2, **Figure 4C**) a premature stop codon was predicted,[10] we subsequently investigated whether these deletions affected any known domain within SlWAT1. To this end, protein domains were predicted using InterPro.[11] For wild-type SlWAT1 two EamA domains were found. Most EamA domain-containing

[10]http://www.softberry.com/berry.phtml

[11]https://www.ebi.ac.uk/interpro/

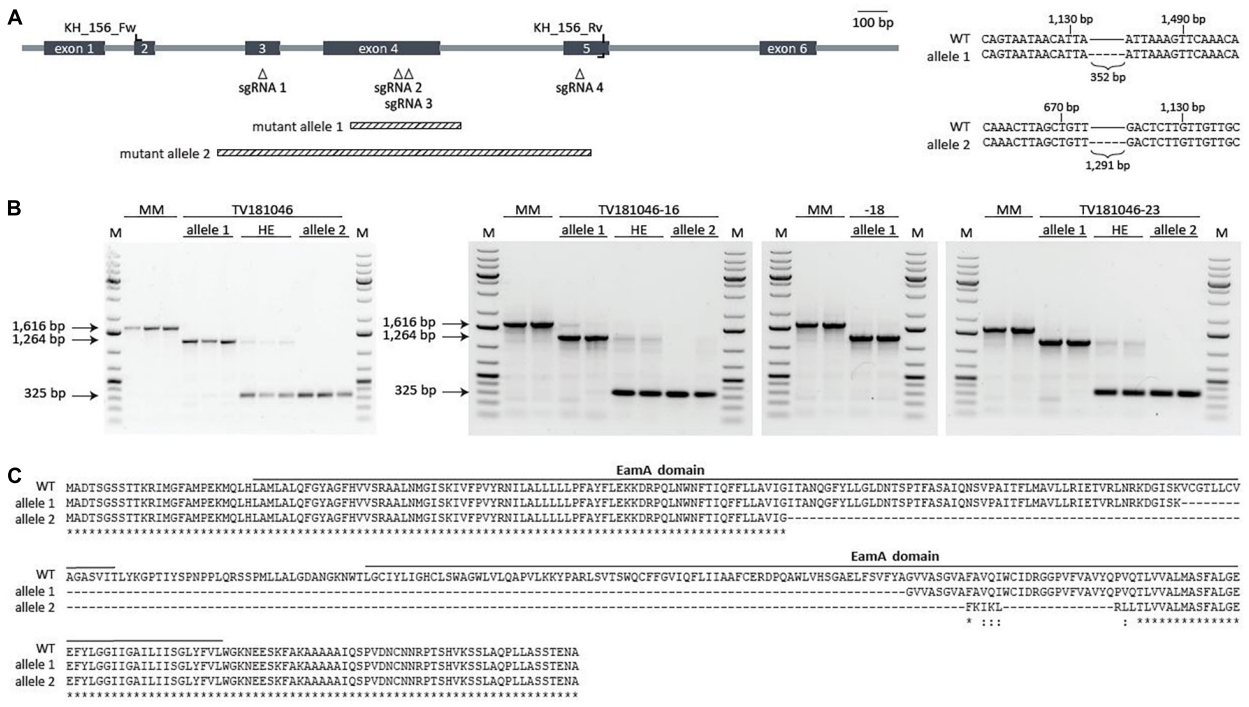


FIGURE 4 | CRISPR T2 family TV181046 and its T3 progeny carry a bi-allelic mutation in *SlWAT1*. **(A)** Schematic overview of *SlWAT1* (Solyc04g080940) indicating location of the sgRNAs, primers used for genotyping and the two mutant alleles (right). Sequencing revealed a 352 and 1,291 bp deletion for mutant alleles 1 and 2, respectively, compared with *SlWAT1* wild-type (WT) (right). **(B)** Gel electrophoresis (1% TAE, ethidium bromide) of gene-specific PCR with (wild-type band at 1,616 bp) showing the two mutant alleles as well as heterozygous (HE) plants for the T2 (left) and the T3 (right) plants with a 1 kb ladder (M). **(C)** Protein alignments of SlWAT1 wild-type (WT) with mutant alleles 1 and 2 showing a 121 and 197 amino acid deletion, respectively. Mutant alleles were translated into protein using http://www.softberry.com/berry.phtml. CLUSTAL multiple sequence alignment was done using https://www.ebi.ac.uk/Tools/msa/muscle/. Solid bars indicate predicted protein domains annotated as EamA domain (https://www.ebi.ac.uk/interpro).

proteins are classified as metabolite transporters that usually carry two copies of this domain (Jack et al., 2001). For the SlWAT1 mutant alleles both predicted EamA domains were affected (**Supplementary Figure 2**). As *AtWAT1* is located in the tonoplast (Ranocha et al., 2010), and also because many EamA domain-containing proteins carry multiple transmembrane domains, we further predicted the transmembrane domains for wild-type SlWAT1, mutant allele 1 and mutant allele 2. Wild-type SlWAT1 was predicted to contain ten transmembrane domains which was described before for WAT1 in Arabidopsis and cotton as well (Ranocha et al., 2010; Tang et al., 2019). For mutant allele 1 only seven out of ten transmembrane domains were found and for mutant allele 2 only four (**Supplementary Figure 2**). Collectively, our data suggested that both mutant alleles carry a deletion which affects known domains in *SlWAT1,* presumably leading to loss of its biological function. This allows us to further study these knock-out CRISPR lines for loss of susceptibility to *V. dahliae.*

Irrespective of the type of mutation, all plants of the T2 and the T3 generation displayed severe growth and development defects (in absence of *V. dahliae* inoculation); the germination rate was low, seedlings were small and light in color, and overall plant growth remained severely compromised (**Figure 5** and **Supplementary Figure 3**). To quantify the size difference, we determined canopy area of mock-inoculated plants for all genotypes in the absence of *V. dahliae* inoculation. For MM Plants, canopy area of mock-inoculated plants was heavily reduced when compared with mock-inoculated plants measured at 21 dpi. While the canopy area of mock-inoculated MM plants was 300 cm^2 on average, the canopy area of most mock-inoculated CRISPR T2 and 3 plants was less than 10 cm^2. However, the observed aberrations alleviated slowly during further plant development and even though the CRISPR plants remained smaller than MM plants, they developed flowers and set fruits (**Supplementary Figure 3**).

To test for loss of susceptibility, plants of the T2 and the T3 generation were inoculated with *V. dahliae*. Stunting based on canopy area was calculated between mock- and *V. dahliae*-inoculated plants for each of the genotypes. Stunting of *V. dahliae*-inoculated T2 plants was significantly reduced to on average 7.1% for line TV181046 when compared with *V. dahliae*-inoculated MM plants with on average 65.5% stunting (**Figure 6A** and **Supplementary Figure 4**). In the T3 families, stunting of TV181046- 16–, –18, and –23 was significantly reduced to 41.7, 1.4, and 26.2% on average, respectively, compared with *V. dahliae*-induced stunting of 60.0% on average in MM plants. Due to the stunting calculations being based on the average of mock-inoculated plants and due to variation in plant size observed in the mutant lines, the differences in stunting of *V. dahliae*-inoculated plants were pronounced in the mutant lines when compared with the MM plants. To quantify the effect on *V. dahliae* proliferation, fungal biomass was

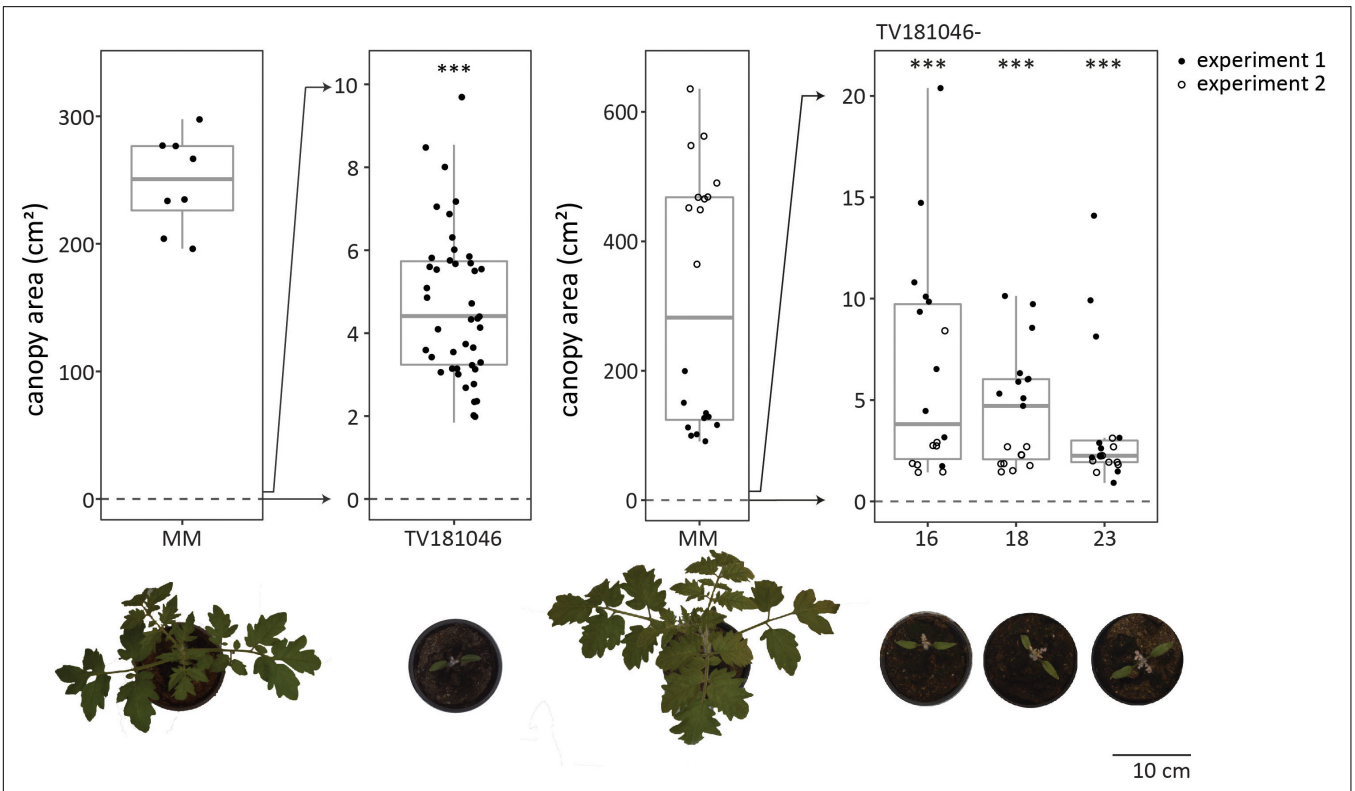


FIGURE 5 | CRISPR T2 family TV181046 and its T3 progeny display severe growth and development defects. Canopy area of mock-inoculated plants at 21 dpi for T2 CRISPR line TV181046 (left) and T3 CRISPR lines TV181046-16, –18, and –23 (right). Data from one or two independent experiments with $n \geq 8$ (*t*-test compared with MM with $^{***}p = 0.001$).

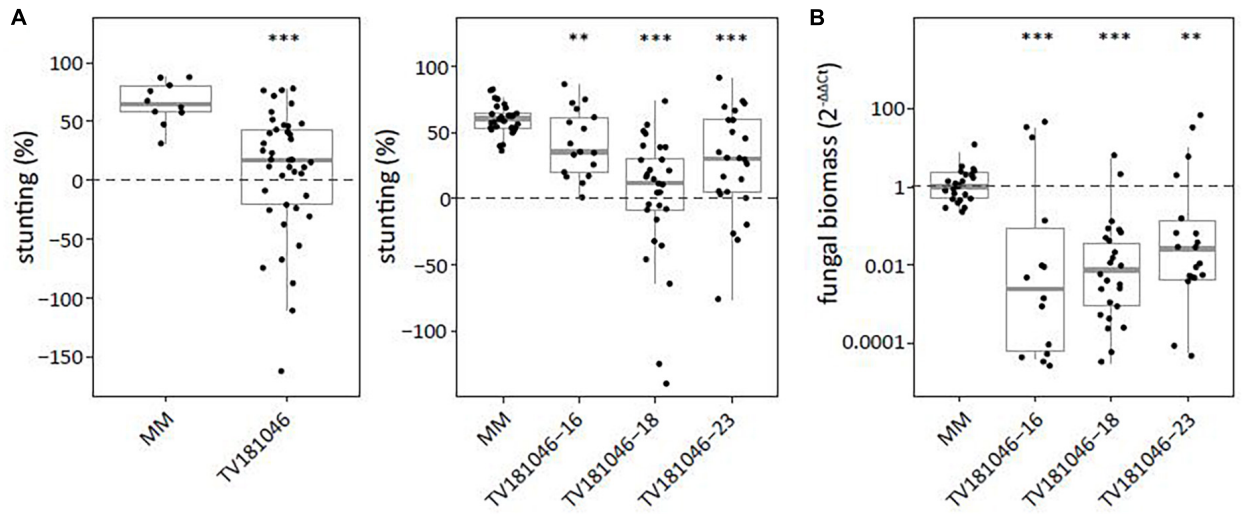


FIGURE 6 | Targeted knockout of *SlWAT1* lead to loss of susceptibility to *V. dahliae.* **(A)** Stunting (%) of *V. dahliae* inoculated T2 (left) and T3 (right) plants when compared with the average stunting of mock-inoculated plants at 21 days post inoculation (dpi). Box plots represent data with $n \geq 9$ plants per experimental repeat (*t*-test when compared with MM with $^{**}p < 0.01$ and $^{***}p < 0.001$). **(B)** Fungal biomass of *V. dahliae*-inoculated T3 CRISPR plants of all three lines in stems at 21dpi. This was calculated as the ratio of the *V. dahliae* ITS gene amplification in comparison with the tomato reference *SlRUB* gene (**Supplementary Table 1**) and normalized to *V. dahliae*-inoculated MM plants using $2^{-\Delta\Delta Ct}$ on a log10 scale with $n \geq 9$ repeat per experimental repeat (*t*-test on ΔCt when compared with MM with $^{**}p < 0.01$ and $^{***}p < 0.001$).

determined in stems of *V. dahliae*-inoculated plants at 21 dpi for each genotype. In the *V. dahliae*-inoculated plants of all CRISPR T3 families, fungal biomass was significantly reduced to around 1% of the biomass in the *V. dahliae*-inoculated MM plants (**Figure 6B**).

Targeted Deletion in *SlWAT1* Leads to Loss-of-Susceptibility to *V. albo-atrum* and *Fusarium oxysporum* f. sp. *Lycopersici*

As *S* gene-mediated resistance can lead to broad-spectrum resistance to multiple pathogens (Wang et al., 2018), we also challenged T3 CRISPR plants with *V. albo-atrum* and *Fol.* For both pathogens, inoculated T3 CRISPR plants showed significantly reduced stunting when compared with inoculated MM plants (**Figure 7A**). Moreover, fungal biomass was significantly reduced in *V. albo-atrum*- and *Fol*-inoculated T3 CRISPR plants of all three lines when compared with inoculated MM plants (**Figure 7B**).

DISCUSSION

For vascular pathogens such as *V. dahliae,* for which only few sources of monogenic resistance are known, crop protection mainly relies on alternative strategies. The impairment of *S* genes has gained increasing attention in resistance breeding over the last years (Pavan et al., 2010; Gawehns et al., 2013; van Schie and Takken, 2014), particularly in the light of recent advances in genome editing in plants (Andolfo et al., 2016; Langner et al., 2018; Zaidi et al., 2018; Yin and Qiu, 2019). Here, we show that targeted deletion of *SlWAT1* using CRISPR-Cas9 led to loss of susceptibility to *V. dahliae* in tomato. Plants of T3 CRISPR lines showed reduced disease symptoms upon challenge with *V. dahliae* as well as reduced fungal biomass when compared with susceptible MM plants (**Figure 6**). The loss of susceptibility to *V. dahliae*, as observed in plants of the CRISPR lines, could not be demonstrated consistently in plants carrying the RNAi silencing construct (**Figures 1**, **2**). This can likely be attributed to the relatively high degree of residual *WAT1* expression in most plants of the T2 and T3 RNAi lines, which likely compromised the efficacy of silencing too much to monitor effects on *V. dahliae* infection.

In *WAT* studies in Arabidopsis and cotton, reduced Verticillium wilt symptoms and reduced fungal proliferation were observed in knock-out mutants or upon transient silencing of *WAT1*, respectively (Denancé et al., 2013; Tang et al., 2019). Remarkably, the loss of susceptibility in Arabidopsis *wat1* mutants was further extended to other vascular pathogens including bacteria and fungi (Denancé et al., 2013). *S* gene-mediated broad-spectrum resistance to multiple pathogens was described before (Wang et al., 2018), and highlights the potential of using impaired *S* genes for the control of multiple pathogens simultaneously. In fact, we also demonstrated loss of susceptibility of knock-out *slwat1* mutants to another pathogenic *Verticillium* species, *V. albo-atrum,* as well to another vascular pathogen, *Fol* (**Figure 7**). Also for these pathogens, disease symptoms and fungal biomass were significantly reduced when compared with susceptible MM plants. Together, this indicates that the function of *WAT1* in susceptibility to different vascular pathogens seems to be conserved across

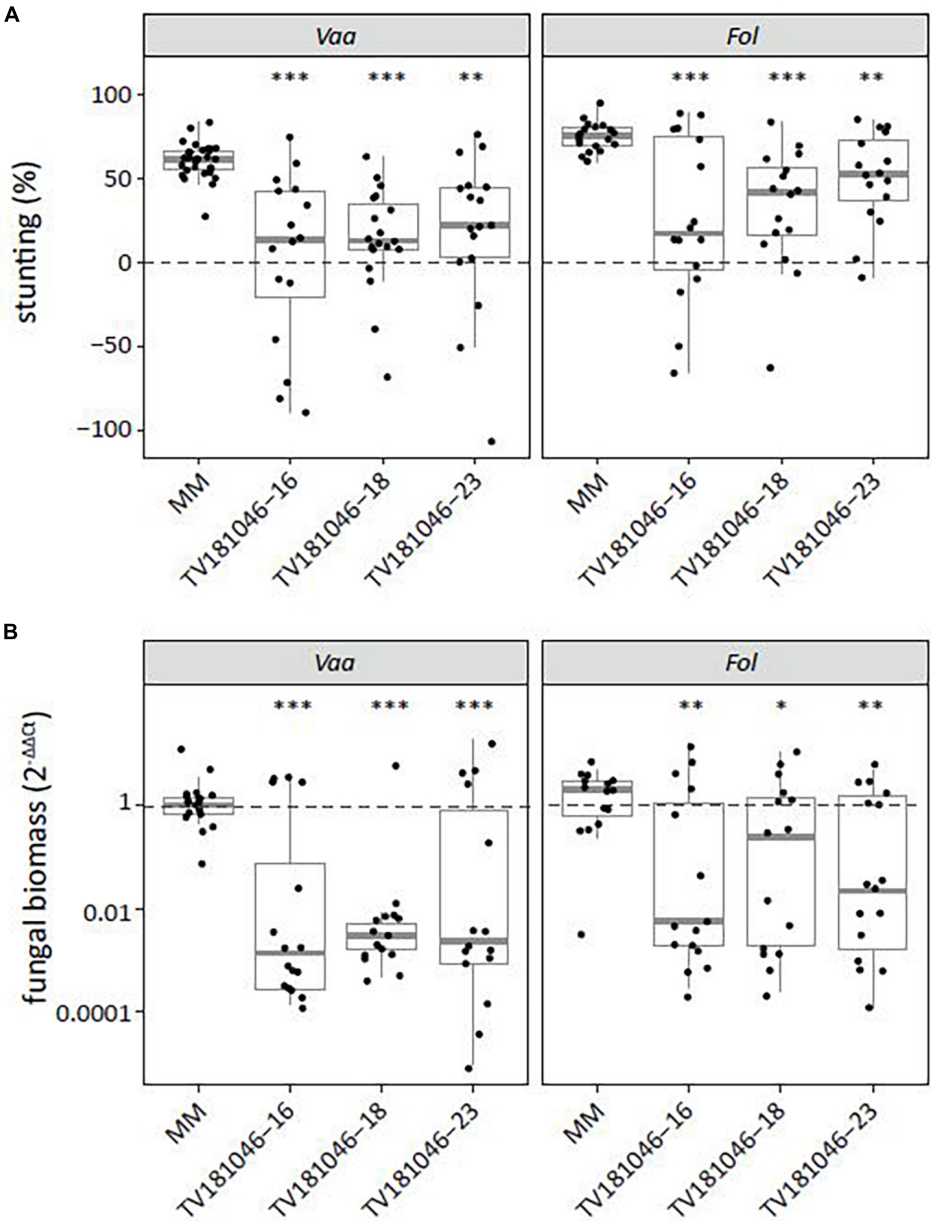


FIGURE 7 | Knockout of *SlWAT1* lead to loss-of-susceptibility to *V. albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. **(A)** Stunting (%) of *V. albo-atrum* (*Vaa*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) inoculated T3 plants when compared with the average stunting of mock-inoculated plants at 21 days post inoculation (dpi). Box plots represent data with $n \geq 7$ plants per experimental repeat (*t*-test when compared with MM with $^{**}p < 0.01$ and $^{***}p < 0.001$). **(B)** Fungal biomass of *Vaa*- and *Fol*-inoculated T3 CRISPR plants at 21 dpi. This was determined on genomic DNA targeting the *ITS* gene (*Vaa* and *Fol*) relative to the tomato reference gene *SlRUB* (**Supplementary Table 1**) and then normalized to *Vaa* and *Fol* inoculated MM plants using $2^{-\Delta\Delta Ct}$ on a log10 scale with $n \geq 7$ per experimental repeat (*t*-test on ΔCt when compared with MM with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

plant species, and therefore impairment of *WAT1* might offers an approach to combat different vascular pathogens in multiple crops.

To date, the function of *WAT1* in so called "vascular immunity" remains to be elucidated. *WAT1* was originally identified in a cell wall mutant screening in zinnia (*Zinnia elegans*) (Pesquet et al., 2005; Ranocha et al., 2010) and the homolog of Arabidopsis was shown to be a tonoplast-localized auxin transporter (Ranocha et al., 2010). In Arabidopsis *wat1* mutants, cell wall-related phenotypes in stems were described with altered cell elongation and reduced secondary cell walls of fiber cells, hence its name *Walls Are Thin 1*. Furthermore, *wat1* mutants showed altered contents of auxin (indole-3-acetic acid, IAA), tryptophan and salicylic acid (SA) (Ranocha et al., 2010; Denancé et al., 2013). The IAA content in roots was reduced in *wat1* mutants while the SA content was found to be elevated when

compared with wild-type plants (Ranocha et al., 2010), which is in line with the previously described antagonism of auxin and SA in plant immunity and development (Wang et al., 2007; Robert-Seilaniantz et al., 2011). SA does not seem to play a role in basal plant defense against *V. dahliae* as different Arabidopsis mutants with a deficiency in SA signaling, such as *enhanced disease susceptibility* (*eds1-2* and *eds5-1*), *nonexpresser of PR genes* (*npr1-1* and *npr1-3*), and *phytoalexin deficient 4* (*pad4-1*), show similar symptoms and levels of fungal biomass as control plants upon *V. dahliae* infection (Pantelides et al., 2010; Fradin et al., 2011). In contrast, a role was assigned to auxin in *V. dahliae* susceptibility as two auxin receptor mutants, *auxin signaling F-box 1* and *3* (*afb1* and *afb3*), as well as auxin transporter mutant *auxin resistant 4* (*axr4*) display reduced symptoms and less fungal biomass upon challenge with *V. dahliae* (Fousia et al., 2018). For another vascular wilt pathogen, *F. oxysporum*, two transcription factor mutants, *auxin response factor 1* and *2* (*arf1* and *arf2*), showed significantly less disease levels although fungal biomass was not quantified (Lyons et al., 2015). Collectively, auxin seems to play a crucial role in *V. dahliae* susceptibility to vascular wilt fungi, and therefore auxin-related genes may be further studied to test their potential as susceptibility factors for *V. dahliae.*

Even though *SlWAT1* CRISPR plants of tomato showed loss of susceptibility to *V. dahliae*, *V. albo-atrum*, and *F. oxysporum*, the targeted deletion in *SlWAT1* was accompanied by severe growth defects. It may be argued that the significantly reduced stunting and reduced fungal biomass accumulation in the *SlWAT1* CRISPR plants is an indirect consequence of the dramatically impaired growth and development of these mutants. In our experiments, we have performed fungal inoculations on 10-day-old seedlings. Since the observed developmental aberrations alleviated slowly during further plant development, it may be worthwhile to inoculate tomato *SlWAT1* CRISPR plants at a later time point than the control plants, when both plants have a similar overall appearance, for example by inoculating 1-month-old *SlWAT1* CRISPR plants. However, this approach obviously has the downside that mutant and control plants will not be tested at the same age.

Impairment of *S* genes is known to cause pleiotropic side effects in some cases (Clough et al., 2000; Sun et al., 2016), and also for *WAT1* such effects were described in other plant species. For Arabidopsis *wat1* mutants, no abnormalities were found in early stages of development, but older plants were stunted when compared with wild-type plants (Ranocha et al., 2010). Transient silencing of *WAT1* in cotton resulted in reduced root length and shorter first internodes (Tang et al., 2019). Such growth defects can certainly be attributed to the imbalance between auxin and SA. Firstly, it is well known that auxin plays an essential role in many aspects of plant development (Korver et al., 2018) and its downregulation, as shown in Arabidopsis *wat1* mutants, might negatively affect growth. Secondly, Arabidopsis *wat1* mutants also showed higher SA levels, which is known to affect plant growth as observed in the *constitutive expressor of PR genes 5* (*cpr5*) mutant which shows high SA levels accompanied by severe dwarfism (Bowling et al., 1997). Evidently, pleiotropic effects of impaired *S* genes are not desirable for breeding purposes, as it might affect yield but also overall development (Hückelhoven et al., 2013; Engelhardt et al., 2018). Additionally, special attention should also be given to resistance against other pathogens as an altered hormone balance, as observed in *WAT1*-mediated resistance (Denancé et al., 2013), can influence resistance to other pathogens (Thomma et al., 1998). Therefore, alternatives for obtaining mutants without such pleiotropic effects need to be explored. For example, potential natural allelic variants of *WAT1* in wild germplasm that can no longer be exploited by the pathogen, but that do not display pleiotropic effects, could be used for breeding. Alternatively, mutant populations can be used to identify *wat1* mutants omitting the severe growth defects. Certainly, mutants with smaller targeted deletions or even single base pair changes could also be studied, to find essential domains that are only required by *Verticillium* spp. for disease development, but that are not involved in tomato growth and development. Lastly, targeted modification in the promoter region of a *S* gene could circumvent pleiotropy by preventing binding the pathogen effector, as it was demonstrated by the *xa13*-mediated resistance against bacterial blight (Chu et al., 2006; Zaka et al., 2018).

In the case of *WAT1*, two Arabidopsis *wat1* mutant alleles that each carry a T-DNA insertion have been described (Ranocha et al., 2010). The T-DNA insertion located 55 bp upstream of the ATG translation start codon leads to lack of *WAT1* expression in the *wat1-1* mutant line. In contrast, the *wat1-2* mutant line that carries the T-DNA insertion 28 bp downstream of the stop codon has about 50% residual *WAT1* expression when compared with wild-type plants. Compared with *wat1-1*, a less strong phenotype was displayed by *wat1-2* plants (Ranocha et al., 2010). In our study, the tomato *WAT1* RNAi plants had a similar canopy area to the control MM plants until 6 weeks after sowing (**Figure 2C**). This finding may indicate that the residual *WAT1* expression in the tomato RNAi lines is sufficient to prevent negative effects on plant growth. Unfortunately, the *WAT1* expression levels were not sufficiently reduced to hamper *Verticillium* infection in most RNAi tomato plants, since a significant reduction in stunting upon *V. dahliae* inoculation was only observed in the T2 family TV181034 (**Figure 2**) and the derived T3 family TV181034-46 (**Figure 3**). Variation in stunting was shown between individual T2 and T3 plants, with some plants showing a very low level of stunting (**Figures 2**, **3**), which may be associated with variation in residual *WAT1* expression. Therefore, in further studies, it could be worthwhile to determine the residual level of *WAT1* expression and correlate that with stunting levels of individual RNAi plants in order to assess a direct correlation between these characteristics. Such direct correlation justifies the further search for a natural *wat1* alleles or the generation of mutations in the promoter of the *WAT1* gene that can associate reduced *WAT1* expression with reduced *Verticillium*-induced stunting.

Summarizing, it remains challenging to identify *WAT1* alleles in tomato, as well as in other crops, that cannot be exploited by *Verticillium* spp. for disease development, yet that do not negatively impact plant growth and development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KH, HS, BT, and YB conceived the study and wrote the manuscript. KH designed and performed the experiments and analyzed the data. DS, SC, and MO helped in collecting data. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.721674/full#supplementary-material

Supplementary Figure 1 | Gel electrophoresis (1% TBE, Gelred, image colors inverted) of gene-specific PCR with primers KH_156 on primary transformants (T1) of plants transformed with *SlWAT1* CRISPR-Cas9 construct. Wild-type PCR product (1,616 bp) indicated with an arrow and highlighted are mutants #10, #19, #23, and #28 that were transferred to the greenhouse, and of which seeds were only obtained from #19 (TV181046).

Supplementary Figure 2 | Predicted transmembrane domains for wild-type SlWAT1 (left), mutant allele 1 (right), and mutant allele 2 (bottom). Graphs were generated with TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Supplementary Figure 3 | CRISPR T2 family TV181046 and its T3 progeny TV181046-16, –18, and –23 display severe growth and development defects. Pictures of MM and T3 CRISPR WAT1 plants at different time points.

Supplementary Figure 4 | Canopy area pictures mock- and *V. dahliae*-inoculated (strain JR2) plants for CRISPR T2 family TV181046 and T3 families TV181046-16, –18, and –23 at 21 dpi compared to MM plants of respective experiment.

Supplementary Table 1 | Primers used in this study.

Supplementary Table 2 | Overview of primary transformants (T1) with presence/absence of silencing construct (NPTII/35S), relative *SlWAT1* expression normalized to control plants at 1 ($2^{-\Delta\Delta Ct}$) and plants from which T2 seeds were obtained.

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Concurrent Disruption of Genetic Interference and Increase of Genetic Recombination Frequency in Hybrid Rice Using CRISPR/Cas9

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Manipulation of the distribution and frequency of meiotic recombination events to increase genetic diversity and disrupting genetic interference are long-standing goals in crop breeding. However, attenuation of genetic interference is usually accompanied by a reduction in recombination frequency and subsequent loss of plant fertility. In the present study, we generated null mutants of the *ZEP1* gene, which encodes the central component of the meiotic synaptonemal complex (SC), in a hybrid rice using CRISPR/Cas9. The null mutants exhibited absolute male sterility but maintained nearly unaffected female fertility. By pollinating the *zep1* null mutants with pollen from *indica* rice variety 93-11, we successfully conducted genetic analysis and found that genetic recombination frequency was greatly increased and genetic interference was completely eliminated in the absence of *ZEP1*. The findings provided direct evidence to support the controversial hypothesis that SC is involved in mediating interference. Additionally, the remained female fertility of the null mutants makes it possible to break linkage drag. Our study provides a potential approach to increase genetic diversity and fully eliminate genetic interference in rice breeding.

Keywords: genetic diversity, genetic interference, genetic recombination, *ZEP1*, synaptonemal complex, hybrid rice, genome editing

INTRODUCTION

Plant breeding aims to develop superior varieties to suit the needs of farmers and consumers (Moose and Mumm, 2008). However, innovations in breeding materials strongly depend on creating novel allele combinations that bring together advantageous alleles and remove linked, disadvantageous alleles. This is traditionally limited by the number of crossovers (COs) during meiosis (Taagen et al., 2020). To generate sufficient genetic diversity, breeders and geneticists are exploring approaches to increase the CO frequency, alter CO distribution, or induce COs between non-homologous chromosomal regions (Mieulet et al., 2018; Blary and Jenczewski, 2019).

The synaptonemal complex (SC) is a meiosis-specific structure involved in CO formation and chromosome segregation (Gao and Colaiácovo, 2018). SC assembly starts from early prophase I and forms a tripartite structure at pachytene, which consists of axial or lateral

elements, transverse filaments, and central element (Moses, 1969; de Boer and Heyting, 2006; Fraune et al., 2016). SC dynamics are tightly regulated by its components for the proper SC assembly (Gao and Colaiácovo, 2018). Among them, TF proteins locate at the central of SC and act important roles in bridging the parallel homologous axes (Dubois et al., 2019). Usually, the N-terminal domain of the TF protein is positioned in the middle of the SC, while its C terminus is located next to the lateral elements (Anderson et al., 2005; Schild-Prüfert et al., 2011). Such special organization pattern is critical for the function of the SC. The first reported TF protein gene is *ZIP1*, which was identified in budding yeast (Sym et al., 1993; Storlazzi et al., 1996). *zip1* mutants exhibit defects in SC formation and genetic recombination; however, its homologous genes in other organisms appear to play opposite roles on CO frequency. *Sycp1* encodes a TF protein in mouse, and *Sycp1*$^{-/-}$ mice shows 90% of COs disappeared (de Vries et al., 2005). Mutation of *c*(3)*G*, the TF protein gene in *Drosophila*, causes the loss of all COs (Page and Hawley, 2001). Unexpectedly, the *syp-1* mutants in the roundworm *Caenorhabditis elegans* exhibit a severe reduction in COs (MacQueen et al., 2002), while knockdown of *SYP-1* by RNA interference (*RNAi*) increases the CO frequency (Libuda et al., 2013). The *ZIP1* orthologs have also been identified in plant species including *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*; Higgins et al., 2005; Wang et al., 2010; Golubovskaya et al., 2011; Khoo et al., 2012; Barakate et al., 2014). Suppression of *ZYP1* in *Arabidopsis thaliana* or in barley (*H. vulgare*) led to reduced CO frequency (Higgins et al., 2005; Barakate et al., 2014). In contrast with *Arabidopsis* and barley, the *Tos17* insertion mutant lines of *ZEP1* in rice had more COs than the wild type (WT; Wang et al., 2010; Wang K. et al., 2015).

Genetic interference, a phenomenon where the occurrence of one crossover (CO) inhibits the formation of other COs nearby on the same chromosome pair, imparts a remarkable level of regulation of the number and distribution of COs per chromosome in eukaryotes (Sun et al., 2017; Otto and Payseur, 2019). Therefore, reduction or disruption of genetic interference can be considered to increase genetic diversity in crop breeding (Taagen et al., 2020). Presently, a number of studies have revealed that the strength of genetic interference increased as the extent of synapsis increased (Libuda et al., 2013; Zhang et al., 2014; Wang K. et al., 2015). However, in some organisms, evidence indicates that genetic interference is exerted prior to the SC assembly (Bishop and Zickler, 2004; Fung et al., 2004). Partly because of the lack of null TF mutants, the exact role of SC in CO interference is still enigmatic. Most recently, the null mutants of *zyp1* have been generated in *Arabidopsis thaliana*, and cytological and genetic analysis reveals that ZYP1 is required for CO interference (Capilla-Pérez et al., 2021; France et al., 2021). However, researches of null mutants of TF protein genes in cereal crops are scarce to date.

In the present study, we selected the inter-subspecific hybrid rice variety Chunyou84 (CY84), an elite inter-subspecific hybrid rice from a cross between the maternal Chunjiang 16 A (16 A), a *japonica* male-sterile line, and the paternal C84, an *indica-japonica* intermediate-type line, as the acceptor and used the CRISPR/Cas9 technology to generate *zep1* null mutants. By genetic analysis, we revealed that genetic interference is completely eliminated in the absence of *ZEP1*, indicating an important role of SC in mediating interference in rice. In addition, the way of generating *zep1* null mutants with increased genetic recombination frequency and eliminated genetic interference is a promising approach to increase genetic diversity in rice breeding.

MATERIALS AND METHODS

Vector Construction

The CRISPR-Cas9 vector for complete knockout of *ZEP1* was constructed by the isocaudomer ligation method, as described in Wang C. et al. (2015). The annealed *ZEP1* g++/*ZEP1* g-- oligonucleotides (**Supplementary Table 1**) were ligated into the SK-sgRNA vector that digested with *AarI*. Then, the sgRNA of *ZEP1* (digested with *KpnI* and *BglII*) was assembled into the pC1300-*Actin*:Cas9 binary vector (digested with *KpnI* and *BamHI*) to obtain the vector pC1300-*Actin*:Cas9-sgRNA^{ZEP1} for generation of *zep1* null mutants.

Rice Transformation and Growth Conditions

The binary vector pC1300-*Actin*:Cas9-sgRNA^{ZEP1} was introduced into *Agrobacterium tumefaciens* (Strain EHA105). The *Agrobacterium* was then transformed into calluses derived from CY84 to generate transgenic lines. Fifteen independent transgenic plants of pC1300-*Actin*:Cas9-sgRNA^{ZEP1} were obtained and grown in summer in the transgenic paddy fields of the China National Rice Research Institute in Hangzhou, China.

Mutant Screening

Genomic DNA of transgenic plants was extracted from approximately 50 mg fresh leaf tissue *via* the cetyltrimethylammonium bromide (CTAB) method. PCR was performed with KOD FX DNA polymerase (Toyobo, Japan) to amplify the fragments surrounding *ZEP1* target site. Primers *ZEP1* F and *ZEP1* R are listed in **Supplementary Table 1**. The mutants were detected according to the published Hi-TOM procedure (Liu et al., 2019).

Cytological Analyses

Panicles at meiosis stage were harvested and fixed in Carnoy's solution (ethanol:glacial acetic acid, 3:1) for more than 24 h at room temperature. Microsporocytes were squashed on a slide with a needle and covered with a coverslip. Then, slides were frozen in liquid nitrogen, and the coverslips were removed rapidly with a blade. Chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vector Laboratories, Burlingame, CA, United States) as described in (Wang et al., 2009). Fluorescence microscopy was conducted using an Olympus BX61 fluorescence microscope fitted with a micro charge-coupled device camera.

Fluorescence Immunolocalization

Fresh young rice panicles were fixed in 4% (w/v) paraformaldehyde for 45 min at room temperature. Anthers at proper meiosis stage were squashed with a needle in PBS solution and covered with a coverslip. After soaking in liquid nitrogen and removing the coverslip, slides were then incubated in a humid chamber at 37°C for 4 h with anti-REC8 (Mouse) and anti-ZEP1 (rabbit) polyclonal antibodies (diluted 1:500 in TNB buffer: 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent). After three rounds of washing in PBS, Texas red-conjugated goat anti-mouse antibody and fluorescein isothiocyanate-conjugated sheep anti-rabbit antibody (1:1,000) were added to the slides. The chromosomes were counterstained with DAPI in an antifade solution (Vector Laboratories, Burlingame, CA, United States). Fluorescence microscopy was performed as described above.

Genotyping *via* Hi-TOM

Single nucleotide polymorphisms (SNPs) markers were developed on the basis of the whole-genome sequences of C84 and chunjiang16A (Wang et al., 2019). The primers are listed in **Supplementary Table 1**. The Hi-TOM method was performed for the genotyping as previously described in (Liu et al., 2019).

Interference Strength Analysis

The analysis of interference strength was described in Wang K. et al. (2015). For each pair of adjacent intervals, the coefficient of coincidence analysis compared the observed frequency of CO occurring in both intervals with the frequency expected if the CO occurred independently in the two intervals, with the interference strength calculated as (1-observed/expected). The expected number of chromosomes with CO occurring in both intervals (XY and YZ) was calculated as: (recombination frequency between X and Y) × (recombination frequency between Y and Z) × (number of chromosomes examined). See the details in **Supplementary Tables 2** and **3**.

Genetic Recombination Analysis

The WT (CY84) and *zep1-KO* plants were crossed with the *indica* variety 93-11 to generate the segregation population. The hybrid seeds were soaked in deionized water at 37°C in the dark for 2 days and then transferred to a net floating on deionized water for further 5 days. The seedlings were cultured in a half-strength Kimura B nutrient solution (pH 5.4; Liu et al., 2020). The genotypes of each plant in the segregation population were identified by Hi-TOM. The genotypes of each female gamete of CY84 and *zep1-KO* plants were deduced and listed in **Supplementary Tables 4**–**7**. Then, they were used for the frequency and distribution of genetic recombination analysis. For each pair of adjacent markers (X and Y), the recombination frequency was calculated as: (number of chromosomes with one CO between X and Y/number of chromosomes examined).

RESULTS

Selection of Gene-Editing Target Site and Acquisition of *ZEP1* Mutants

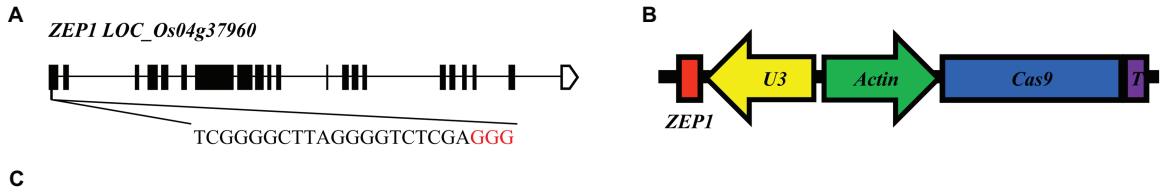
ZEP1 has a full length of 9.478-kbp, distributed in 21 exons and 20 introns and encodes a transverse filament protein containing 869 amino acids (**Figure 1A**). ZEP1 comprises coiled-coil proteins with globular domains at its N termini from amino acids 1 to 63 and C termini from amino acids 714 to 869. The C-terminal globular domain of 156 residues putatively binds DNA, while the N-terminal globular domain of 63 residues is basic (Wang et al., 2010). The *Tos17* insertion mutant lines of *ZEP1* used in previous studies are partial loss-of-function mutants (Wang et al., 2010; Wang K. et al., 2015), which still contain the N-terminal residues. To generate *zep1* knockout mutants, we employed the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system to edit *ZEP1* and chose the 19th to the 38th nucleotides in the first exon near the initiation codon as our target site (**Figure 1A**). We successfully constructed the CRISPR-Cas9 vector pC1300-*Actin*:Cas9-sgRNA^{ZEP1} (**Figure 1B**) and then introduced it into the elite inter-subspecific hybrid rice variety Chunyou84 (CY84) *via Agrobacterium*. A total of fifteen independent transgenic plants were obtained in the T_0 generation with six homozygous mutations (#1, #4, #7, #9, #11, and #12) and five biallelic mutations (#2, #3, #6, #13, and #14; **Figure 1C**).

Null Mutants of *ZEP1* in Rice

To clearly uncover the editing mutations of *ZEP1* on genetic recombination frequency and genetic interference, we selected the 1-bp insertion homozygous mutants for further researches. As shown in **Figure 2A**, the 1-bp insertion caused premature termination of *ZEP1* transcription and ultimately encoded a severely truncated protein containing 48 residues, of which only 12 residues were consistent with that of WT. Because of the basic of ZEP1 N-terminal globular domain, we speculated the 1-bp insertion mutants are *ZEP1* knockout lines, which differ from the previous partial loss-of-function *zep1* mutants (Wang et al., 2010; Wang K. et al., 2015). To confirm the absolute absence of ZEP1, we conducted immunostaining by using anti-REC8 and anti-ZEP1 antibodies. REC8 is a component of the cohesion complex and is required for sister chromatid cohesion, axial element formation, and homolog pairing and is used as a marker to monitor early meiotic events during prophase I here (Zhang et al., 2006). In the WT plants, ZEP1 signals were robust at pachytene, while in the mutants, no signals except the background were detected in any observed meiocytes ($n = 500$; **Figure 2B**). These results implied that *ZEP1* was completely knocked out in the mutants. Hereafter, we renamed these mutants as *zep1-KO*.

Effects of *ZEP1* Null Mutation on Sterility

Fertility is an important agronomic trait for genetic recombination analysis and plant breeding (Wang K. et al., 2015). There were no discernible differences in plant morphology between the WT



C

Code	alleles	Sequence	Mutation
Wild type	-	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTCGAGGGGTTCCGA	-
#1	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#2	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTC--GAGGGGTTCCGA	-2 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#3	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTtcgccggatatatgacgctaggaA	+10 bp
#4	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#5	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTCGAGGGGTTCCGA	WT
#6	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGT-TCGAGGGGTTCCGA	-1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#7	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#8	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTCGAGGGGTTCCGA	WT
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#9	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#10	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTCGAGGGGTTCCGA	WT
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTttCGAGGGGTTCCGA	+2 bp
#11	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#12	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#13	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTA-------CGAGGGGTTCCGA	-7 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp
#14	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGT----------TCCGA	-10 bp
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtcaAGGGGTTCCGA	+1 bp, SNP
#15	a1	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTCGAGGGGTTCCGA	WT
	a2	ATGCAGAAGCTGGGTTTATCGGGGCTTAGGGGTCTtCGAGGGGTTCCGA	+1 bp

FIGURE 1 | Genome editing of *ZEP1* in hybrid rice. **(A)** *ZEP1* gene structure and gRNA target site. **(B)** The structure of CRISPR-Cas9 vector targeting *ZEP1*. **(C)** Mutations in *ZEP1* editing lines in the Chunyou84 (CY84) background. The protospacer adjacent motif sequence is highlighted in red. Red lowercase letters and dashed lines indicate inserted or deleted nucleotides, respectively.

and the *zep1-KO* lines (**Figure 3A**), but the anthers of *zep1-KO* plants were light white while those of the WT were bright yellow (**Figure 3B**). We further confirmed the suspected male sterility by 1% I_2-KI solution staining and observed no staining in the pollens from the *zep1-KO* plants (**Figure 3C**). Therefore, unlike the previously described partial fertility of the *zep1* mutants (Wang et al., 2010; Wang K. et al., 2015), complete knockout of *ZEP1* caused absolute male sterility. In contrast to the anthers, the *zep1-KO* pistils seemed unaffected, similar to those of WT plants (**Figure 3B**). To determine whether the female gametes were fertile, we pollinated the *zep1-KO* and the control WT plants with pollen from WT plants. Both the WT and the *zep1-KO* plants could set seeds (**Figure 3D**), with a seed-setting rate of 38.1 ± 5.8% and 34.4 ± 8.5% for the WT and *zep1-KO*,

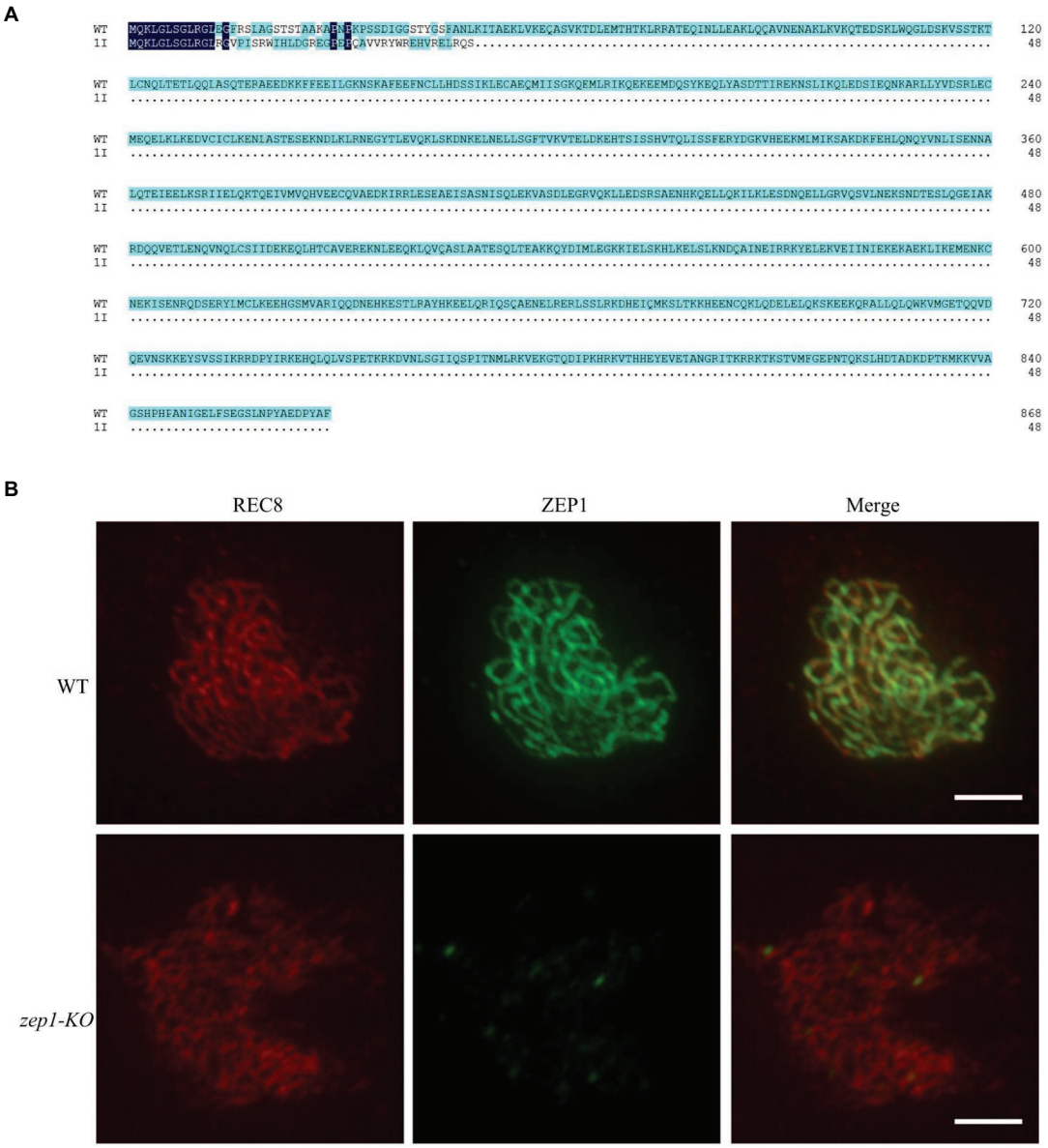


FIGURE 2 | Generation of *zep1* null mutants. **(A)** Amino acid sequence alignment of ZEP1 protein of WT and mutants. The *ZEP1* gene encodes a transverse filament protein containing 869 amino acids, while 1 bp insertion caused the premature transcription termination of *ZEP1*. **(B)** Immunostaining of REC8 (red) and ZEP1 (green) at pachytene stage in the WT and *zep1-KO* plants. Bar = 5 μm. REC8 was used to indicate the meiotic chromosomes.

respectively. These results suggested that the female meiocytes of *zep1-KO* mutants were not severely affected.

The complete male sterility in the *zep1-KO* plants might result from defects in meiosis. To test this hypothesis, we investigated the chromosome behaviors of male meiocytes by DAPI staining. In WT plants, all 12 bivalents in the pollen mother cells (PMCs) lined up on the equatorial plate at metaphase I (**Figure 4A**). However, univalents, bivalents, and multivalents were observed in the *zep1-KO* PMCs, numbering 4.40 ± 1.96, 8.37 ± 1.30, and 1.53 ± 0.97 ($n = 30$), respectively (**Figure 4**). Therefore, the presence of univalents and multivalents likely caused the male sterility of the *zep1-KO* lines.

Effects of *ZEP1* Null Mutation on CO Formation

To explore whether the female recombination frequency was affected by disruption of *ZEP1*, we firstly crossed the WT (the CY84 hybrid) and *zep1-KO* lines with the *indica* rice variety 93-11 and then examined the genetic recombination events of WT × 93-11 and *zep1-KO* × 93-11 F_1 individuals *via* genotyping. To this end, we developed 10 nearly uniformly distributed single nucleotide polymorphism (SNP) markers along each of chromosomes 1 and 8 (**Figure 5A**) that were polymorphic between the parental lines of the CY84 hybrid. Then, we genotyped 364 individual F_1 progeny of WT × 93-11 and 525 individual F_1

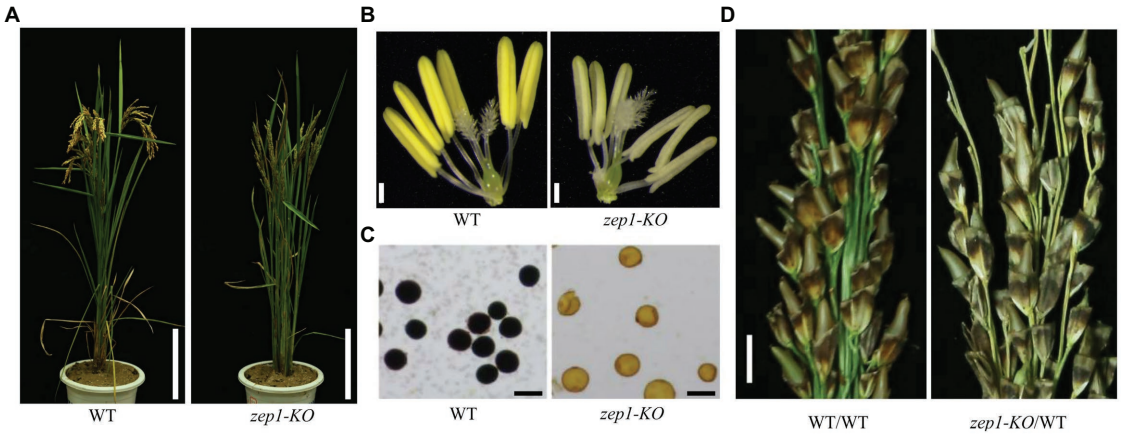


FIGURE 3 | Effects of *ZEP1* knockout mutation on sterility. **(A)** Comparison of WT and *zep1-KO* plants at maturity. Bar = 20 cm. **(B)** Comparison of anthers and pistils of WT and *zep1-KO* plants at heading date. Bar = 1 mm. **(C)** Iodine-iodide kalium staining of pollen from the WT and *zep1-KO* plants. Bar = 5 μm (pollen). **(D)** Cross the WT and *zep1-KO* plants (as maternal plant) with pollen from WT plants. Bar = 0.5 cm.

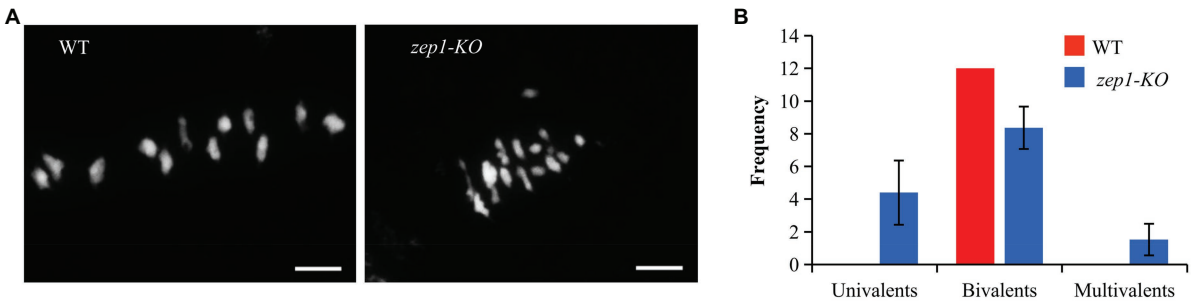


FIGURE 4 | Chromosome behaviors of male meiocytes in WT and *zep1-KO* plants. **(A)** DAPI staining of the meiotic chromosomes of pollen mother cells (PMCs) from the WT and *zep1-KO* plants. Bar = 5 μm. **(B)** Frequency distribution of univalents, bivalents, and multivalents in the pollen mother cells (PMCs) from WT and *zep1-KO* plants. ($n = 30$).

progeny of *zep1-KO* × 93-11 using those markers. After this, we tracked the recombination events that had occurred in the WT or *zep1-KO* parental plants (CY84 hybrid background) based on a previous method (Wang K. et al., 2015), using our SNP markers to deduce the genotype of each chromatid following meiosis and thus infer the number of COs that had occurred to produce each chromatid. In the WT plants, chromosome 1 chromatids with zero, single, double, triple, and quadruple COs occurred at frequencies of 20, 34, 34, 11, and 1%, respectively. By contrast, the *zep1-KO* mutants exhibited a decreased frequency of zero (8%), single (25%), and double (28%) COs but a higher frequency of triple (20%), quadruple (14%), quintuple (3%), sextuple (1%), and septuple (1%) COs (**Figure 5B**). Similar results were detected on chromosome 8, with 19% zero, 34% single 25% double, 16% triple, 4% quadruple, and 2% quintuple COs occurred in *zep1-KO* mutants, while the frequencies of zero, single, double, and triple COs occurred in WT plants were 23, 49, 25, and 2%, respectively (**Figure 5C**).

We further calculated the recombination frequency of *zep1-KO* and WT plants. The average number of COs per chromatid on chromosomes 1 and 8 in WT plants was 1.40 and 1.06, respectively, while that of *zep1-KO* mutants was significantly higher, with 2.33 and 1.57 COs per chromatid on chromosomes 1 and 8, respectively (**Figure 6**). The recombination frequency was elevated in all identified intervals in the *zep1-KO* lines compared to that of the WT, varying from 1.01- to 3.57-fold (**Figure 6**). In particular, the recombination frequency of the DE interval on chromosome 1 and the EF interval on chromosome 8, both of which span the centromere, was elevated by 1.83- and 3.29-fold, which was higher than the average recombination frequency of entire chromosomes: 1.63- and 1.58-fold for chromosomes 1 and 8, respectively (**Figure 6**).

Effects of *ZEP1* Null Mutation on Genetic Interference

To assess whether genetic interference was affected by the null mutation of the *ZEP1* gene, we conducted coefficient of coincidence analysis according to the method described for

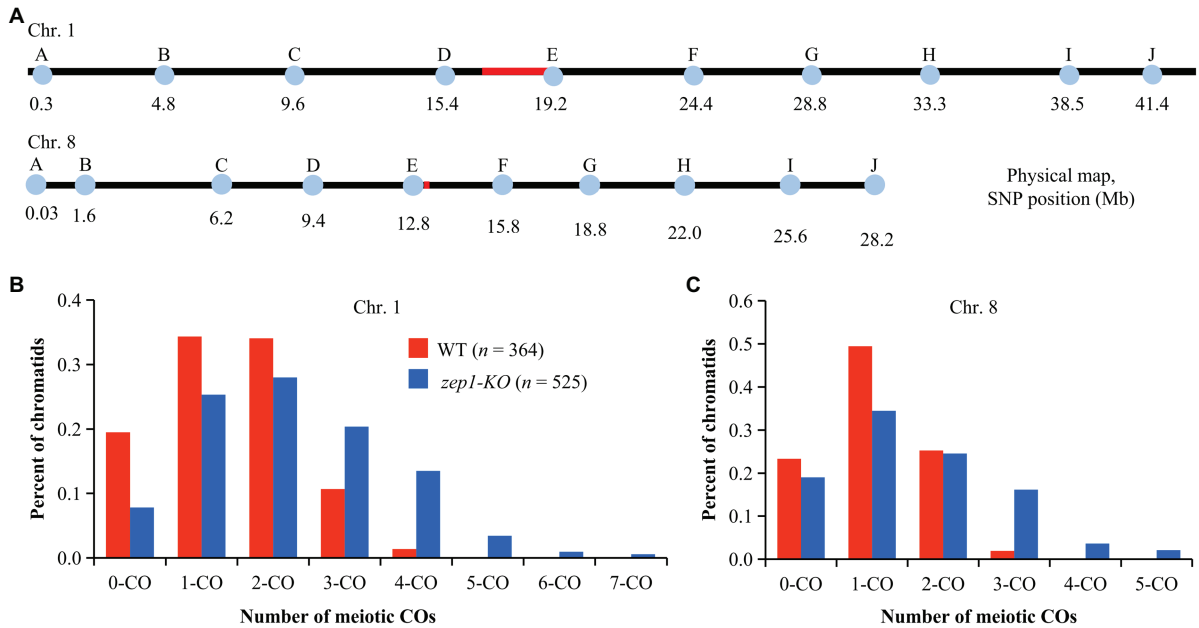


FIGURE 5 | Analysis of chromatids in WT plants and *zep1* null mutants. **(A)** Physical positions of the SNP markers along chromosomes 1 and 8. **(B)** Percentage of total chromatids (*n*) for different types of COs on chromosome 1. **(C)** Percentage of total chromatids (*n*) for different types of COs on chromosome 8.

C. elegans (Libuda et al., 2013). In the analysis, the strength of interference (*I*) equal to 1 is indicative of complete interference, while an *I* equal to zero is indicative of an absence of interference (Fernandes et al., 2018). In WT plants, the strength of interference (*I*) in eight adjacent interval pairs of chromosome 1 ranged from 0.37 to 1.00, indicating the existence of strong genetic interference between neighboring COs. By contrast, the *zep1-KO* mutants showed reduced *I* for those interval pairs, with *I* values of −0.18 to 0.14 (**Figure 7A**). For chromosome 8, the *I* value of the four measured interval pairs in the WT ranged from 0.48 to 0.71, whereas that in *zep1-KO* lines ranged from −0.17 to 0.05 (**Figure 7B**). These results suggest that genetic interference is likely fully eliminated in *zep1-KO* plants.

DISCUSSION

ZEP1 is the central element protein of the SC that regulates genetic recombination frequency in rice (Wang et al., 2010). The partial loss of function of this gene can increase genetic recombination frequency and attenuate CO interference (Wang K. et al., 2015), but the effects of *ZEP1* null mutations on CO formation and interference have not been reported comprehensively yet. The N-terminal domain of the ZEP1 is located in the middle of the SC, and this special organization pattern is critical for SC assembly (Anderson et al., 2005; Wang et al., 2010). In this study, we successfully generated null mutation of *ZEP1* in hybrid rice by genome editing. Genetic analysis of the null mutants revealed that genetic recombination frequency was greatly increased, and CO interference was completely eliminated in the absence of *ZEP1* in CY84 variety background. Compared with the partial loss of function of *ZEP1* mutants (Wang et al., 2010; Wang K. et al., 2015), the new null allele in this study showed stronger phenotype than those of previous weak alleles, indicating the residuary N-terminal domain of the ZEP1 in previous weak alleles might still play some roles in meiosis, although the SC cannot be assembled in the weak mutant.

Interference is a phenomenon that reduces the likelihood of a CO occurring adjacent to another CO along regions of a chromosome (Taagen et al., 2020). However, the relationship between the SC and genetic interference has long been a controversy in organisms. In budding yeast, the data of synapsis initiation complex (*SIC*) are assembled prior to SC and their proper position in the absence of SC formation demonstrated an aspect of interference that is independent of synapsis (Fung et al., 2004). By contrast, in *Caenorhabditis elegans*, it was found that partial depletion of the central region proteins of SC attenuated genetic interference (Libuda et al., 2013). The partial loss of function of *ZEP1* mutants in rice showed similar results like that in *Caenorhabditis elegans* (Wang K. et al., 2015). Nonetheless, the exact role of SC in CO interference is still enigmatic because of the lack of null TF mutants. Recently, the *zyp1* null mutants in *Arabidopsis thaliana* were generated. Cytological and genetic analysis of the null mutants revealed that ZYP1 is required for CO interference (Capilla-Pérez et al., 2021; France et al., 2021). In this study, we found that genetic interference was eliminated in some detected genomic regions in our obtained rice *ZEP1* null mutants. The phenotype of *ZEP1* weak and null alleles in rice supports that the strength of genetic interference increased as the extent of synapsis increased.

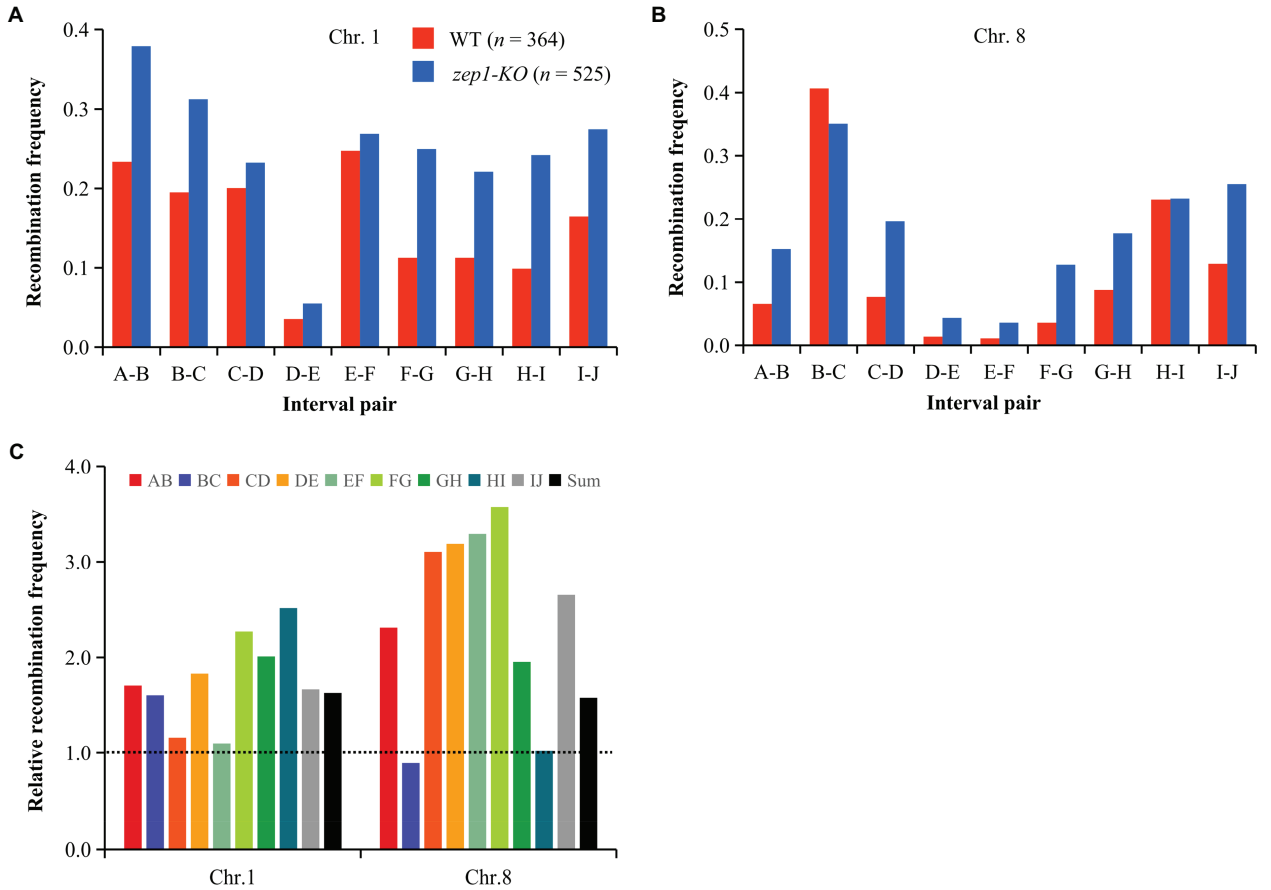


FIGURE 6 | *zep1* null mutation increases recombination frequencies. **(A)** Recombination frequencies measured between adjacent SNP markers on chromosomes 1. **(B)** Recombination frequencies measured between adjacent SNP markers on chromosomes 8. **(C)** Relative recombination frequency (*zep1-KO*/WT) measured between adjacent SNP markers on the chromosomes 1 and 8.

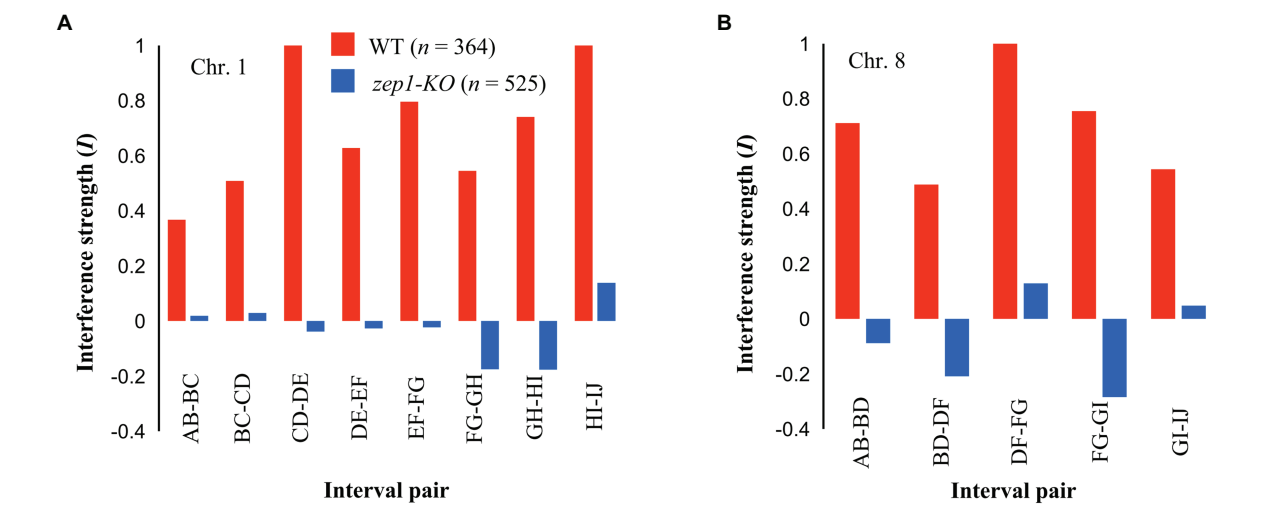


FIGURE 7 | *zep1* null mutation disrupts crossover (CO) interference. Interference strength (*I*) values for different adjacent interval pairs of chromosomes 1 **(A)** and 8 **(B)** in WT and *zep1-KO* plants.

Meiotic crossovers shuffle chromosomes to produce unique combinations of alleles that are transmitted to offspring (Mieulet et al., 2018). However, meiotic crossovers are tightly regulated by numerous of genes, typically one to three per chromosome (Fernandes et al., 2018). To combine favorable alleles into elite varieties, breeders and geneticists are exploring approaches to increase meiotic crossovers. Mutation of anti-crossover factors, such as FANCM, RECQ4, and FIGL1 in plants, induces a large increase in crossover frequency (Crismani et al., 2012; Girard et al., 2015; Séguéla-Arnaud et al., 2015; Mieulet et al., 2018). Reducing CO interference alters CO distribution, which also shows potential to enhance crop breeding efficiency (Taagen et al., 2020). However, it is very difficult to elevate genetic recombination frequency and eliminate genetic interference simultaneously in breeding process. Here, we used CRISPR/Cas9 to knock out *ZEP1*, encoding a component of the SC, in hybrid rice. The edited mutants concurrently exhibited disrupted CO interference and an elevated recombination frequency. Our study provides an effective approach to enrich genetic diversity and accelerate rice breeding. Of course, as the background play important roles in the formation of COs, more studies in different varieties are required to test its application potential in the future.

CONCLUSION

In summary, we used CRISPR/Cas9 to completely knock out the *ZEP1* gene, encoding the central component of the SC, in hybrid rice. The mutants exhibited eliminated genetic interference and elevated genetic recombination frequency on chromosomes 1 and 8 in CY84 variety background. Our study of female gamete revealed the important role of the SC in mediating genetic interference and limiting COs. Very recently, null mutants of *ZYP1* in *Arabidopsis* were generated and a virtual absence of CO interference had been detected (Capilla-Pérez et al., 2021; France et al., 2021). Therefore, all of these studies support the long-term debating role of SC in mediating CO interference. Moreover, it is potential to break linkage drag and enrich genetic diversity by using *zep1* null mutants during rice breeding.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KW managed the project. CL, YC, YH, GD, XW, TS, JL, and MW performed the experiments. YH, QL, and CL analyzed the data. CL and YH wrote the manuscript. KW and ZC revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.757152/full#supplementary-material

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frontiers in Plant Science

REVIEW
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Multiplex Genome-Editing Technologies for Revolutionizing Plant Biology and Crop Improvement

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Multiplex genome-editing (MGE) technologies are recently developed versatile bioengineering tools for modifying two or more specific DNA loci in a genome with high precision. These genome-editing tools have greatly increased the feasibility of introducing desired changes at multiple nucleotide levels into a target genome. In particular, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) [CRISPR/Cas] system-based MGE tools allow the simultaneous generation of direct mutations precisely at multiple loci in a gene or multiple genes. MGE is enhancing the field of plant molecular biology and providing capabilities for revolutionizing modern crop-breeding methods as it was virtually impossible to edit genomes so precisely at the single base-pair level with prior genome-editing tools, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Recently, researchers have not only started using MGE tools to advance genome-editing applications in certain plant science fields but also have attempted to decipher and answer basic questions related to plant biology. In this review, we discuss the current progress that has been made toward the development and utilization of MGE tools with an emphasis on the improvements in plant biology after the discovery of CRISPR/Cas9. Furthermore, the most recent advancements involving CRISPR/Cas applications for editing multiple loci or genes are described. Finally, insights into the strengths and importance of MGE technology in advancing crop-improvement programs are presented.

Keywords: multiplex genome editing, CRISPR/Cas9, CRISPR/Cas12, plant science, crop improvement

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INTRODUCTION

Plant scientists have long been searching for biological tools to generate site-specific mutations in the plant genome for research. Until the emergence of genome-editing tools, it was almost impossible to recognize and alter specific genomic sequences at a chosen locus. Recently, nucleases have been engineered that recognize and cleave DNA at target genomic sites, introducing double strand breaks (DSBs) that are subsequently repaired by either error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. During the repair process, precise DNA fragment deletion, insertion, or replacement can occur, resulting in gene editing, mutation, or replacement (Hsu et al., 2013; Miglani, 2017; Mishra et al., 2018).

Using nucleases to simultaneously and precisely introduce two or more DSBs at specific loci within a single genome in a single round of mutagenesis will hereafter be called multiplex genome editing (MGE) (Cong et al., 2013). MGE generates rapid desirable changes in specific but different genes or quantitative trait loci (QTLs), resulting in the development of new plant genotypes carrying multiple mutations in one generation. The capability to create such genomic changes will allow researchers to understand and study the interactions between members of a gene family that have redundant functions, and it may provide an opportunity to analyze epistatic relationships among closely related genetic pathways (Xing et al., 2014). Furthermore, these multiple genomic modifications or edits could provide the most practical platform for metabolic pathway engineering in plants. MGE can be used to facilitate many desirable crop improvements, such as the production of seedless plant species and better disease resistance (Naim et al., 2018; Tripathi et al., 2019), as well as to skip extensive segregation in vegetatively propagating crop plants, such as sugarcane (Kannan et al., 2018).

Unlike traditional random mutagenesis, MGE tools are designed to target specific genomic regions and to generate specific mutations or variations with much more precision (Belhaj et al., 2015). Accomplishing multiple gene mutations in an elite cultivar formerly required many years and several cycles of crossing and selection. Moreover, the traditional approach for conventional breeding has sometimes been more complicated because of linkage drag, which impedes the transmission of good traits aside from deleterious genes. For instance, developing high-yielding genotypes with premium grain quality traits has always been considered a challenge for plant breeders utilizing conventional breeding approaches (Abdelrahman and Zhao, 2020). However, many researchers have reported using MGE tools to improve traits with such complications (Wang et al., 2014; Li W. et al., 2016; Kannan et al., 2018). MGE tools have become more user-friendly, particularly after the development and adaptation of the CRISPR-Cas system (Jinek et al., 2012; Cho et al., 2013; Cong et al., 2013; Mali et al., 2013). Among genome-editing technologies, including zinc-finger nucleases (ZFNs) (Bibikova et al., 2002; Lloyd et al., 2005; Ainley et al., 2013; Qi et al., 2014) and transcription activator-like effector nucleases (TALENs) (Christian et al., 2010; Li et al., 2011), the CRISPR/Cas system uniquely depends on base complementarity between the guide RNA (gRNA) and the target DNA for recognition, which greatly simplifies the genome-editing process (Jinek et al., 2012). Due to its simplicity for designing multiple target genetic loci, researchers have preferred the CRISPR/Cas system for genome editing in recent years. TALENs have also been successfully utilized for MGE in polyploid plants, including wheat, tobacco, and sugarcane (Wang et al., 2014; Li W. et al., 2016; Kannan et al., 2018). However, while using the multiplex TALENs approach, each unit must be designed separately, which increases the difficulty in delivering different units simultaneously to a plant cell. The CRISPR/Cas system has many advantages over TALENs and ZFNs: it is more efficient in editing target loci, and multiple single-guide RNAs (sgRNAs) can be designed to be expressed at once for simultaneous editing of multiple loci (Cong et al., 2013). Adding more gRNAs to the transformation construct is far simpler than engineering new proteins to improve the trait of choice in plants. By using just one Cas protein with multiple gRNAs, multiple gene mutations have been achieved in various plants, including rice, wheat, maize, tomato, and *Arabidopsis* (Li et al., 2013; Upadhyay et al., 2013; Ma et al., 2015; Yan et al., 2016; Rothan et al., 2019).

Further investigations and developments are ongoing to improve MGE applications for the genetic improvement of crops. To date, MGE using CRISPR/Cas has been efficiently conducted to enhance crop yield, quality, and stress resistance (Xu et al., 2016; Kaur et al., 2018; Li Y. et al., 2019; Zhou et al., 2019). The results of different experiments have shown that different applications and procedures are progressively appearing. Immense technical progress has recently occurred in this research field, utilizing more complex genetic, epigenetic, and transcriptional manipulations for research needs. Recently, Kannan et al. (2018) reported targeted mutagenesis of more than 100 alleles in sugarcane to improve saccharification efficiency without affecting biomass yield. In this review, we discuss different emerging applications that have been developed and adapted for MGE and their potency and associated experiments for crop-improvement programs. We further summarize and provide recent information about the progress on MGE in plants, emphasizing its role in developing plant molecular biology and plant breeding.

TOOLS FOR MGE

TALENs for Targeting Gene Families

When MGE tools were first developed, before there was a CRISPR/Cas system, Wang et al. (2014) reported using a single TALEN pair to edit three homoeoalleles encoding mildew resistance locus proteins (MLOs) in hexaploid bread wheat (*Triticum aestivum* L., 2n = 42, AABBDD). The goal was to develop broad-spectrum and durable *Blumeria graminis* f. sp. *tritici* (*Bgt*) resistant wheat lines. The single TALEN pair recognition sequences were strictly conserved at the *MLO-B1* and *MLO-D1* target sites and has one nucleotide mismatch at the *MLO-A1* target site; they had mutations at the three target sites. The results revealed that of the 27 T0 plants, four mutants carried two edited targets in either homozygous or heterozygous forms, and only one plant harbored triple mutations. Plants with triple mutations at the three different targets showed resistance, with no apparent fungal growth on the leaves, although abundant fungal growth was found on the leaves of wild-type plants, and the plants with combinations of other mutations.

Multiplex editing using TALENs was also employed to produce highly potent biopharmaceutical glycoprotein products. In an attempt to increase their activity, stability, and immunogenicity in plants, Li J. et al. (2016) reported that two *α (1,3)-fucosyltransferase* (*FucT1* and *FucT2*) and two *β (1,2)-xylosyltransferase* (*XylT1* and *XylT2*) genes within *Nicotiana benthamiana* were successfully edited, resulting in plants with improved capacity to produce glycoproteins devoid of plant-specific residues. Conserved sequences in the first exon of each gene family were analyzed, and one sequence-specific TALEN pair for each gene family was designed accordingly.

The researchers tried to knockout the two-gene family members separately and together by cotransformation of the two TALEN pairs. Both *FucT* and *XylT* mutant plants had significant changes in their N-glycan profiles. Notably, this was the only trial of multiplex editing using two different modules of TALEN pairs to target two divergent gene families in one genotype.

To date, the highest record for simultaneous targeting genome editing in the same plant genotype has been reported in sugarcane crops. Kannan et al. successfully edited 107 of 109 gene copies of the sugarcane *caffeic acid O-methyltransferase* (*COMT*) gene family by introducing a single TALEN pair (Kannan et al., 2018). A conserved region of *COMT* was targeted with a single TALEN pair for multiallelic mutagenesis to modify lignin biosynthesis in sugarcane. This improved saccharification efficiency by up to 43.8% and significantly reduced lignin content in the mutant, without significant differences in biomass production compared with the wild type.

These studies made it clear that TALEN-based genome editing allows for efficient multiplex editing of gene families with conserved sequences among different gene copies, and this has been successfully demonstrated in polyploid genomes. However, Li et al. succeeded in editing two different gene families with two different cotransformed TALEN pairs; the editing efficiency of several TALEN pairs for each target was tested before conducting the experiment (Li J. et al., 2016). Furthermore, the procedure is more complicated if more gene families are targeted simultaneously because TALEN technologies require the engineering of DNA-binding domains for individual targeting applications (Lowder et al., 2015).

The CRISPR/Cas9 System Offers Excellent MGE Ability

The CRISPR/Cas9 is much easier and more efficient than other genome-editing tools, particularly for the editing of multiple unrelated genes. Using multiple sgRNAs and expressing only one Cas protein significantly simplifies and enhances the editing efficiency of multiple genes. The ease of plasmid construction in CRISPR/Cas9 experiments further allows the function of multiple associated genes to be investigated that either has negative effects or that improve plant-specific characteristics.

Attempts have been made to improve the CRISPR/Cas system to further simplify and enhance the efficiency of editing multiple genes. The core focus has been to develop an easy and fast toolbox to insert multiple sgRNA expression cassettes into one binary vector. Several sequential rounds of regular cloning steps can be utilized to insert different expression cassettes containing sgRNAs for different targets into a single construct, but this is very laborious and there is a limited number of assembled sgRNAs (Li et al., 2013; Mao et al., 2013; Zhou et al., 2014; Yan et al., 2015). A more advanced cloning method, called Golden Gate cloning, depends on the use of type IIS restriction enzymes in the DNA assembly. Golden Gate cloning has been used to generate non-palindromic sticky ends in DNA fragments carrying sgRNAs. This technique facilitates the cloning of different constructs into binary vectors based on the different toolkits available for CRISPR/Cas9-mediated genome editing. The methods investigated to enhance the cloning of different gRNAs into a single binary vector can be divided based on the technique used to assemble the sgRNAs together in the single delivery construct. These strategies are either based on assembling multiple individual gRNA expression cassettes, each transcribed from RNA polymerase III promoters, such as U6 or U3 (Xing et al., 2014; Lowder et al., 2015; Ma et al., 2015; Wang C. et al., 2015; Yan et al., 2015; Vazquez-Vilar et al., 2016; Zhang et al., 2016; Zhao et al., 2016; Char et al., 2017; Ordon et al., 2017; Tian et al., 2017), or through a single transcript of polycistronic mRNAs that are cleaved into individual gRNAs posttranscriptionally by the CRISPR-associated RNA endoribonuclease Csy4 from *Pseudomonas aeruginosa* (Tsai et al., 2014); the tRNA processing enzymes, naturally present in the host cells (Xie et al., 2015); or self-processing ribozymes (Gao and Zhao, 2014; Tang et al., 2016).

RNA Polymerase III Promoters Drive the Expression of Multiple sgRNAs

Two sets of CRISPR/Cas9 constructs have been developed by Xing et al. (2014) and Ma et al. (2015), where the gRNAs are driven by small RNA polymerase III (**Figure 1A**) and designed for cloning into a binary vector with prepared sgRNA expression cassettes in a single round of Golden Gate cloning using Type IIS restriction enzymes, such as *Bsa*I. This approach has allowed for simultaneous knockout of multiple genes in *Arabidopsis* and rice. Both sets of CRISPR/Cas9 constructs are the most commonly used tools of this type, and both require a specific promoter to drive each sgRNA. Similarly, Lowder et al. (2015) designed an approach for MGE that uses RNA polymerase III to drive sgRNA expression in a successive three-step procedure, which is as follows: an intermediate vector carrying the sgRNAs of interest is constructed first, followed by Type IIS restriction enzyme digestions, recovery of the sgRNA cassettes, and then Golden Gate cloning into a binary vector.

Single Transcript System for the Expression of Multiple sgRNAs

Although the abovementioned method in section RNA Polymerase III Promoters Drive the Expression of Multiple sgRNAs is frequently used for MGE, one limitation is the size of the final construct, which can sometimes be too large and repetitive due to repeating promoter sequences. These repeats can induce frequent recombination events (Vidigal and Ventura, 2015; Ding et al., 2020) and cause plasmid instability in *E. coli* and *Agrobacterium*. This might be why this method has been reported to result in the assembly of two to eight gRNA expression cassettes. Recently, Stuttmann et al. (2021) reported a new CRISPR system to allow multiplexing of up to 24 gRNAs in *Arabidopsis*; they observed that processing polycistronic single transcripts containing multiple gRNAs has a major advantage because they can be produced by a polymerase II promoter, which can produce longer transcripts than a polymerase III promoter (Arimbasseri et al., 2013).

In an early attempt to have a polycistronic CRISPR/Cas9 system for MGE, Tsai et al. developed a vector in which multiple gRNAs can be assembled, each flanked by cleavage sites for the

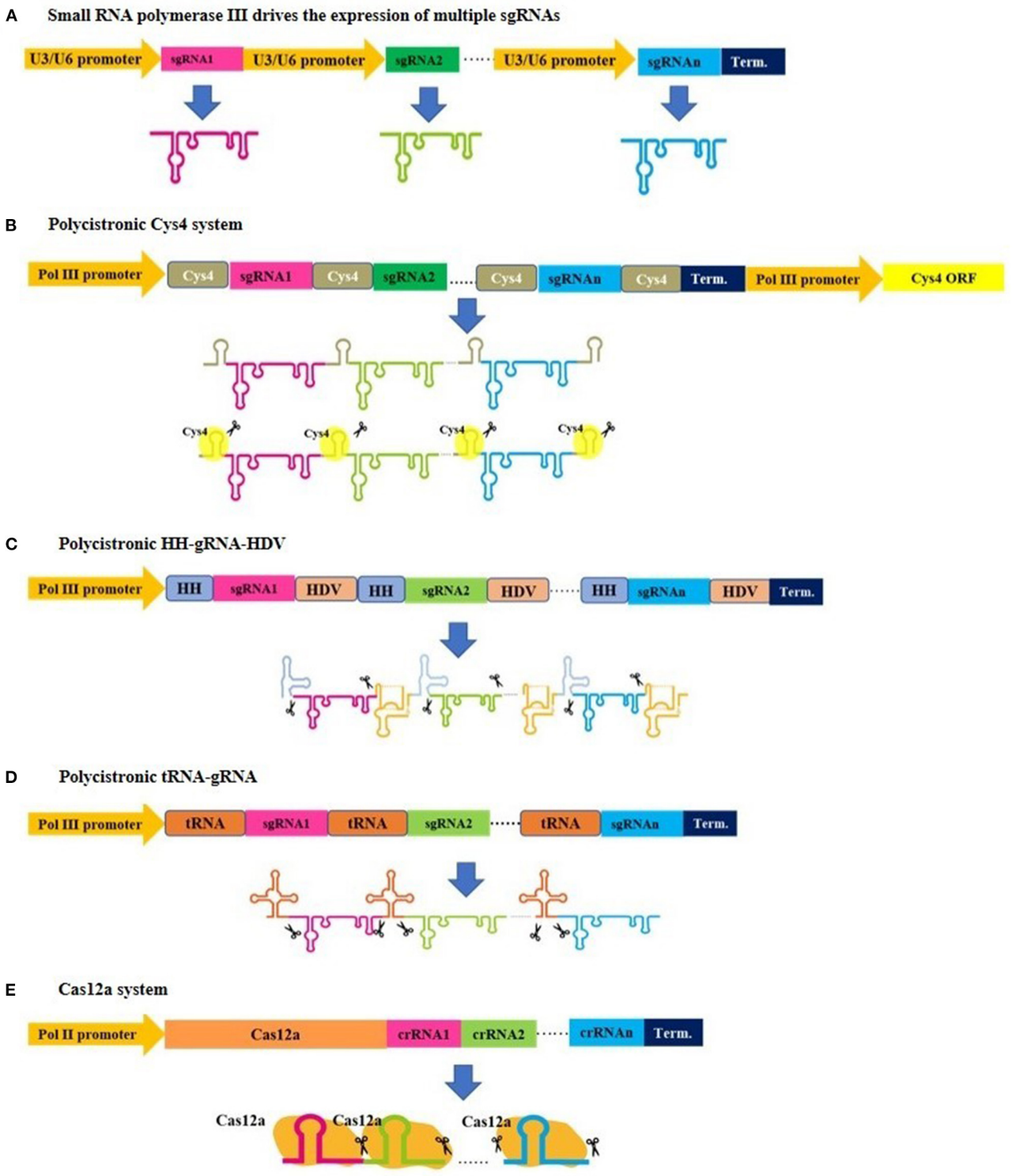


FIGURE 1 | An illustration demonstrating various strategies for expressing multiplex gRNA cassettes in plants. **(A)** Small gRNAs are cloned after U3 or U6 promoters and derived by small RNA polymerase III to generate individual gRNAs. **(B–D)** Small gRNAs are cloned to be transcribed into a single transcript, and subsequent posttranscriptional processing is needed for gRNA separation, where Csy4, tRNAs, and hammerhead ribozyme regulate this separation. Similarly, a single transcript is generated in the **(E)** Cas12a system, but this system has a gRNA self-cleaving feature and does not require additional elements for posttranscriptional processing.

Csy4 RNase of *Pseudomonas aeruginosa* (Haurwitz et al., 2010; Tsai et al., 2014). Based on this, each gRNA must be designed to have a 3′ end with a Csy4 recognition site to allow the coexpressed Csy4 to cleave the long transcript, thereby releasing the gRNAs (**Figure 1B**).

Mimicking the native tRNA-snoRNA43 dicistron (Kruszka et al., 2003) in cells, Xie et al. further designed a polycistronic tRNA-gRNA (PTG) system for simultaneous production of single gRNA transcripts (**Figure 1C**), in which the gRNAs are transcribed by RNA polymerase III and linked together by their tRNA sequences (Xie et al., 2015). The tRNAs are then recognized and processed by endogenous RNases (e.g., RNase P and RNase Z in plants) that excise the individual gRNAs from the transcript. These gRNAs direct Cas9 (driven by RNA polymerase II) to their targets for genome editing. This application has resulted in highly efficient genome editing, although Xie and his group observed a reduction in the efficiency of PTGs with an increasing number of gRNAs, possibly due to multiple sgRNAs competing for binding to Cas9.

In an alternative approach, Tang et al. designed a CRISPR/Cas system to allow the expression of both Cas9 and sgRNAs from a single Pol II promoter using a self-processing hammerhead ribozyme (Haseloff and Gerlach, 1988; Tang et al., 2016). The Cas9 and sgRNAs were linked by ribozyme (RZ) cleavage sites, allowing transcripts to be cleaved by the cis-acting ribozyme (**Figure 1D**). The approach was tested using six target sites in three rice genes, and the mutation frequencies in T0 plants ranged from 53.8 to 100% with a majority of biallelic mutations.

In the same context, Cermák et al. designed several multiplexed gene-editing tools based on the posttranscriptional processing of a single polycistronic mRNA using the CRISPR-associated Csy4 RNA endoribonuclease from *Pseudomonas aeruginosa* (Tsai et al., 2014; Cermák et al., 2017), the tRNA processing enzymes naturally present in the host cells (Xie et al., 2015), and ribozymes (Gao and Zhao, 2014). Furthermore, Cermák and colleagues compared the editing efficiency of these systems for targeting two sites in the *AUXIN RESPONSE FACTOR 8A* (*ARF8A*) gene of tomato, and found that Csy4 had the best efficiency, followed by the tRNA system. The trial results were confirmed with eight gRNAs and in four different tomato genes. The efficiency of the Csy4 system was further demonstrated using up to 12 gRNAs for targeted gene deletions.

The CRISPR/Cas12a (Cpf1) System: An Emerging Tool for MGE

For the aforementioned CRISPR/Cas9 systems, the size of the final construct is one of the most critical limitations in multiplexing experiments. Moreover, the requirement of GC-rich protospacer-adjacent motif (PAM) sequences is a drawback of using Cas9, especially when the number of genes to be edited is large. Furthermore, even in cases where single polycistronic multiple gRNA methods are employed, additional sequences must be added for further processing after transcription. These limitations may be overcome by a newer member of the Cas protein family, Cas12a, formerly known as Cpf1 (Zetsche et al., 2015). Cas12a has a relatively small size, its required PAM sequence allows T-rich regions to be targeted, and it requires a short crRNA (42 nt) which facilitates construction and multiplexing (**Figure 1E**). Moreover, the Cas12a multiplexing system has major advantages over other DNA editing systems. The Cas12a system does not require either the addition of several pol III promoters (only one Pol III promoter is enough to drive several small crRNAs) or the addition of specific sequences for multiple crRNA constructs because it can sufficiently process and mature crRNAs using its ribonuclease activity (Zetsche et al., 2015, 2017; Fonfara et al., 2016). These features make Cas12a an ideal system for MGE. Moreover, Cas12a cleaves the DSB at cohesive ends distal from the PAM site, which allows continuous cleavage of DNA (Zetsche et al., 2015). Swarts and Jinek compared the structure function relationships of Cas9 and Cas12a and their implications in genome editing in detail (Swarts and Jinek, 2018). Importantly, Cas12a was found to be very specific in plant MGE using whole-genome sequencing (Tang et al., 2018). Similar findings were reported while using CIRCLE-seq (Tsai et al., 2017; Lee et al., 2019).

Inactive Cas Proteins for MGE

Mutating specific amino acids in Cas9 and Cas12a abolishes the DNA cleavage activity of these proteins, converting them into inactive (or dead) Cas (dCas). Fusion of dCas with cytosine deaminase (CBE) or adenosine deaminase (ABE) and sgRNAs induces point mutations at the target site. Editing a single nucleotide may be sufficient to alter gene structure to obtain a desirable function (Komor et al., 2016; Eid et al., 2018; Mishra et al., 2020). Shimatani et al. used such technology to induce multiple herbicide-resistance point mutations in rice (Shimatani et al., 2017). Recently, Xu et al. increased the efficiency of this technique by 2- to 5-fold by developing a tool kit that uses high-fidelity dCas9 variants fused to a tandemly arrayed tRNA-modified sgRNA architecture in rice (Xu et al., 2019). Furthermore, oriented transcriptional regulation was achieved by the fusion of dCas enzymes to effector domains, including CRISPR-mediated inhibitor (CRISPRi) and activator (CRISPRa) (Gilbert et al., 2013; Qi et al., 2013). Lowder et al. (2017) described a toolbox for constructing and cloning multiplex CRISPR/Cas9 transcriptional activation and the repression of *Arabidopsis* genes.

REVOLUTIONIZING ROLES OF MGE IN PLANT BIOLOGY

CRISPR/Cas is bringing about a marked transformation in plant biology. MGE using CRISPR/Cas endows scientists with the ability to decipher very difficult biological problems by targeting different genes simultaneously. In this section, of the review, we aim to explore the impacts of the multiplex CRISPR/Cas system. **Table 1** summarizes the efforts taken using MGE for revolutionizing plant biology and crop improvement.

Knock-Out of Multiple Genes at Once

Loss of function or gene knockout has been widely investigated in plant genome-editing studies mainly because of the activation of the naturally occurring non-homologous end joining (NHEJ)

TABLE 1 | Summary presentation of the efforts done using multiplex genome editing for revolutionizing plant biology and crop improvement programs.

MGE application	Target genes/QTLs	Crop	Objective	References
Plant biology				
Knock-out of multiple genes				
	Two targets at ssDNA tomato yellow leaf curl virus (TYLCV) viruses	Tomato	Virus biological control in plants	Ali et al. (2015)
	OsGW2, *OsGW5*, and *OsTGW6*	Rice	Larger grain size	Xu et al. (2016)
	RAS-PDS1 and *RAS-PDS2*	Banana	Demonstration of gene knockout in banana	Kaur et al. (2018)
	OsSWEET11 and *OsSWEET14*	Rice	Broad-spectrum disease resistance in rice	Xu et al. (2019)
	TMS5, Pi21, and *Xa13*	Rice	Hybrid rice production and disease resistance	Li S. et al. (2019)
Engineering efficient plant metabolic pathways				
	OsGSTU, OsMRP15, *and OsAnP*	Rice	Anthocyanin synthesis pathway	Ma et al. (2015)
	Three different sites in the *OsWaxy*	Rice	Decreasing amylose content	Ma et al. (2015)
	Two sites in *slyPDS*	Tomato	Generating photobleached phenotype	Li R. et al. (2018)
	GABA-TP1, *GABA-TP2*, *GABA-TP3*, *CAT9*, and *SSADH*	Tomato	Metabolic engineering and manipulation of the gamma-aminobutyric acid (GABA)	Li R. et al. (2018)
	SGR1, LCY-E, Blc, LCY-B1, *and LCY-B2*	Tomato	Increase the accumulation of lycopene	Li X. et al. (2018)
	GmF3H1, *GmF3H2*, and *GmFNSII-1*	Soybean	Increasing the isoflavone content	Zhang et al. (2020)
Transcriptional regulation				
	Three different sites in the promoter of *production of Anthocyanin Pigment1* gene (*AtPAP1,* encoding a transcription factor) and *miR319* (encoding a microRNA)	*Arabidopsis*	Transcription gene activation	Lowder et al. (2015)
	Promoter regions of the *WRKY30*, *RLP23*, and *CDG1* genes	*Arabidopsis*	Simultaneous activation of their transcription	Li et al. (2017)
	Two sites at locule number (lc) QTL + eight gRNAs targeting the promoters of SlCLV3, S and SP	Tomato	Generating desirable/beneficial regulatory variants	Rodríguez-Leal et al. (2017)
	OsWOX11 and *OsYUC1*	Rice	Transcription gene activation	Gong et al. (2020)
Chromosomal segment restructuring				
	Two targets flanking a 245 kb segment	Rice	Chromosomal segment deletions	Zhou et al. (2014)
	Two targets flanking 1.8 kb segment	Tobacco	Chromosomal segment deletions and inversion	Gao et al. (2015)
	Two targets flanking a 100 bp segment	*Arabidopsis*	Chromosomal segment deletions	Ordon et al. (2017)
	6 targets at a 58 kb region	Medicago	Chromosomal segment deletions	Cermák et al. (2017)
	Different genomic regions ranging from 1.7 to 13 kb	Arabidopsis	Chromosomal segment deletions	Wu et al. (2018)
	Two targets flanking an 18 (kb) segment	Arabidopsis	Chromosomal inversion	Schmidt et al. (2019)
Multiplex base alterations				
	Three sgRNAs targeting *ALS* and *FTIP1e genes*	Rice	Herbicide resistance	Shimatani et al. (2017)
	OsACC-T1, *OsALS*-T1, *OsCDC48*-T3, *OsDEP1*-T1, *OsDEP1*-T2, and *OsNRT1.1B*-T1.	Rice	Herbicide resistance	Li C. et al. (2018)
	AtALS, *AtPDS*, *AtFT*, and *AtLFY* genes and *BnALS* and *BnPDS*	Arabidopsis and Rapeseed	System verification	Kang et al. (2018)
	Two targets *StGBSSI* gene	Potato	impaired amylose biosynthesis in potato	Veillet et al. (2019)
	ZmALS1 and *ZmALS2*	Maize	Herbicide resistance	Li Y. et al. (2019)
	Four targets at *OsWAXY*, *OsCDC48*, *and OsSNB genes*	Rice	System optimization	Wang F. et al. (2020)
	BnALS1 and *BnALS3*	Oilseed rape	Herbicide resistance	Wu et al. (2020)
	Multiple sites at *OsALS1* gene	Rice	Facilitating the directed evolution of plant	Kuang et al. (2020)
	Multiple loci at *OsACC*	Rice	Herbicide resistance	Liu et al. (2020)
	Multiple targets trials	Rice	Prime genome editing optimization	Lin et al. (2020)

(Continued)

TABLE 1 | Continued

MGE application	Target genes/QTLs	Crop	Objective	References
Crop improvement				
Generating diversity and crop domestication				
	Two sites at locule number (*Lc*) QTL + eight gRNAs targeting the promoters of *SlCLV3*, *S* and *SP*.	Tomato	Wild relative domestication	Rodríguez-Leal et al. (2017)
	Six targets at SP, SP5G, SlCLV3 and SlWUS	Tomato	Wild relative domestication	Li T. et al. (2018)
	Six targets at *SELF PRUNING*, *OVATE*, *FRUIT WEIGHT 2.2*, *LYCOPENE BETA-CYCLASE*, *MULTIFLORA* and *FASCIATED/YABBY* genes	Tomato	Domestication of wild *Solanum pimpinellifolium*.	Zsögön et al. (2018)
	GS3, *GW2*, and *GN1A*	Rice	Wild relative domestication (*Oryza glaberrima*)	Lacchini et al. (2020)
	Multiple	Rice	Wild relative domestication (*O. alta*)	Yu et al. (2021)
Increasing crop yield potential				
	OsGW2, *OsGW5*, and *OsTGW6*	Rice	Larger grain size	Xu et al. (2016)
	OsGS3, OsGW2, and *OsGn1a*	Rice	Larger grain size	Zhou et al. (2019)
	GS3, *GW2*, and *GN1A*	Rice	Increasing grain size	Lacchini et al. (2020)
Improving crop product quality				
	4 alleles of *GBSS*	Potato	Produce waxy potato	Andersson et al. (2017)
	OVATE, *FRUIT WEIGHT 2.2* and *LYCOPENE BETA CYCLASE*	Tomato	Fruit shape	Zsögön et al. (2018)
	Several sites at *St16DOX*	Potato	Reducing steroidal glycoalkaloids (SGAs)	Nakayasu et al. (2018)
	Two sites at *α-gliadin* gene	Wheat	Produce low-gluten wheat	Sánchez-León et al. (2018)
	OsGS3, OsGW2, and *OsGn1a*	Rice	Grain shape	Lacchini et al. (2020)
	OsGS3, OsGW2, and *OsGn1a*	Rice	Grain shape	Lacchini et al. (2020)
Enhancing crop resistance/tolerance to biotic/abiotic stress				
	DIPM-1, DIPM-2, and *DIPM-4*	Apple	Disease resistance	Malnoy et al. (2016)
	OsSWEET11 and *OsSWEET14*	Rice	Generating broad spectrum disease resistance	Xu et al. (2019)
	SWEET11, *SWEET13* and *SWEET14*	Rice	Generating broad spectrum disease resistance	Oliva et al. (2019)
	TMS5, *Pi21*, and *Xa13*	Rice	Disease resistance and yield enhancement	Li S. et al. (2019)

repair pathway after Cas9 protein was reported to cause DSBs at the target DNA site. NHEJ is an error-prone repair mechanism that can generate insertion/deletion (Indels) of nucleotides near the cleavage site (Mao et al., 2013; Xie and Yang, 2013; Ma et al., 2015). These Indels mostly cause a frameshift mutation of the codon. Successful implementation of multiple gene knockouts mainly depends on the precise selection of the target loci.

In this regard, abundant studies have described multiple gene knockouts in different plant species for various purposes. Ali et al. (2015) explained the potential of CRISPR/Cas9 utilization in multiplexing two sgRNAs to knockout ssDNA viruses and expanded the possibilities of virus biological control in plants. In another attempt, Xu et al. generated a multiplex knockout to develop broad-spectrum disease resistance in rice by using a multiplex CRISPR/Cas9 system to alter the effector-binding elements (EBEs) of the bacterial blight TAL effector for two susceptibility genes, *OsSWEET11* and *OsSWEET14*, in rice cv. Kitaake (harboring the defeated *OsSWEET13*) (Xu et al., 2019). Furthermore, multiple gene knockouts have been involved in metabolite manipulations. In this regard, Kaur et al. designed a single gRNA to target the conserved regions of the two genes encoding RASPDS (Rasthali phytoene desaturase) of the banana cv. Rasthali (Kaur et al., 2018). PDS is one of the carotenoid biosynthesis pathway enzymes that limit the rate of carotenoid synthesis (Cazzonelli and Pogson, 2010; Bai et al., 2016), and its disruption impairs chlorophyll, carotenoid, and gibberellin biosynthesis, thereby producing albino plants. Moreover, multiplex knockout trials indicated the great promise in overcoming redundancy and polyploidy in double- and higher-order knockouts created by multiplex CRISPR/Cas9 in tomatoes (Li W. et al., 2016).

Engineering-Efficient Plant Metabolic Pathways

Reducing or increasing the levels of specific metabolites through modulation of plant endogenous metabolic pathways has become achievable with the application of MGE. MGE has provided opportunities to investigate the roles of multiple genes in synthetic pathways in plants. For instance, using MGE, Ma et al. confirmed the role of *OsGSTU, OsMRP15, and OsAnP* genes in the anthocyanin synthesis pathway in rice (Ma et al., 2015). Moreover, the authors decreased the amylose content in rice by targeting three different sites in the *OsWaxy* gene.

Double and higher-order loss-of-function created by CRISPR/Cas9 offers great promise in modulating multiple genes in a single pathway or multiple pathways. Li et al. assessed the efficiency of multiplexing in tomatoes using the robust CRISPR/Cas9 system, as described by Ma et al. (2015) and Li X. et al. (2018) by targeting two loci in exons 6 and 7 of the tomato *PDS* gene (*slyPDS*). A strong photobleached phenotype was exhibited among the transgenic lines, and some T0 lines had concurrent homozygous modifications at both target sites. Subsequently, the authors used this system for metabolic engineering and manipulation of the gamma-aminobutyric acid (GABA) shunt metabolic pathway in tomatoes. They targeted five key genes in its pathway, *GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9,* and *SSADH*. The researchers successfully edited five sites, whereas no editing events occurred in the third site, which was thought to be caused by the lack of CG content at this site. Quadruple mutant plants were obtained, which expressed the highest GABA levels in leaves, reaching almost 19-fold higher than that in wild-type plants. Li et al. discussed the relationship between various pathway components and the effects of mutations on certain components in the pathway. These results indicated the power of multiplex CRISPR/Cas9 as a tool for plant metabolic engineering and will improve our understanding of plant polygenic functional outcomes. In a bidirectional strategy, the same group targeted the loss-of-function of five different tomato genes using six gRNAs to increase the accumulation of lycopene while inhibiting its conversion to β- and α-carotene (Li X. et al., 2018).

Some plant metabolites, such as isoflavone, are controlled by multiple genes and involve specific pathways content (Li and Zhang, 2017). These compounds not only benefit human health but also play a critical role in the plant-environmental interactions for plant fitness in certain environments. Zhang et al. designed a single CRISPR/Cas9 binary vector to target three genes encoding key enzymes in the competing metabolic isoflavone biosynthesis pathway with the aim of increasing the isoflavone content in soybean plants (Zhang et al., 2020). The triple mutant (T3 homozygous plants) plant leaves had two times the level of isoflavone compared with that of wild-type plant leaves. The results of this study indicated the possibility of using MGE for loss-of-function competing pathway genes to increase the availability of specific plant substances or metabolites for another pathway.

Transcriptional Regulation

Controlling gene expression has long been a goal of plant scientists for deciphering plant gene function and improving crop traits. CRISPR-mediated gene regulation, designed by engineering a nuclease-inactivated Cas9 (dCas9) protein with a transcriptional activation domain (VP64) or repressor domains (SRDX), has been very powerful in activating or repressing the endogenous expression of plant genes of interest (Lowder et al., 2017; Li Z. et al., 2019; Malzahn et al., 2019). With the help of gRNA, the dCas9 fusion protein can specifically bind to the promoter region and manipulate the expression of downstream genes.

To date, MGE-mediated transcription manipulation has mainly been focused on promoters implicated in gene repression, activation, and epigenetic modification. An increase of ∼80-, 37-, and 192-fold was reported when targeting the promoter regions of the *WRKY30*, *RLP23,* and *CDG1* genes for simultaneous activation of their transcription in *Arabidopsis* (Li et al., 2017). Meanwhile, several gRNAs simultaneously targeting the same promoter in plants using dCas9-VP64 displayed synergistic effects (Lowder et al., 2015, 2018; Vazquez-Vilar et al., 2016). To determine the efficiency of CRISPR/Cas9 in controlling the gene expression of protein-coding and non-coding genes in *Arabidopsis*, additive transcription activation was observed by Lowder et al., who targeted three different sites in the promoter of the production of anthocyanin pigment1 gene (*AtPAP1,* encoding a transcription factor) and *miR319* (encoding a microRNA; Lowder et al., 2015). Gong et al. (2020) targeted two different genes, *OsWOX11* and *OsYUC1*, involved in crown root development and auxin biosynthesis in rice, respectively. They designed multiple gRNAs to target different sites in the promoter regions upstream of these genes, and the results indicated that targeting two different regions within 350 bp upstream of the TSS is most effective for dCas9-TV-based transcriptional activation.

With the flexibility and expandability of CRISPR/Cas9, it is possible to introduce several regulatory mutations of *cis*-regulatory elements (CREs), such as promoter enhancers and silencer sequences that regulate gene transcription, which could lead to a range of quantitative transcriptional and phenotypic changes. Rodríguez-Leal et al. (2017) provided a prime example of the utilization of a multiplex CRISPR/Cas9 system to fine-tune gene expression by targeting eight sites in the promoter regions of different tomato genes. The results have demonstrated that alteration of gene expression based on editing the CREs of key developmental transcription factors promises an attractive approach for generating desirable/beneficial regulatory variants, offering great potential for crop molecular breeding.

Chromosomal Segment Restructuring

Chromosomal rearrangements include transposition, duplication, deletion, inversion, or translocation of nucleic acid segments (Gray, 2000). Chromosomal rearrangements occur regularly in plants (Udall et al., 2005; Szinay et al., 2012; Li J. et al., 2016; Zapata et al., 2016; Schmidt et al., 2019). The introduction of two DSBs in a single chromosome by multiple editing mainly leads to deletions (Siebert and Puchta, 2002) and sometimes inversions (Qi et al., 2013; Zhang et al., 2017), whereas two breaks on different plant chromosomes could lead to the occurrence of reciprocal translocation (Pacher et al., 2007).

Induction of deletions, inversions, and translocation using MGE has been employed in mammals and reviewed elsewhere (Cheong et al., 2018). Most of the reports about chromosomal segment restructuring in plants focused on introducing deletions. Deletions smaller than 100 bp were detected at high frequencies in the *N. benthamiana* T0 and *Arabidopsis* T2 populations, whereas deletions larger than 120 bp were still feasible, but at lower frequencies in *Arabidopsis* (Ordon et al., 2017). Durr et al. (2018) aimed to improve the propagation of deletions occurring in somatic cells by direct plant regeneration. Furthermore,

different investigators have reported the generation of heritable deletions of genomic regions using CRISPR/Cas9 in *Arabidopsis*, *Medicago,* and rice (Zhou et al., 2014; Gao et al., 2015; Cermák et al., 2017; Wu et al., 2018). More recently, a unique study on inversion formation in *Arabidopsis* was reported, where an 18 kilobase (kb) segment was successfully inverted and transmitted to the next generation (Schmidt et al., 2019). The impact of these chromosome restructuring methods in the field of plant biology is remarkable because it is now feasible to redesign chromosomes according to specific needs (Schmidt et al., 2020).

Multiplex Base Alterations

This application utilizes the CRISPR/Cas system to modify individual bases at multiple sites simultaneously for a gain of function. The establishment of this technology first relied on the fusion of cytosine deaminase or adenosine deaminase to the artificially mutated DNA nickase nCas9, which forms the cytosine editor (CBE) and adenine editor (ABE), respectively. In addition, the use of uracil glycosylase inhibitory protein (UGI) can increase the stability of uracil in DNA, resulting in enhanced editing efficiency. Combined with the multiple-targeting feature of sgRNAs, base editing can be achieved at multiple sites of the genome. Both CBE and ABE have been successfully applied in various model plants and crops (Chen et al., 2019). Most multiplexing base editing researches have focused on herbicide resistance (Shimatani et al., 2017; Kang et al., 2018; Li C. et al., 2018; Li Y. et al., 2019; Veillet et al., 2019; Wu et al., 2020). The reported maximum number of multiplexed sgRNAs in a single construct is four targets in rice, two targets at *OsWAXY,* and one at *OsCDC48* and *OsSNB* (Wang F. et al., 2020). Shimatani et al. (2017) reported a system with three sgRNAs targeting *ALS* and *FTIP1e* that was designed to generate double mutants resulting in the resistance to two herbicides. Moreover, using gRNA libraries, base editors can generate high-density substitutions in target regions, thus facilitating the directed evolution of plant genes (Kuang et al., 2020; Li et al., 2020; Liu et al., 2020). The development of different base-editing platforms was reviewed in detail in Mishra et al. (2020). Ren et al. developed a CRISPR/Cas9 toolkit, extensively enabling efficient base editing at desired sites in the rice genome (Ren et al., 2017). Multiple genomic sites could be targeted for single-base modification. These toolkits were further improved to increase the possibility of generating gain-of-function and loss-of-function mutants for several traits of agronomic importance through base editing (Ren et al., 2018). More recently, Li et al. (2020), created a system that enables the creation of saturated targeted endogenous mutagenesis editors (STEMEs) that allow simultaneous C to T and A to G conversions at specific target sites in plants, where both CBE and ABE are fused together with cas9 nickase and UGI.

A more advanced editing technique that efficiently produces all possible base conversions without the mandate of DSBs or donor DNA was developed, and it is called prime editing (Anzalone et al., 2019). This system uses a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase programmed with a prime editing guide RNA (pegRNA), allowing new genetic information to be written at a specific locus (Lin et al., 2020). Although the efficiency of prime editors is lower than that of base editors, the system is still a versatile and appealing new tool for creating all 12 types of single-point mutations, mixtures of several substitutions, and indels. Gao and her group used this system to develop mutant rice plants carrying G to T mutations, multinucleotide substitutions, and a number of desired nucleotide deletions (Lin et al., 2020). To date, it is very difficult to achieve these types of mutations using any other current editing tool.

MGE IN CROP IMPROVEMENT

Multiplex genome-editing is an innovative approach that accelerates the crop improvement cycle and makes the expected outcome more feasible and achievable. This technology provides effective solutions to overcome the limitations of plant breeding approaches, particularly for providing required variants that cause characteristics desired by breeders at a fast pace without labor-intensive phenotypic and molecular evaluation of breeding germplasm. Although MGE applications are relatively new, the technology has been widely used to improve a diverse range of crops to gain in yield, quality, and nutritional value, herbicide resistance, and biotic and abiotic stress tolerance. In this section, we describe the revolution in crop improvement programs by introgression of this technology for editing plant genotypes to accomplish different breeding objectives.

Generating Diversity and Crop Domestication Using MGE

Plant breeders aiming to develop new genotypes always seek to improve specific plant characteristics, and to achieve their goal, they unintentionally decrease genetic diversity among the crop genotypes. Furthermore, most of the studies on genetic diversity and QTLs depend on naturally occurring mutations that rarely occur in gene-regulatory regions such as promoters and upstream open-reading frames (uORFs) (Rodríguez-Leal et al., 2017), whereas traditional breeding often requires more effort to screen and utilize QTLs carrying favorable mutations.

The CRISPR/Cas9 may allow new genetic diversity to be generated by targeting specific genome loci, which may lead to the identification of new genes. In this context, Rodríguez-Leal et al. demonstrated that genome-editing tools can introduce new *cis*-regulatory alleles with the aim of identifying new alleles that provide beneficial quantitative traits (Rodríguez-Leal et al., 2017). A CRISPR/Cas9-based system was reported that targets the promoter sequence of genes to regulate three major productivity traits, namely, fruit size, inflorescence branching, and plant architecture in tomatoes. The results demonstrated a wide range of variations that could be achieved by altering the expression of the target genes.

Multiplex genome-editing further accelerates the domestication of crop wild relatives by modifying domestication genes. Domestication genes with marked effects on specific key phenotypes have been reported in barley (Komatsuda et al., 2007), maize (Wang H. et al., 2015), rice (Civán and Brown, 2017), and tomato (Li T. et al., 2018). Zsögön et al. designed a multiplex CRISPR–Cas9 strategy to target six genes in wild

Solanum pimpinellifolium that are important for productivity in the present tomato crop breeding lines (Zsögön et al., 2018). The mutants were modified in their fruit number, size, shape, nutrient content, and plant architecture in a single generation and within a single transformation event. In the same context, with the aim of maintaining favorable characteristics of wild ancestors of *Solanum pimpinellifolium,* Li T. et al. (2018) edited four lines of four wild tomato accessions, all of which were resistant to bacterial spot disease and two of which were salt-tolerant, using the Csy4 multi-gRNA CRISPR/Cas9 system to target coding sequences, *cis*-regulatory regions, or uORFs of four genes associated with morphology, flower and fruit production, and ascorbic acid synthesis. The generated Cas9-free genotypes had a domesticated phenotype with parental disease resistance and salt tolerance. African rice, *Oryza glaberrima*, is a wild relative of the cultivated Asian rice *Oryza sativa*. African rice has specific characteristics to withstand diverse environmental stresses but has low yield ability. Lacchini et al. (2020) disrupted domestication loci of the cultivated African landrace Kabre, which resulted in mutants with significantly improved grain yield. Furthermore, domesticating allotetraploid rice was demonstrated by targeting six agronomically important traits of *O. alta* (an allotetraploid perennial wild rice) (Yu et al., 2021).

Increasing Crop Yield Potential by MGE

High-yielding ability under different environments is the core trait behind developing new crop genotypes. Therefore, it is necessary to introduce multiple QTLs into an elite genotype to withstand different environmental stresses without yield penalties. These QTLs are generally located in different genetic backgrounds, and there is no certain guarantee that they will perform the best when they are transferred to other genetic backgrounds. Even in conventional crop breeding, this transfer requires multiple rounds of crossing and selection, which cost labor, time, and money, and linkage drag might often interrupt efforts with unfavored genes. MGE allows knocking out of multiple genes with negative effects on yield simultaneously from genotypes with specific resistance to environmental stress in a single generation. Xu et al. (2016) targeted three major genes, namely *OsGW2*, *OsGW5,* and *OsTGW6*, which negatively regulate rice grain weight. Double (gw5tgw6) and triple (gw2gw5tgw6) T1 mutants showed larger grain sizes than wild-type rice. Zhou et al. (2019) targeted negatively regulated genes for grain size, width, weight, and number, namely *OsGS3, OsGW2,* and *OsGn1a*, respectively, which directly affected grain yield, in three elite rice varieties. Similar results were obtained by Lacchini et al. (2020) after targeting the same genes (*OsGS3, OsGW2,* and *OsGn1a*) in a mutated African rice cv. Kabre with a disrupted *HTD1* gene (short stature compared to the wild type).

Improving Crop Product Quality by MGE

Since yield is the most important trait for breeders, the adoption of new genotypes by farmers is strongly driven by crop quality traits (Dalton, 2004; Wang et al., 2012; Abdelrahman and Zhao, 2020). High-yielding varieties with improved quality are more acceptable to consumers, which in turn increases commercial value for the crop. Quality characteristics differ among crops; however, physical properties, eating quality, and nutritional properties are core traits.

Most of the studies that focus on the domestication of crop plants consider quality genes, among others, during planning for multiplex editing of different genes. Zsögön et al. (2018) aimed to create a novel genotype derived from tomato wild-type *S. pimpinellifolium* by generating loss-of-function mutations in six genes using MGE. Among the edited genes, three genes were related to the quality traits of fruit shape, fruit weight, and lycopene content (*OVATE*, *FRUIT WEIGHT 2.2*, and *LYCOPENE BETA CYCLASE*, respectively). Mutants with loss-of-function in the *OVATE* gene had an oval fruit shape, while no changes in fruit sizes were reported for *FRUIT WEIGHT 2.2* mutants. Meanwhile, mutants of *LYCOPENE BETA CYCLASE* had 100% more lycopene without a negative effect on β-carotene or lutein compared with the wild type. The level of lycopene, which is a kind of carotenoid, is one of the most important nutritional quality traits of tomato fruit. Li et al. successfully increased lycopene in tomato fruits to ∼5.1-fold by using multiplex CRISPR/Cas9 genome editing of five genes that promote the synthesis of lycopene (*SGR1* gene) and genes that catalyze the cyclization of lycopene (*LCY-E*, *LCY-B1*, and *LCY-B2*, as well as *Blc*) (Li X. et al., 2018). The strategy was to promote lycopene biosynthesis while inhibiting its conversion into β-carotene and α-carotene. However, the increased number of mutant genes did not result in a concomitant increase in lycopene (Li X. et al., 2018). Instead, the *SGR1* single mutant exhibited the highest lycopene level followed by the plants with triple mutations in *SGR1*, *Blc,* and *LCY-E*.

Multiplex genome-editing has been successfully implemented for altering physical and nutritional quality properties. Lacchini et al. (2020) improved the grain shape of an *Oryza glaberrima* landrace when multiplexed editing of the *OsGS3, OsGW2,* and *OsGn1a* genes was performed. Kim et al. (2017) designed nine crRNAs to simultaneously target two homologous genes, *FATTY ACID DESATURASE 2-1A* (*FAD2-1A*, Glyma10g42470) and *FAD2-1B* (*Glyma20g24530*), in the soybean genome using CRISPR/Cas12a. *FAD2* encodes an enzyme that is responsible for the conversion of monounsaturated oleic fatty acid to a polyunsaturated linoleic fatty acid. Sánchez-León et al. (2018) designed two sgRNAs to target the *α-gliadin* gene using CRISPR/Cas9 to produce low-gluten wheat.

Moreover, several studies have reported improvements in the eating quality of crops using MGE. In potato (*Solanum tuberosum*), reducing steroidal glycoalkaloids (SGAs) is a requisite in breeding programs, as the accumulation of SGAs causes unfavorable taste. Nakayasu et al. (2018) multiplexed several sgRNAs to knock out a single gene (*St16DOX*; encoding a steroid 16α-hydroxylase in SGA biosynthesis) using hairy root culture and succeeded in developing SGA-free hairy roots of tetraploid potato (Nakayasu et al., 2018). In another study aiming to enhance the potato starch quality, Andersson et al. used three sgRNAs to target the four alleles of the potato granule-bound starch synthase (*GBSS*) gene to produce waxy potato (Andersson et al., 2017). Knocking out the four alleles caused a reduction in GBSS enzyme activity, leading to alteration in starch composition

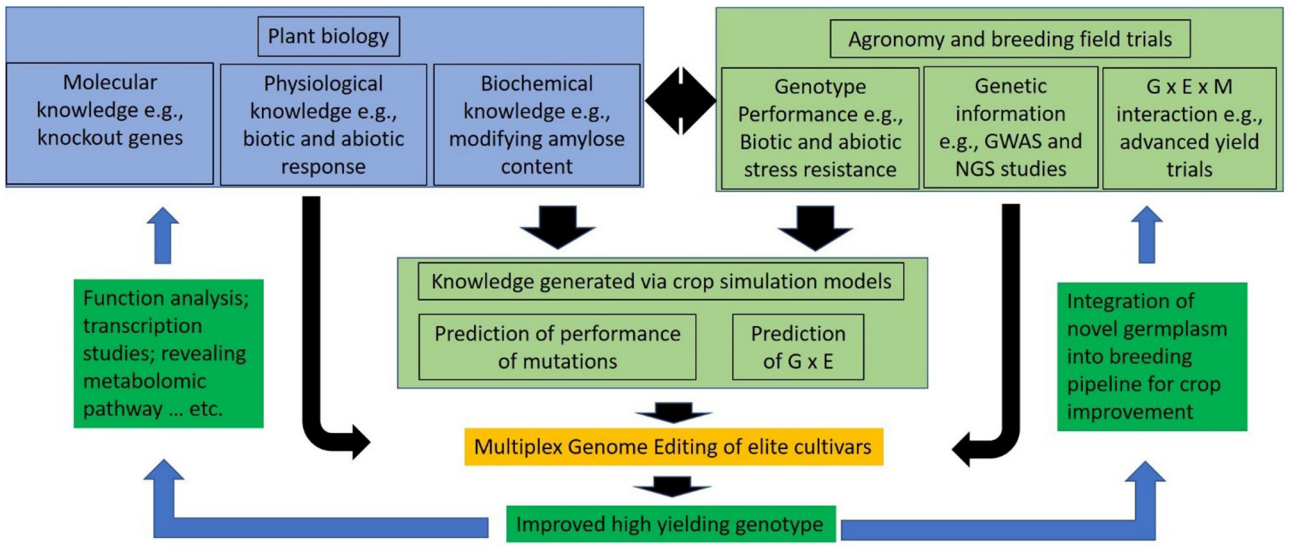


FIGURE 2 | The role of multiplex genome editing (MGE) is explained: the development of novel germplasm is accelerated that either increases knowledge in plant biology or assists crop improvement programs.

with a concomitant increase in the amylopectin/amylose ratio. Furthermore, mutants with only one functional allele still produced a significant amount of amylose.

Enhancing Crop Resistance/Tolerance to Biotic/Abiotic Stress by MGE

Biotic and abiotic stress threatens food security, causing a reduction in crop production. Abiotic stress is more prominent during global environmental changes. Drought, salinity, and extreme temperatures are the main abiotic stressors that cause biological and biochemical effects on plant growth and development. Most of the gene editing for abiotic stress tolerance has been simplex targeting, i.e., one gene related to one constraint. Zafar et al. reviewed the efforts that have been made for the development of mutations for abiotic stress (Zafar et al., 2020). Ethylene-responsive factors (ERFs) are proteins that enhance plant tolerance to different abiotic stressors by activating stress-responsive genes. However, multiplex gene editing for biotic stress was more feasible because of the ease of targeting diseases susceptibility (*S*) genes, which confer a negative effect on disease resistance. Recently, Oliva et al. used CRISPR–Cas9-mediated genome editing to introduce mutations in the promoters of three *SWEET* genes (*SWEET11*, *SWEET13,* and *SWEET14*) of rice cv. Kitaake, IR64, and Ciherang-Sub1 for generating broad-spectrum bacterial blight resistance genotypes (Oliva et al., 2019). With the same objectives, Xu et al. (2019) generated mutations in two EBEs of the *S* genes *OsSWEET11* and *OsSWEET14* in rice cv. Kitaake. The resulting mutants were stable and consistently exhibited broad-spectrum resistance to most bacterial blight strains, with few exceptions in two generations of rice lines. Malnoy et al. (2016) mutated *DIPM-1, DIPM-2*, and *DIPM-4* to increase apple resistance to fire blight disease. Disease genes were also multiplexed with other traits to broaden the scope of the multiplexing objectives. Li S. et al. (2019) introduced mutations into the *TMS5*, *Pi21*, and *Xa13* genes in rice. Triple T1 mutants showed characteristics of thermosensitive genic male sterility (TGMS) with an enhanced blast and bacterial blight resistance. MGE will pave the way toward sustainable development of disease resistance by targeting more *S* genes in different plant species.

CONCLUSION AND FUTURE PERSPECTIVES

Conventional plant breeding has limitations in meeting food security goals for ever-growing populations around the world. However, practical operations of screening germplasm for genetic variations, developing breeding populations, mutation breeding, and hybridization are usually labor-intensive and time-consuming. Genome editing is an innovative technology that is considered an important tool for plant biology studies and for crop improvements. Using MGE to simultaneously target different genomic regions has accelerated both basic and applied researches. Different applications of multiplex technology have been developed that allowed robust, routine CRISPR reagent delivery systems in plants. All these improvements made it easier to utilize MGE technology for enhancing plant biology knowledge by knocking out multiple genes to disrupt gene function and decipher their roles in metabolic pathways. Furthermore, multiplex CRISPR-mediated gene editing provides a gene regulation tool to activate or repress the expression of multiple genes simultaneously. With multiplex CRISPR/Cas9, it is possible to reformat and rearrange chromosomal segments based on the needs of biologists. The utilization of MGE may increase the effect of off-target occurrence as the number

of targets increased (McCarty et al., 2020). Several online toolboxes for off-target detection and expectations have been developed and updated for eliminating the percentage of off-target occurrence (Cradick et al., 2014; Kang et al., 2020; Naeem et al., 2020; Störtz and Minary, 2021).

Applications of multiplex base editing have facilitated gain-of-function investigations; however, base editing generates mainly C–T and A–G conversions to date. More recently, prime editing has advanced base editing so that new genetic information can be written into specified DNA loci. This technology will expand the capabilities of MGE that may allow the gain-of-function of multiple genes simultaneously. With the identification and utilization of CRISPR systems, breeding schemes have become faster and more precise. Generating mutations with MGE allows diversity for selection and yield enhancement considering quality aspects. Improving specific traits without any other alteration to others may become possible in the near future.

Multiplex genome-editing will be enhanced by skipping long laborious and complicated plant regeneration and tissue culture steps by allowing CRISPR reagents to be delivered directly into plant meristems or pollen tissues, as reported by Maher et al. (2020), which presented a successful example of tissue culture-free plant gene editing. Furthermore, it is now also possible to write new genetic information to a specific locus in the genome (Lin et al., 2020). Despite diverging social perspectives, it is believed that multiplex CRISPR technology will play an important role in agricultural sustainability by developing resilient germplasm.

Implementation of MGE is necessary for understanding the dynamics of synthetic metabolic pathways. In addition, exploring enzyme functionalities in different pathways and their relationship with other omics information is crucial. Additionally, more attention should be devoted to utilizing this technology for the exploration of interactions among different metabolic pathways and gene regulatory, signal transduction, and protein-protein interaction networks in response to biotic and abiotic stress.

Advanced knowledge regarding plant biology, agronomy, and breeding supports crop improvement programs. Through the utilization of such knowledge and new modeling approaches to predict the performance of the genetic variants (Wang H. et al., 2020), MGE will accelerate the development of elite crop cultivars with precise genomic modifications (**Figure 2**). MGE will increase and facilitate new plant biology discoveries and/or the development of new elite germplasm.

AUTHOR CONTRIBUTIONS

MA and KZ designed and discussed the content. MA and ZW wrote the first draft of the manuscript. JR and KZ critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Employing CRISPR/Cas Technology for the Improvement of Potato and Other Tuber Crops

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New breeding technologies have not only revolutionized biological science, but have also been employed to generate transgene-free products. Genome editing is a powerful technology that has been used to modify genomes of several important crops. This review describes the basic mechanisms, advantages and disadvantages of genome editing systems, such as ZFNs, TALENs, and CRISPR/Cas. Secondly, we summarize in detail all studies of the CRISPR/Cas system applied to potato and other tuber crops, such as sweet potato, cassava, yam, and carrot. Genes associated with self-incompatibility, abiotic-biotic resistance, nutrient–antinutrient content, and post-harvest factors targeted utilizing the CRISPR/Cas system are analyzed in this review. We hope that this review provides fundamental information that will be useful for future breeding of tuber crops to develop novel cultivars.

Keywords: double-stranded DNA breaks, genome editing, sequence-specific nucleases, CRISPR/Cas system, tuber crops

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INTRODUCTION

The Cell Repair Mechanism of Double-Strand DNA Breaks

Cells have various inherent mechanisms for the repair of double-stranded DNA breaks (DSBs), which are caused by endogenous and exogenous genotoxic agents (Wang and Xu, 2017; Vats et al., 2019). Cell type, cell state, and nature of the DSBs influence multiple DNA repair mechanisms. The failure and misrepair of DSBs can result in mutations and occur genome instability, and even can lead to cell death (Mcvey and Sang, 2008). However, during some essential physiological processes, DSBs are used to generate genetic diversity, such as the immune repertoire generation and the recombination of alleles during meiosis (Le Guen et al., 2015). Moreover, the loss of genetic diversity would results in an evolutionary dead end. So the DNA repair mechanism needs to cause active maintenance of genome stability and also generate genetic diversity in populations. Therefore, the high accuracy of DNA repair processes is an important phenomena in all organisms (Guirouilh-Barbat et al., 2014).

The DSB repair uses three general type of strategies. Non-homologous end joining (NHEJ) is generally regarded as error-prone, and results in mutations such as smaller insertions, deletions, and substitutions at the DSB site. It is the dominant and most active pathway in eukaryotes for repairing DSBs in all phases of the cell cycle (Anzalone et al., 2020). Homologous recombination (HR) is error-prone often with point and fragment mutations, insertions, and deletions (Rodgers and McVey, 2016). It is believed that HR is the foundation of genome engineering. In HR, the identical sister chromatids are served as templates to repair DSBs in cells during the late S/G2

phases of the cell cycle (Wang and Xu, 2017; Afzal et al., 2020). The HR, in theory, can lead to knock-in, protein-domain swapping, new gene functions, or alteration in gene regulation. HR is commonly considered as an error-free process, whereas NHEJ is considered to be error-prone process. However, Bétermier et al. (2014) and Guirouilh-Barbat et al. (2014) concluded that HR and NHEJ have a double effect, which on the one hand, they are essential for genomic stability maintenance and diversity, but on the other hand can damage the maintenance of genomic integrity. Microhomology-mediated end joining (MMEJ) is an error-prone repair system that repairs DNA breaks through using of substantial microhomologous sequence (4–25 bp) and always lead to small insertions or deletions (Mcvey and Sang, 2008; Uranga et al., 2021). The MMEJ is an active repair system during the G1and early S phases of the cell cycle (Taleei and Nikjoo, 2013).

Genome Editing

Genome editing (GE), a recent technological innovation in the life sciences, is an example of techniques that are used to explore biological phenomena (Vats et al., 2019). In this technique, DNA is modified through cell-repair mechanisms that introduce precise breaks in the genome at specific sites (Sun et al., 2016). Genome-edited plants are not similar to conventional transgenic plants, as they may not incorporate foreign DNA. This distinction makes genome editing a unique and powerful breeding tool that has promising applications in agriculture development, especially when genome-edited crops are not adjusted as genetically-modified (GM) plants. This technique requires the introduction of sequence-specific nucleases (SSNs) into plants through the following three major methods of transformation: *Agrobacterium*-mediated transformation (Zhang et al., 2020), biolistic-mediated transformation (Hamada et al., 2018; Rustgi and Luo, 2020), and polyethylene glycol (PEG)-mediated protoplast transfection (Sant'Ana et al., 2020). Potato and most tuber crops are members of the dicotyledonous family and the most popular method for genetic transformation of dicotyledonous plants is *Agrobacterium*-mediated gene transfer.

From both a scientific and a regulatory perspective, it is beneficial if the integration of DNA in the edited plant genome is avoided (Andersson et al., 2018). In general, several strategies have been developed for obtaining transgene-free CRISPR/Cas9-edited tuber crops. They are the selection of transgene-free genome-edited plants by Mendelian segregation in T1 generation of potato obtained by using *Agrobacterium*-mediated delivery method (Ye et al., 2018; Enciso-Rodriguez et al., 2019) CRISPR/Cas9 cytidine base editor through the *Agrobacterium*-mediated delivery method (Veillet et al., 2019b), transient expression of CRISPR/Cas9 construct by the PEG-mediated transfection method (Andersson et al., 2017; Chatukuta and Rey, 2020), and ribonucleoprotein (RNP)-based genome editing (Andersson et al., 2018; González et al., 2020). In the case of Mendelian segregation, one of the limiting factors is the need of hybridization and selection of transgene-free plants from T1 segregating generations which are time consuming and laborious. The alternative delivery methods, including biolistic, PEG-mediated protoplast transfection, and RNP- based methods have been considered to be easy ways to result in non-transgenic mutants. However, these approaches always show high sensitivity and are limited to some species due to bottlenecks in the regeneration process.

Genome editing provides agriculture productivity for the world population, which has reached 7.8 billion and is estimated to exceed 10 billion by 2055 (FAO, 2017; Li and Yan, 2020). Up until 2018, GE crops have been planted approximately 191.7 million acres throughout 26 countries and adopted by approximately 17 million farmers (Yu et al., 2019; Przezbórska-Skobiej and Siemiński, 2020). The legal status between genetically modified organisms (GMO) and genome-edited products is still in discussion. However, the United States and Japan have regulatory systems at the highest political levels, whereas European countries adopted a strict policy toward genome-edited products (Menz et al., 2020). So that scientists are giving evidence that genome editing is different from genetic modification because the DNA editing method is the same as the conventional breeding method or natural biological evolution (Turnbull et al., 2021).

Genome Editing With Sequence-Specific Nucleases

Current advancement of SSNs, such as meganuclease I-SceI (1944), zinc-finger nucleases (ZFNs,1996–2003), transcription activator-like effector nucleases (TALENs, 2009–2010), and Cas nuclease (160 kDa protein, 2013-present) offer alternative approaches for trait improvement in crop plants (Butler et al., 2015; Schiml et al., 2015; Neela and Fanta, 2019; Turk, 2019). All SSNs have been widely used in the genome editing studies for trait improvement of various plant species such as *Arabidopsis*, tobacco, and maize (reviewed in Zhang et al., 2018).

Zinc-Finger Nucleases

Zinc-finger nucleases consist of two functional fused domains, including a non-specific DNA-cleavage domain and a DNA-binding domain composed of Cys_2His_2 zinc fingers, which target three base pairs and a non-specific catalytic domain of the FokI endonuclease (Bibikova et al., 2002; Nargesi et al., 2021). ZFNs were first used in model plant *Arabidopsis thaliana* (Lloyd et al., 2005). Since then, ZFNs has become increasingly popular for the genome modifications in various crop species, such as rice (Cantos et al., 2014) and maize (Gao et al., 2010). However, ZFNs have several limitations, including complexity of the contact between zinc fingers and DNA, an inherent drawback, and challenges in authenticating such proteins for a particular DNA locus of context.

Transcription Activator-Like Effector Nucleases

Similar to ZFNs, TALENs are fusions of transcriptional activator-like effectors (TALE) repeats built from arrays of 33–35 amino acid modules and the FokI restriction enzyme from the plant pathogen *Xanthomonas* (Schiml et al., 2015; Ye et al., 2018; Afzal et al., 2020). The Fok1 nuclease is a common functional part between ZFNs and TALENs. However, designing TALENs is more

affordable than ZFNs (Nargesi et al., 2021). The TALENs are simpler to construct and authenticate and thus provide a cost effective and faster method of genome editing. The challenges associated with synthesis, protein design, and corroboration remain as obstacles to more extensive use in genome-editing applications (Bétermier et al., 2014).

Clustered Regularly Interspaced Short Palindromic Repeats

Clustered regularly interspaced short palindromic repeats (CRISPR/Cas) is an essential genome-editing technique based on the type II prokaryotic adaptive immune system from *Streptococcus pyogenes* Cas9 (SpCas9) (Schiml et al., 2015; Veillet et al., 2019a). Jennifer Doudna and Emmanuelle Charpentier first adapted gene-editing by using CRISPR/Cas9 system to function in the test tube in 2012, and were awarded the Nobel Prize for a significant contribution to Chemistry in 2020. In nature, bacteria and archaea use the RNA-guided endonucleases as part of an adaptive immune system to direct the degradation foreign nucleic acids (Anzalone et al., 2020). Although SpCas9 is predominantly used for plant functional genomics various other Cas enzymes from different bacteria have been successfully utilized as an alternative for genome editing system in plants, including the *Staphylococcus aureus* Cas9 (SaCas9) (Kaya et al., 2016), *Streptococcus thermophilus* (CRISPR1, CRISPR2, CRISPR3, and CRISPR4) (Sapranauskas et al., 2011), *Francisella novicida* Cas12a (Cpf1, FnCas12a) (Wu et al., 2020), *Brevibacillus laterosporus* (BlatCas9) (Gao et al., 2020), and *Leptotrichia shahii* (LshCas13a, Cas13a) (Veillet et al., 2019a).

The CRISPR/Cas9 genome-editing system is a two-component complex composing of single-guide RNA (gRNA) and Cas9 enzyme. The customizable gRNA, a small piece of the pre-designed RNA sequence of 20 bases, binds to Cas9 and specifies a target sequence (locus) within the genome. The Cas9 enzyme acts as a pair of molecular scissor that cut the two strands of DNA at a specific location in the genome defined by the gRNA sequence, then section of DNA can be modified by adding or removing (Hongbao et al., 2018; Veillet et al., 2020). Schematic representation of plant genome editing with CRISPR/Cas is shown in **Figure 1**. In August 2013, three studies were published in the same issue of Nature Biotechnology showing that Cas9 works in plant cells. One of the genes considered for editing was phytoene desaturase gene (PDS3) in the model plants *A. thaliana* and *N. benthamiana* and in the crop plant *O. sativa* by transient expression system in protoplasts with the plant codon-optimized SpCas9 (pcoCas9) (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). In these studies other genes including flagellin sensitive 2 gene (AtFLS2) in *Arabidopsis* (Li et al., 2013), three rice genes (*OsBADH2*, *Os02g23823* and *OsMPK2*), and a wheat gene (*TaMLO*) (Shan et al., 2013) have been targeted. Higher mutation efficiency was demonstrated with the use of plant codon optimized Cas9.

The CRISPR/Cas9 system has been mainly used to generate knockout mutants by single gene editing. The development and advantage of base editors has expanded the scope of genome editing by introducing base substitutions without requiring donor DNA or induction of a DSB (Nadakuduti et al., 2018; Choi et al., 2021). Thus far, two kinds of base editors have been developed. Cytosine base editors (CBEs) catalyze the conversion of C and G base pairs to T and A base pairs, and adenine base editors (ABEs), which catalyze A and T to G and C conversions (Anzalone et al., 2020). For CBE, the Cas9 nickase (nCas9) or catalytically dead Cas protein (dCas9) is fused with a cytidine deaminase that catalyzes the deamination of cytosine (C) in an arrow window of the non-targeted strand. Deamination converts the original C to U, which is recognized as T during DNA replication, ultimately resulting in a C and G to T and A single-base substitution (Bharat et al., 2020). Base editing in the CRISPR/Cas9 system for various plant species was reviewed by Li et al. (2021). For example, Veillet et al. (2019a, 2020) developed a *S. aureus* cytosine base editor in the granule-bound starch synthase *StGBSSI* (PGSC0003DMG400012111) and *StDMR6-1* (PGSC0003DMG400000582) genes through a SanCas9 (D10A) that mediated transition (C to T) and transversion (C to G) mutations in potato.

Constant improvements in CRISPR/Cas systems have enabled more ambitious applications that aim to improve plant productivity or develop other desirable traits. One long-standing aim has been the induction of targeted chromosomal rearrangements (crossovers, inversions, or translocations) (Enciso-Rodriguez et al., 2019). Chromosome breaks in dicentric chromosomes whose centromeres are separated by >20 kb are repaired via pathways that rely mainly on sequence homology (HR, SSA) (Cook et al., 2021).

CRISPR/Cas is a flexible, simple, effective and affordable system, and it does not require any protein engineering steps in the CRISPR/Cas system construction (Amancio et al., 2017; Lino et al., 2018). As a consequence, these advantages make the CRISPR/Cas a more usable genome-editing system compare with ZFNs and TALENs. However, CRISPR/Cas still has some disadvantages and limitations, such as PAM restriction and the need for more diversity in CRISPR tools to mediate different simultaneous catalytic activities (Veillet et al., 2019a). The option of using the CRISPR/Cas tool and the general editing strategy depends on some factors such as cell type, cellular environment, a form of the agent, method of delivery each forces different constraints, and different propensities for unwanted genome modification events (Anzalone et al., 2020). All these methods including ZFNs, TALENs, and CRISPR/Cas have been applied for trait improvement in *Arabidopsis*, barley, *Brachypodium*, maize, tobacco, rice, soybean, tomato, wheat, and potato (reviewed in the references) (Malzahn et al., 2017; Nathalia and Patron, 2017; Afzal et al., 2020). The use of CRISPR/Cas9 *in vivo* and *in vitro* for crop improvement via nutritional enhancement and production of biotic and abiotic stress-tolerant plants have also been described (Afzal et al., 2020).

Tuber Crops

Starchy root and tuber crops (RTC), including potato, cassava, sweet potato, yam, and other minor healthiest

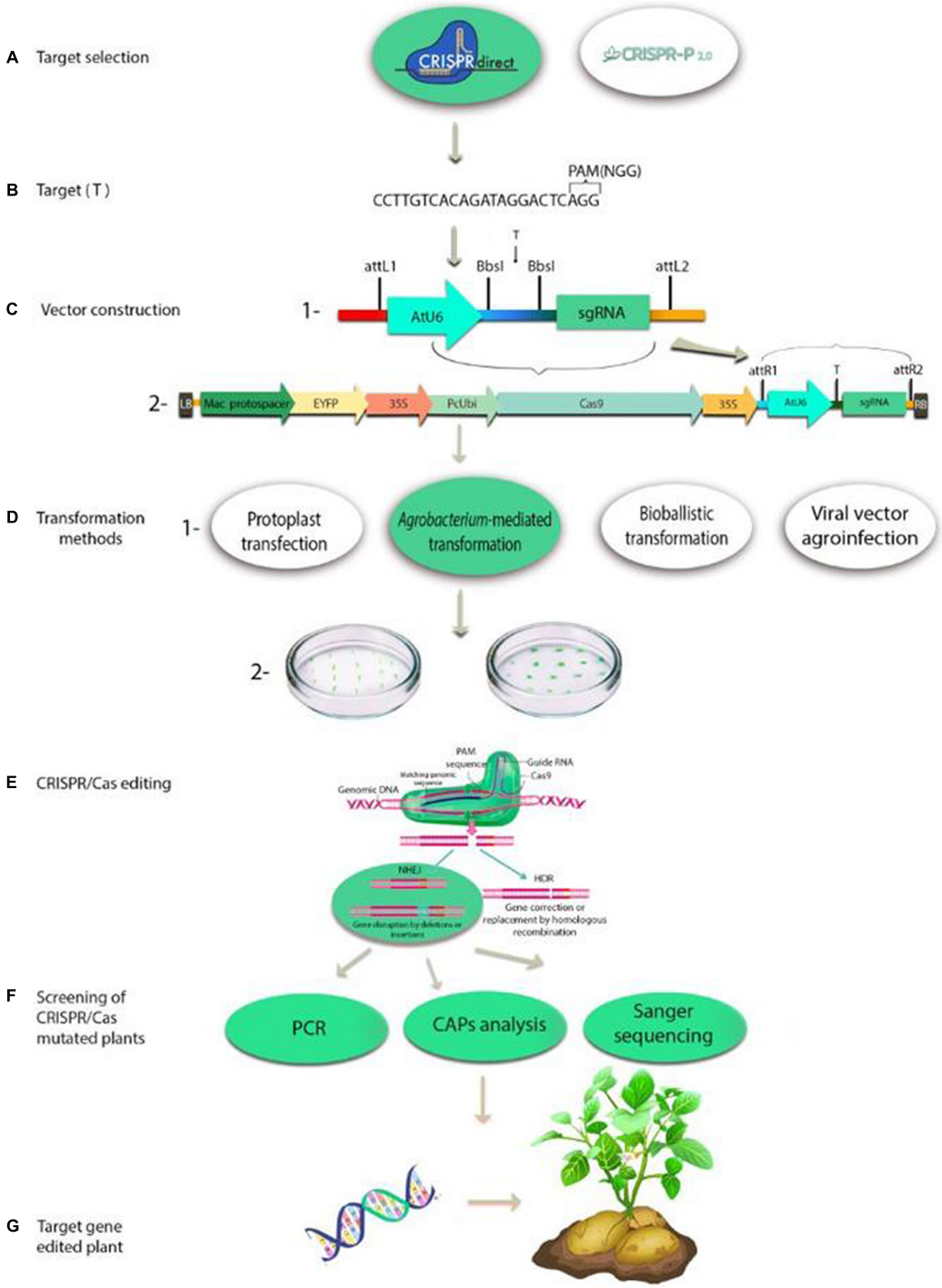


FIGURE 1 | Schematic representation of plant genome editing with CRISPR/Cas: **(A)** Examples of software tools available for CRISPR sgRNA design; **(B)** The 20-bp target sequence with protospacer adjacent motif (PAM); **(C)** (1) Representation of cloning the 20-bp target sequence into the pair of BbsI sites in gRNA cloning vector; (2) Representation of cloning the gRNA expression construct into all-in-one binary vector by the recombination of attL and attR sites; **(D)** (1) Plant transformation methods are used for delivery of CRISPR/Cas9 system: protoplast transfection with polyethylene glycol (PEG) or via electroporation; *Agrobacterium*-mediated transformation; bioballistic transformation and viral vector agroinfection. (2) Examples of plant tissues used as the explants for transformation; **(E)** Diagrammatic representation of the CRISPR/Cas editing system: CRISPR/Cas induces double-stranded breaks in genomic DNA strands. The PAM sequence is primarily required for the Cas9 to cleave DNA. A stretch of 20 bases of sgRNA defines the binding specificity. Two methods repair the DSBs in DNA: non-homologous end-joining (NHEJ), which is not precise and permanently results in a gene knockout and homology-directed repair (HDR), which is activated in the presence of a template and results in knockin or gene replacement; **(F)** Screening of CRISPR/Cas mutated plants by polymerase chain reaction (PCR) analysis, cleaved amplified polymorphic sequences (CAPs) analysis and Sanger sequencing; **(G)** Target gene-edited plant in a potato example.

root vegetables are essential to the agriculture and food security of many countries, and are a dietary supplement of 2.2 billion people in developing countries[1]. The nutritional composition of some RTCs have been reviewed (Chandrasekara and Josheph Kumar, 2016).

Cassava, potato, and sweet potato account for 90% of the world's tuber crop production at an annual level of approximately 836 million tons. The primary producer of the RTCs is Asia, followed by Africa, Europe, and America. In 2017, 494.6 million tons of RTC were produced worldwide (Neela and Fanta, 2019). This review covers the technological advancement, limitations, and future prospects of the CRISPR/Cas system and summarizes the application of this system in potato and other RTCs such as sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta Crantz*), yam (*Dioscorea* spp.), and carrot (*Daucus carota* subsp. *sativus*).

POTATO AND APPLICATION OF CRISPR/Cas SYSTEM

Potato (*Solanum tuberosum* L.) is one of the most consuming crops globally after rice (Oryza s*ativa* L.), wheat (*Triticum* spp.), and maize (*Zea mays* L.) (Amir et al., 2018; Gonzales et al., 2020; Reyniers et al., 2020; Das et al., 2021). Most potato cultivars are tetraploid (2n = 4x = 48), highly heterozygous, and extremely genetically diverse with a very high single nucleotide polymorphism (SNP) frequency (Johansen et al., 2019b). Potato is grown on approximately 18.6 million ha in 150 countries, with an estimated production of 400 million tons annually (Singh, 2008). Potato occupies a wide eco-geographical range, including the northern latitudes of North America, Europe, and Asia (the long-day photoperiod) after originated in the equatorial Andean region of South America (a short-day photoperiod) (Gonzales et al., 2020). Potato grows in a temperature range of 15–29°C and an altitude range of 1500–3200 m a.s.l (Minda et al., 2018). The annual rainfall range requirement of the potato crop is 400–1500 mm, while the pH requirement is 5–6.5. Potato can be grown below 5 up to 4.5 pH levels.

Potatoes have potential of extraordinarily high yield and are an excellent source of more carbohydrates, proteins, minerals, and vitamins than other potential food crops (Hameed et al., 2018). These aspects have received increasing attention from scientific and many research studies have been performed on yield increase, various stress adaptation, and post-harvest conservation and processing (Sevestre et al., 2020). Unique morphological, developmental, and compositional features have been characterized for nearly 70,000 species of potato. The nuclear and organelles genome of a homozygous doubled-monoploid potato breeding line (*S. tuberosum* group Phureja DM1-3 516 R44) and a heterozygous diploid line (*S. tuberosum* group Tuberosum RH89-039-16) have been sequenced by the Potato Genome Sequencing Consortium in 2011 and predicted 39 031 protein-coding genes. This provides a platform for genetic improvement, including molecular and gene editing research in potato[2]. Historically, conventional breeding techniques have been used to increase agronomic yield, processing, and improve storage quality. Unfortunately, the conventional breeding and genetic analysis of cultivated potato has some challenging related to tetrasomic inheritance, high heterozygosity, and self-incompatibility (Nadakuduti et al., 2018). In this study, we reviewed the recent developments of CRISPR/Cas system for the potato and discuss the future perspectives (**Table 1**).

CRISPR/Cas System for the Enhancement of Potato Breeding

Plant genetic resources are the most essential natural resources in the world. Many crops species are recognizable as hybrids that have been domesticated from the wild type polyploidy plants or generated by various conventional or modern biotechnological breeding program (Alix et al., 2017; Bevan et al., 2017). The conventional mutagenesis-based breeding processes are time-consuming and laborious, especially for polyploid crop breeding (Liu et al., 2021). Re-domestication of diploid potatoes applying modern agricultural technologies has made good progress in unlocking the genetic diversity of potatoes. However, most diploid potato species have gametophytic self-incompatible, which has been a limiting factor for the development of inbred lines in potatoes. The genetic basis of self-incompatible mechanism in flowering plants, including the *Solanaceae* family was reviewed in the some researches (Kao and McCubbin, 1996; Silva and Goring, 2001). These technologies include genetic engineering, molecular marker technology, *cis*-genesis, and more recently gene editing (Beumer et al., 2021).

Self-incompatibility is genetically determined by the highly polymorphic S-locus, which consists of strongly linked genes, termed as the pistil-expressed *S-RNase* (S-locus *RNase*) and the pollen-expressed F-box proteins (*SLF* or S locus F-box). The pollen tube growth is one of the main self-incompatible indicators, and a multi-allelic RNase in the pistil blocks incompatible pollen tube growth. To generate self-compatible diploid potato lines, the CRISPR/Cas9 system was used to target the first and second exons of the *S-RNase* gene and self-compatibility was confirmed in T1 lines (Ye et al., 2018; Enciso-Rodriguez et al., 2019). Ye et al. (2018) revealed that a self-incompatible diploid potato could be reinvented with a heterozygous mutation by knocking out the *S-RNase* alleles, and only a single mutation was transmitted to the T1 generation. Further research revealed that self-compatibility was achieved in the *S-RNase* knockout T0 lines, which contained bi-allelic and homozygous deletions/insertions in self-incompatible genotypes, and transmitted self-compatibility to T1 progeny with a potential transgenerational deletion (Enciso-Rodriguez et al., 2019). This study also confirmed that *S-RNase* is located within a pericentromeric region of 6.1 and 18.9 Mb of chromosome 1 in the physical map.

[1]https://www.nri.org/development-programmes/root-and-tuber-crops-in-development/overview

[2]https://www.nature.com/articles/nature10158#citeas

TABLE 1 | Application of CRISPR/Cas system in potato (*Solanum tuberosum* L.).

Plant	Method of delivery	Technology	Target gene (s)	Trait associated with the genes	Type of mutation	Results	References
CRISPR/Cas system for the enhancement of breeding							
Wild type S. Chacoense, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pKSE401 vector	CRISPR/Cas9 system	*S-RNase* (*S*-locus *RNase*) gene	Self-incompatibility	Single and heterozygous mutations	Self-compatibility was observed and transmitted to T1 progeny	Ye et al., 2018
Cvs. DRH-195 and DRH-310 F1 (inbred lines), Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pHSE40 vector	CRISPR/Cas9 system	*S-RNase* (*S*-locus *R*Nase) gene	Self-incompatibility	Bi-allelic and homozygous deletions and insertions; inversions in a biallelic configuration; Chimeric mutations; Chromosome doubling	Self-compatibility was observed and transmitted to T1 progeny	Enciso-Rodriguez et al., 2019
CRISPR/Cas system for the enhancement of resistance biotic stress							
Cvs. Desiree and King Edward, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pDIRECT_22C vector	CRISPR/Cas9 system	*MLO* (*StMLO1*), *HDS*, AtTTM2, StDND1, StCHL1, and DMR6	Late blight	A high number of tetra-allelic deletion; very short deletions and insertions	Tetra-allelic deletion mutants showed enhanced late blight resistance	Kieu et al., 2021
Cv. Desiree, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pP3, pCI, pNIb, and pCP vectors	CRISPR/Cas13a system	PVYO, PVYN, and PVYN:O; targeting the P3, CI, NIb, and CP regions.	Potato virus Y (PVY)	Not given	Lower levels of the virus PVY in systemic leaves	Zhan et al., 2019
Cv. Chicago, Potato (*Solanum tuberosum* L.)	PEG-mediated transfection method of protoplasts	CRISPR/Cas9 system	Coilin gene	Biotic stress (ordinary strain PVY-O) and abiotic (salt and osmotic) stresses.	Deletion	Higher resistance and visible differences in the edited lines F25 and D15	Makhotenko et al., 2019
Cvs. Desiree and MSX914-10 (X914-10), Potato (*Solanum tuberosum* L.)	*Agrobacterium* and GVR-mediated transformation; 35S T-DNAvector, pMDC32 and pLSL vectors	CRISPR/Cas9 system	Acetolactate Synthase1 (*StALS1*)	Herbicide Resistance	Insertion and deletion	Germline inheritance with transmission percentages ranging from 87 to 100%	Butler et al., 2015
Cvs. Desiree and MSX914-10 (X914-10), Potato (*Solanum tuberosum* L.)	*Agrobacterium* and GVR-mediated transformation;	CRISPR/Cas9 system; TALENs	Acetolactate Synthase1 (*StALS1*)	Herbicide Resistance	Point mutation	Reduced herbicide susceptibility phenotype	Butler et al., 2016
Tomato cv. WVA106, and cv. Desiree, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; CBE binary vector	CRISPR/Cas9 system	Acetolactate Synthase1 (*StALS1*)	Chlorsulfuron-resistant	Substitutions	Chlorsulfuron resistant mutants	Veillet et al., 2019b
CRISPR/Cas system for the enhancement of resistance abiotic stress							
Diploid clones, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pJET1.2 vector	CRISPR/Cas9 system	*StCDF1* and *StFLORE*	Tuber development and drought response	Not given	Enhanced resilience drought tolerance; *StCDF1* is a non-redundant regulator of tuberization	Gonzales et al., 2020
Cv. Atlantic, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; VK005-14-g2 vector	CRISPR/Cas9 system	Alternative oxidase gene (*StAOX*)	High light stress	Substitutions	Inhibition of cyanide-resistant respiration.	Hua et al., 2020
CRISPR/Cas system for the enhancement of nutrient contents in potato							
Cv. Kuras, Potato (*Solanum tuberosum* L.)	PEG-mediated transfection method of protoplasts	Transient expression of CRISPR/Cas system	Granule-bound starch synthase (*StGBSS*)	Tuber starch quality	Insertions	Full knockout of GBSS enzyme activity	Andersson et al., 2017
Cv. Kuras, Potato (*Solanum tuberosum* L.)	RNPs delivery in protoplasts	CRISPR-Cas9 Ribonucleoproteins (RNPs)	Granule-bound starch synthase (*StGBSS*)	Tuber starch quality	Insertions	Transgene-free full knockout of GBSS enzyme activity plants	Andersson et al., 2018
Cv. Sayaka, Potato (Solanum tuberosum L.)	*Agrobacterium*-mediated transformation; pMR vector	CRISPR/Cas9 system	Granule-bound starch synthase (*StGBSS*)	Tuber starch quality	Deletions and insertions	Transformants containing dMac3 showed a higher frequency of mutations	Kusano et al., 2018

(Continued)

TABLE 1 | (Continued)

Plant	Method of delivery	Technology	Target gene (s)	Trait associated with the genes	Type of mutation	Results	References
Cv. Desiree, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pDIRECT_22a vector	CRISPR/Cas9 system	Starch synthase (SS6)	Tuber starch quality	Deletions and insertions	Deciphered function of SS6	Sevestre et al., 2020
Cvs. Desiree and Wotan, Potato (*Solanum tuberosum* L.)	PEG-mediated transfection method of protoplasts	CRISPR/Cas9 system	Granule-bound starch synthase (*StGBSS*)	Tuber starch quality	Deletions and large insertions	Increased editing efficiency of the target gene by using StU6 promotor	Johansen et al., 2019b
Cvs. Desiree and Furia, Cv. Desiree, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; CBE binary vector	CRISPR/Cas9 system	Granule-bound starch synthase (*StGBSS*)	Tuber starch quality	small indels,	Mediated transition (C to T) and transversion (C to G) mutations	Veillet et al., 2019a
Cv. Desiree, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; CBE binary vector	CRISPR/Cas9 system	Granule-bound starch synthase (*StGBSS*) and *StDMR6-1*	Tuber starch quality	Small insertions or deletions	Mediated transition (C to T) and transversion (C to G) mutations	Veillet et al., 2020
CRISPR/Cas system for reduction of anti-nutrient contents in potato							
Cv. Mayqueen, Potato (*Solanum tuberosum* L.)	*Agrobacterium*-mediated transformation; pMgP237-2A-GFP vector	CRISPR/Cas9 system	*St16DOX*	Steroidal glycoalkaloids (SGAs) in potato hairy roots	Deletion, Insertion	Abolition of *St16DOX* wild-type sequences in tetraploid potato	Nakayasu et al., 2018
CRISPR/Cas system for the reduced of post-harvest factors affecting potato							
Cv. Desiree, Potato (*Solanum tuberosum* L.)	Protoplasts	CRISPR/Cas9 system	Polyphenol Oxidases catalyze (*StPPO2*)	PPOs activity in the tuber	Small and larger deletions	Lines with a reduced PPO activity and enzymatic browning	González et al., 2020

CRISPR/Cas System for Enhancement of Resistance to Biotic Stress

Biotic stress in plants is occurred by living organisms, commonly viruses, bacteria, fungi, nematodes, insects, arachnids, and weeds (Singla and Krattinger, 2016; Hashem et al., 2019). Potato crops are suffering several pests and diseases, such as late blight (caused by the oomycete pathogen *Phytophthora infestans*), Colorado potato beetle (*Leptinotarsa decemlineata*), bacterial wilt (caused by *Ralstonia solanacearum*), early blight (caused by the fungus *Alternaria solani*), and potato blacklegs (bacterial infection) (Kieu et al., 2021)[3]. Various pathogen infections affect potato yield with high economic losses of 30–64% every year (Torrance and Talianksy, 2020), even it is reached 88% (Kolychikhina et al., 2021). So the identification and characterization of disease resistance genes in the major crops genome have been a main focusing issue for the research community.

CRISPR/Cas has been used to enhance resistance to potato late blight, which remains the most significant disease of potato cultivation globally (Hameed et al., 2018). Late blight is a fungal disease that can destroy the leaves, stems, and tubers of potato plants (Tsedaley, 2014) and exhibits rapid multiplication and dissemination. Tiwari et al. (2021) published late blight resistance genes (*R3a*, *RGA2*, *RGA3*, *R1B-16*, *Rpi-blb2, Rpi,* and *Rpi-vnt1*) in potato. Susceptibility genes (*S*-genes) are important for increasing pathogen resistance (Tiwari et al., 2021). Potato pathogen susceptible genes *StMLO1* (Mildew locus O), *HDS* gene homologue (DMG400008050), *AtTTM2* gene homolog (DMG400025117), *StDND1* (DMG400001441), *StCHL1* (DMG400000711), and other two *DMR6* homologues (DMG400000582 and DMG401026923) were edited with CRISPR/Cas9. The mutant lines encoding *StMLO1, HDS,* and *AtTTM2* genes were as susceptible to late blight as wild type, whereas *StDND1, StCHL1*, and *StDMR6-1* tetra-allelic deletion mutants exhibited enhanced resistance to late blight (Kieu et al., 2021).

Potato virus X (PVX, genus potexvirus), potato virus Y (PVY, genus potyvirus), potato virus A (PVA), potato virus M (PVM), potato virus S (PVS), and potato leafroll virus (PLRV, genus polerovirus) have been recorded in all potato growing zones and are probably the most diverse and economically harmful viruses infecting the world's potato production (Halabi et al., 2021). The PVY have positive-sense (+), single-stranded RNA genome, with an average size of 9.7 kb in length. Upon translation, the RNA genome encodes a single large polyprotein of around 3061 amino acid residues. The single large polyprotein is processed by three virus-specific proteases domains into 11 mature proteins, namely P1, HC-Pro, P3, P3N-PIPO, 6K1, VPg, CI, 6K2, NIa (VPg plus Pro), NIb (viral replicase), and CP (capsid protein) (Quenouille et al., 2013; Cui and Wang, 2016). Zhang et al. successfully applied the CRISPR/Cas13a system by programming sgRNA (named pP3, pCI, pNIb, and pCP) specific for the RNA genome of three PVY strains (PVY^O, PVY^N, and $PVY^{N:O}$). When transgenic lines with high transgene expression levels were assessed for PVY resistance with the PVY^o virus, PVY mosaic symptoms

[3]https://cipotato.org/potato/potato-pests-diseases/

were detected in leaves of infected WT plants at 25 days post-inoculation, whereas, no disease symptoms were detected in any of the transgenic plants. ELISA and qRT-PCR results indicated that transgenic plants accumulated much lower levels of the virus in systemic leaves than WT plants after PVY inoculation (Zhan et al., 2019).

One of the genes associated with plant stress is coilin. The mechanisms associated with the protective response to stress are unclear. Coilin is a major structural scaffold protein necessary for Cajal bodies, which are dynamic subnuclear compartments involved in the biogenesis of ribonucleoproteins. Makhotenko et al. (2019) applied the CRISPR/Cas9 system on the coilin gene in the potato cultivar Chicago. The edited lines (named F25 and D15) showed significantly higher resistance than control plants to biotic (ordinary strain PVY-O) and abiotic (salt and osmotic) stresses (Makhotenko et al., 2019).

Development of herbicide-resistant crops has resulted in remarkable changes to sustainable development of agronomy, one of which is the adoption of practical, effortless, and scientific crop-production systems with green technology (Vencill et al., 2012). The recent advantages of CRISPR/Cas9-mediated gene editing allows to target endogenous genes such as acetolactate synthase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), cellulose synthase A catalytic subunit 3 (CESA3), and splicing factor 3B subunit 1 (SF3B1) (Han and Kim, 2019). ALS, also called acetohydroxyacid synthase, is found in plants and microorganisms and catalyzes the biosynthesis of various amino acids, including valine, leucine, and isoleucine (Ay et al., 1998). Resistance to ALS inhibitors is often attributed to an amino acid substitution in ALS (Svitashev et al., 2015). One of the first applications of the CRISPR/Cas system for ALS gene editing in potato was performed by Butler and colleagues (Butler et al., 2015, 2016). The StALS1 gene of the diploid and tetraploid potato was modified using CRISPR/Cas and TALEN with transformation of a binary T-DNA vector [conventional T-DNA and modified geminivirus (GVR) T-DNA]. Transformed events modified with GVRs introduced point mutations that supported a more herbicide-resistant phenotype and inheritable mutations, while events transformed with conventional T-DNAs had no detectable mutations and had same appearance as wild type. The *ALS* gene was also successfully and efficiently targeted in tomato and potato plants by CBE using *Agrobacterium*-mediated transformation. This resulted in chlorsulfuron-resistant mutant plants, as reported transgene-free edited plants were generated in the first offspring (Veillet et al., 2019b).

CRISPR/Cas System for Enhancement of Resistance to Abiotic Stress

The extreme levels of abiotic stresses such as drought, flooding, salinity, mineral deficiency, low or high temperatures, high light, post-harvest problems, and accumulation of reducing sugars during cold storage have negative impact in crop yield (Le Gall et al., 2015). Abiotic stresses usually lead to superoxide overproduction in mitochondria (Dourmap et al., 2019). The mitochondrial respiratory chain in higher plants consists of the ATP-coupling cytochrome pathway (CP) and the cyanide (CN)-resistant respiratory pathway. The relationship of this chain to abiotic stress has been studied (Watanabe et al., 2010; Afzal et al., 2020). Plant mitochondria have an alternative oxidase (AOX), which is encoded by a small family of nuclear genes (Saha et al., 2016). AOX catalyzes the ATP-uncoupling cyanide (CN)-resistant pathway and can command the synthesis of important signaling molecules, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and nitric oxide (NO). AOX is present in plants, fungi, protozoa, and some invertebrates. At the beginning of the 20th century, the AOX was first discovered from thermogenic plants during anthesis (Saha et al., 2016). Later it was found that it exists in the inner mitochondrial membrane of plants, fungi, protozoa, and some invertebrates (Araújo Castro et al., 2017). AOX activity can protect cells from reactive oxygen species (ROS) such as O_2^-, H_2O_2, and OH, particularly during different abiotic stress (Wang and Vanlerberghe, 2013). A CRISPR vector for the functional gene *StAOX* of the potato cyanide-resistant respiratory pathway was designed to examine the role of cyanide-resistant respiration in potato against high-light stress. The results indicated that high-light stress induced expression of AOX and accelerated cyanide-resistant respiration, and that loss of *StAOX* directly led to inhibition of cyanide-resistant respiration (Hua et al., 2020).

CRISPR/Cas9 technology has been widely applied for plant genomics research, with a focus on not only loss-of-function and gain-of-function analysis, but also on plant gene association analysis. In the potato genome, the association of transcription factor *StCDF1* with the antisense transcript *StFLORE* to enhance drought tolerance was detected by mutation of the *StFLORE* promoter with CRISPR/Cas9 system. Although indicated that the overexpression of *StFLORE* or the downregulation of *StCDF1* increased tolerance to drought through the regulation of stomata size and number, and *StFLORE* is regulated by *StCDF1* (Gonzales et al., 2020).

CRISPR/Cas System for Enhancement of Nutrient Content in Potato

Several studies have described improvements in the nutritional value of potato tubers, for example by enhancing essential amino acid compounds of seed protein (lysine, methionine, cysteine, and tyrosine contents), triacylglycerols, and vitamin E by overexpressing *AmA1*, *PrLeg*, *WRINKLED1, DIACYLGLYCEROL ACYLTRANSFERASE* 1, *OLEOSIN*, *At-HPPD* and *At-HPT* genes (Raina and Datta, 1992; Chakraborty et al., 2000, 2010; Crowell et al., 2008; Goo et al., 2013; Xu et al., 2020). Potato is also one of the major crops grown for starch production. Starch granules of potatoes have typical microscopic structures with different shapes and sizes, neutral taste, and good paste clarity (Singh et al., 2016). The starch content in potato, sweet potato, cassava, yam and maize endosperm consists of 65–90% of the absolute dry weight (Tigabu and Desta, 2018). Technology for starch isolation from natural sources is relatively simple and the range of industrial starch-based products is quite extensive and diverse. Starch extracted from potatoes has many utilization in food and technical-industrial applications, as potato starch has a variety of preferable molecular and intermolecular

features (purity, molecular weight, phosphorylation, branching) (Khlestkin and Eltsov, 2021). Starch contains two major different carbohydrate, namely amylose (20–30%) and amylopectin (70–80%). Due to the low solubility of amylose, amylose solutions are generally unstable and easily to retrograde, gel, and turn opaque. In contrast, amylopectin is highly soluble in water, which characteristically makes amylopectin solutions very stable and clear (Visser et al., 1997). Changing the ratio of these two components significantly alters the properties of the starch. Therefore, starch has been the target of numerous genetic modification studies for a variety of purposes. Mutations in granule-bound starch synthase (*GBSS*) and two starch synthase (SS) genes (*SSII* and *SSIII*) resulted in starch with short-chain amylopectin and gelatinization (Khlestkin et al., 2017). In potato, the GBSS enzyme is encoded by a single locus (GBSSI) with four alleles in cultivated potato. Granule-bound starch synthase (GBSS) plays an essential role in amylose synthesis (Miao et al., 2014). In cultivated potatoes, the GBSS enzyme is encoded by a single locus (*GBSSI*) with four alleles. Waxy or amylose-free (amf) starch is a new type of starch made uniquely out of amylopectin molecules. Due to its superior viscosity and elasticity, amf starch has found applications in many areas of the food industry, such as stabilizers and thickeners. Waxy potato has been generated by silencing *GBSSI* gene function using traditional mutational breeding (Visser et al., 1991; Kuipers et al., 1994; Muth et al., 2010), RNAi (Andersson et al., 2003) and modern gene editing technology (Andersson et al., 2018; Veillet et al., 2019b). The *StGBSS* gene in the cultivar Kuras has been targeted for full knockout of GBSS enzyme activity using transient expression of CRISPR/Cas9 components in protoplasts, either as DNA plasmids or as ribonucleoprotein (RNP) complexes. As a result, the starch was confirmed as being of amylopectin quality in the line with all four alleles mutated (Andersson et al., 2018). In the study of Kusano, to the high-level expression of the CAS9 gene, two traditional enhancers were used for the efficient mutation of GBSSI. One of these enhancers was a translational enhancer dMac3 sequence (161 nucleotides), which consisting of a portion of the OsMac3 mRNA 5′-untranslated region (UTR). Another one was 5′-UTR from the alcohol dehydrogenase (ADH) gene. As the result, transformants containing dMac3 showed a higher frequency of mutations compare with transformants containing ADH enhancer and no enhancer sequence (Kusano et al., 2018).

Furthermore, multiple of unknown proteins associated with potato starch granules and multiple protease inhibitors were identified. Among these proteins, a still unknown isoform of starch synthase (*SS6* gene coordinates on chromosome 7) demonstrated a great potential to be a key enzyme of the starch biosynthetic pathway in potato (Helle et al., 2018). Knockout mutagenesis of SS6 resulted in deletions and the function of SS6 was deciphered. The properties of the starch generated by this enzyme has great potential in industrial applications (Sevestre et al., 2020). CRISPR/Cas9 editing efficiency may be improved by using endogenous plant-specific U6 promoters, which contributes to increased sgRNA levels. Replacement of the regularly used *Arabidopsis* AtU6-1 promoter with endogenous potato StU6 promoters resulted in enhanced editing efficiency of the GBSS gene, with editing frequencies of 30–70% in protoplasts and 35% full allelic gene editing (Johansen et al., 2019b).

CRISPR/Cas System for Reduction of Anti-nutrient Content in Potato

Another research priority for scientists and breeders is to improve potato tuber quality by removing anti-nutritional compounds, such as steroidal glycoalkaloids, acrylamide, and food toxins (Hameed et al., 2018). Potato accumulates the poisonous and bitter-tasting solanidane glycoalkaloids α-solanine and α-chaconine. These substances are harmful to many living things (Akiyama et al., 2021). It is known that *St16DOX* is the single gene responsible for steroid 16α hydroxylation in SGA biosynthesis and is an ideal research objective for generation CRISPR/Cas9-mediated mutagenesis to SGA-free potato plants. The knockout of *St16DOX* gene resulted in multiple mutations, including chromosomal fragment deletion at different sites, which resulted in abolition of St16DOX wild-type sequences in tetraploid potato (Nakayasu et al., 2018).

CRISPR/Cas System for the Reduced of Post-harvest Factors Affecting Potato

The post-harvest storage of potato tubers is also one of the key factors that determine the processing quality and final products. This long-term storage always accompanies with various storage diseases, such as soft rot, black dot, and *Fusarium* dry rot, which significantly affect tuber quality and makes it unsuitable for further processing (Barley Disease Control, 2009; Hameed et al., 2018). Potatoes are usually subjected to cold storage (4–8°C) to achieve a more sustainable supply to consumers throughout the year. However, cold temperature increases the reducing sugars accumulation in potato tubers. Upon high-temperature processing, these reducing sugars react with free amino acids to produce a brown and bitter-tasting products, and elevated levels of acrylamide, which is considered a potential carcinogen (Clasen et al., 2016). Enzymatic browning of potatoes is a major problem that arises during harvest and post-harvest procedures such as transport, storage, distribution, and blanching. In enzymatic browning, polyphenol oxidases (PPOs) catalyze the rapid polymerization of o-quinones from natural phenols. As a result, the chemical reaction leads to the formation of dark-colored precipitates in fruits and vegetables with negative effects on color, taste, flavor, and nutritional value (Yoruk and Marshall, 2010).

Four genes are mainly responsible for PPO activity in the potato tuber. The *StPPO2* (PGSC0003DMG400018916) gene is the principal contributor to PPO total protein content (55% of the total enzyme), followed by *StPPO1* (PGSC0003DMG400029575; 25–30%) and *StPPO4* (PGSC0003DMG400018917) and *StPPO3* (PGSC0003DMG400018914) (both < 15%). According to some reports, the down-regulation of multiple *StPPO* genes may harm the other functions of the enzyme in the plant. González et al. (2020) developed a CRISPR/Cas-based genome-editing approach to target *StPPO2*. Mutations induced in the four alleles of *StPPO2* gene showed a significant reduction (up to 69%) in tuber PPO activity and enzymatic browning of (73%) in compared to the

control (González et al., 2020). In this study, we also summarized CRISPR/Cas studies for other tuber crops and discuss the future perspectives (**Table 2**).

SWEET POTATO, YAM, CASSAVA, CARROT, AND APPLICATION OF CRISPR/Cas SYSTEM

Sweet Potato

Sweet potato (*Ipomoea batatas*) is one of the main starch-rich tuber crops and is a source of carbohydrates, β-carotene, vitamin A, C, B, and E complex, calcium, and iron. Cultivated forms of sweet potato have 2n = 6x = 90 chromosomes and are widely grown in 111 countries, particularly Southeast Asia is the major contributor, following by Oceania, and China. Sweet potato originated from either the Central or South American lowlands (Saranraj et al., 2019). Based on nutritional qualities and sensory acceptability, the color of sweet potato flesh varies from white, yellow, purple, and orange (Neela and Fanta, 2019). In contrast to other staple food crops, sweet potato possess special attributes, such as adaptability to wider topography, good productivity in short duration, and balanced nutritional composition (Trancoso-Reyes et al., 2016). To improve starch quality in sweet potato, the genes encoding starch biosynthesis (*IbGBSSI*- and *IbSBEII*) were subjected to CRISPR/Cas9-based mutagenesis by Wang et al. (2019). Most of mutations were nucleotide substitutions that cause amino acid changes and, less frequently, to stop codons. This study demonstrated the efficiency of CRISPR/Cas9 technology for improvement of starch qualities in sweet potato (Wang et al., 2019).

Yam

Yam (*Dioscorea* spp.) is a multi-species tuber crop that supplies food and income to millions of people around the world, particularly in Africa. The "yam belt" in West Africa includes Nigeria, Benin, Togo, Ghana, and Cote d'Ivoire and produces 92% of the 72.6 million tons of worldwide global yam production (NARIT; http://narit.or.th/images/category/dioscorea-spp-yam-4f349d). Yams are consumed raw, as a cooked soup, or used as a powder and flour in food preparations. Yam tubers are not only considered as a fundamental carbohydrate with relatively high protein and ascorbic acid content, but also have many bioactive components, such as mucin, dioscin, dioscorin, allantoin, choline, polyphenols, diosgenin, and vitamins such as carotenoids and tocopherols (Iwu et al., 1990). The phytoene desaturase gene (PDS) is essential for chlorophyll biosynthesis and is involved in converting phytoene into the carotenoid precursors phytofluene and carotene (Mann et al., 1994). PSD knockout mutations have an albino phenotype and thus are usually used as a visual marker to approve genome editing in various plants, including rice (Banakar et al., 2020), *Arabidopsis, M. truncatula*, *N. benthamiana* (Li et al., 2013; Wolabu et al., 2020), and potato (Butler et al., 2020). The CRISPR/Cas9 system was successfully used to target the PDS gene in yam. The efficiency of yam-derived promoters was identified by expression of gRNAs for PDS gene editing. Promoter DaU6.5 performed the best efficiency, while DaU6.2 and DaU6.3 had similar efficiency as revealed by fluorescence scores (Syombua et al., 2021).

Cassava

Cassava (*Manihot esculenta*) is one of the most major staple crops for around 800 million people in tropical and sub-tropical regions of the world (Alicai et al., 2016). Africa presents over 50% of the world cassava production of 233.8 million metric tons. Cassava brown streak disease (CBSD) is the most devastating cassava disease in the Eastern, Central, and Southern Africa. Nowadays, West Africa including Nigeria, the largest cassava producer in the world, is also suffering from this disease (Alicai et al., 2019).

Cassava brown streak disease is caused by two species of positive-sense RNA viruses belonging to the family Potyviridae and genus Ipomovirus, specifically CBSV and Ugandan cassava brown streak virus (UCBSV) (Winter et al., 2010). The CBSV genome contains a polyprotein of 2902 amino acids that is proteolytically cleaved into 10 mature proteins (Mbanzibwa et al., 2009). Diseases caused by the family Potyviridae require the interaction of viral genome-linked protein (VPg) and host eukaryotic translation initiation factor 4E (eIF4E) isoforms. The eIF4E protein family assumes a fundamental par in the initiation of cap-dependent mRNA translation. It is known that cassava encodes the five eIF4E proteins such as eIF4E, eIF(iso)4E-1, eIF(iso)4E-2, novel cap-binding protein-1 (nCBP-1), and nCBP-2. Protein–protein interaction experiments consistently demonstrate that VPg proteins associate with cassava nCBPs. CRISPR/Cas9-mediated genome editing was applied to generate ncbp-1, ncbp-2, and ncbp-1/ncbp-2 mutants in cassava cultivar 60444. Compared to wild-type, the ncbp-1/ncbp-2 double mutants showed more resistance to CBSD severity in root and stem (Gomez et al., 2018). Among the viruses that infect cassava, cassava mosaic disease (CMD) is the most dangerous and widespread in the cassava-growing field. At least seven cassava mosaic geminivirus (CMG) species, including South African cassava mosaic virus (SACMV) limit crop production in Africa (Patil and Fauquet, 2010). The complete nucleotide sequences of infectious clones of SACMV were determined and this enabled the selection of putative host interacting genes for different investigations (Berrie et al., 2001). CRISPR/Cas9 technology was used in cassava with the aim of engineering resistance to SACMV. The sgRNAs of AC2 gene that is coding for the multifunctional TrAP protein and AC3 gene that is coding for the REn protein were targeted, but a clear disease-resistance phenotype was not observed in mutant lines (Mehta et al., 2019).

Plant gene association analysis as described above in potato plants has also been applied in cassava. For example, the association of SACMV with the ubiquitin E3 ligase gene (*MeE3L*) was studied by CRISPR/Cas9-mediated system of cassava gene *MeE3L* in SACMV-infected cassava protoplasts (Chatukuta and Rey, 2020).

TABLE 2 | Application of CRISPR/Cas system in sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta Crantz*), yam (*Dioscorea* spp.), and carrot (*Daucus carota subsp. sativus*).

Plant	Method of delivery	Technology	Target gene (s)	Trait associated with the genes	Type of mutation	Results	References
Sweet potato (*Ipomoea batatas*)							
Cvs. Xushu22 and Taizhong6, Sweet potato (*Ipomoea batatas*)	*Agrobacterium*-mediated transformation; *IbSBEII-sgRNA2* and *IbSBEII-sgRNA12* vectors	CRISPR/Cas9 system	Granule-bound starch synthase and starch branching enzyme (*IbGBSSI* and *IbSBEII*)	Tuber starch quality	Deletions, substitutions, and insertions	Knockout of *IbGBSSI* reduced amylopectin, while the knockout of *IbSBEII* increased the amylose percentage	Wang et al., 2019
Yam (*Dioscorea* spp.)							
Cv. *D. alata*, Yam (*Dioscorea* spp.)	*Agrobacterium*-mediated transformation	CRISPR/Cas9 system	Phytoene desaturase gene (*DrPDS*)	Albino	Deletion and insertion	Promoter DaU6.5 performed best, while DaU6.2 and DaU6.3 yielded similar efficiency for expressing gRNAs	Syombua et al., 2021
Cassava (*Manihot esculenta*)							
Model cv.60444, Cassava (*Manihot esculenta*)	*Agrobacterium*-mediated transformation; pCAMBIA2300 vector	CRISPR/Cas9 system	Novel cap-binding protein (*nCBP*-1 and *nCBP*-2) genes	Cassava brown streak disease (CBSD)	Deletions and insertions	Reduced CBSD severity in root and stem	Gomez et al., 2018
Cassava (*Manihot esculenta*)	*Agrobacterium*-mediated transformation; pJET1.2 vector	CRISPR/Cas9 system	African cassava mosaic virus genes (*AC2* and *AC3*)	African cassava mosaic virus	Single-nucleotide insertion, and substitution	Resulted in the lack of resistance	Mehta et al., 2019
Cvs. model cv.60444, *M. esculenta* T200 and *M. esculenta* TME3, Cassava (*Manihot esculenta*)	PEG-mediated transfection method of protoplasts	CRISPR/Cas9 system	Ubiquitin E3 ligase gene (*MeE3L*)	South African cassava mosaic virus (SACMV)	Single-nucleotide insertion	Accumulation of SACMV DNA was increased in the mutant lines	Chatukuta and Rey, 2020
Carrot (*Daucus carota* subsp. *Carota)*							
Carrot (*Daucus carota* subsp. *Carota*)	*Agrobacterium*-mediated transformation; pYPQ154, pYPQ166, and pYPQ167	CRISPR/Cas9 system	*F3H*	Anthocyanin compound	Small Indels, and long chromosome fragment deletions	Individual transgenic calli, which differing in degree of discoloration from white, mosaic to purple. No transgenic plant was regenerated	Klimek-Chodacka et al., 2018
Cvs. Kurodagosun and Deep purple, Carrot (*Daucus carota* subsp. *Carota)*	*Agrobacterium*-mediated transformation; *VK005* vector	CRISPR/Cas9 system	*DcPDS* and *DcMYB11*3-like genes	Albino and purple depigmentation	Insertion, deletion, and substitution	Generated Albino-type and purple depigmented plants	Xu et al., 2019

Carrot

Carrot (*Daucus carota* subsp. *carota* L.) is an economically important toot vegetable crop. Carrots are grouped into the carotene or western (*D. carota* ssp. *sativus* var. sativus) and the anthocyanin or eastern groups (*D. carota* ssp. *sativus* var. atrorubens Alef.). Purple carrot cultivars accumulate rich cyanidin-based anthocyanins, while orange, yellow, and red carrot cultivars accumulate rich carotenoids in the taproots (Xu et al., 2017). Carotenoids and anthocyanins both provide various health benefits to humans. Anthocyanins are an enormous group of more than 500 pigments abundant in the plant kingdom that accumulate in the cell vacuole of certain some organs. Many of the genes that encode enzymes involved in anthocyanin biosynthesis have been well characterized. For the first time, CRISPR/Cas9-mediated loss of function of the gene F3H, which is critical for anthocyanin biosynthesis, was reported by Klimek-Chodacka et al. (2018). They studied three codon-optimization variants of SpCas9 genes and demonstrated that AteCas9 has high efficiency in carrot cells for producing mutations, followed by zCas9, and Cas9p. The knockout of F3H gene affected the discoloration of calli, however, no transgenic plant was regenerated.

Albino and purple depigmentation mutants were regenerated when DcPDS and DcMYB113-like genes in orange and purple carrot were targeted by CRISPR/Cas9, respectively (Xu et al., 2019). The function of PDS genes is described above, while the DcMYB113-like changes critical functions in anthocyanin biosynthesis, and knockout mutations were expected to cause depigmentation phenotype. In addition, four different promoters (AtU3b, AtU3d, AtU6-1, and AtU6-29), which individually drive four sgRNA expression cassettes, were tested for mutation efficiency rate. The highest efficiency of mutagenesis was noticed in the loci targeted by AtU6-29-driven sgRNAs in both *DcPDS* and *DcMYB113*-like knockout T0 plants (Xu et al., 2019).

CONCLUSION

CRISPR/Cas-mediated genome editing technology can selectively modify any part of a genome to target genes controlling stress tolerance and nutritional quality of tuber crops. The simplicity and versatility of this technology make it a powerful tool for precise crop improvement via generation of knockout mutations in the form of insertions, deletions, and substitutions. The development of base editors, which generate base substitutions without requiring donor DNA or DSB induction, has expanded the possibilities of genome editing. The cultivated potato and most tuber crops have a complex genetic structure due to autotetraploidy and heterozygosity. Genetic improvement of these crops presents numerous challenges using traditional breeding techniques. To this end, the genome sequences of potato and other tuber crops provides a platform for genetic improvement via gene-editing technology. As mentioned above the CRISPR/Cas9 system with increased mutagenesis frequency has been established by using the improved CRISPR/Cas9 vector containing a translational enhancer sequence dMac3 in potato (Kusano et al., 2018) and through the use of codon optimized U6 promoter according species (Johansen et al., 2019a; Syombua et al., 2021).

Although significant progress in the last 5 years has made it possible to increase the efficiency and target specificity of CRISPR technology by using marker gene such as PDS, more work remains to be done to improve this technology for agriculturally important traits of commercial cultivars. In potato and other tuber crops, it is possible use the fluorescence marker-assisted selection for transgene-free genome-edited plants by the co-expression of fluorescence marker genes, as has been done for rice, tomato, and Arabidopsis (Aliaga-Franco et al., 2019). All the more already investigated the potentials of green fluorescent protein as a marker to select transgenic hybrid potato tubers (Palumbo and Veilleux, 2007) and red fluorescent protein to identify the localization of key enzymes of carotenoid biosynthesis after stable transformation in potato (tubers) (Nick, 2013). However, the fluorescence marker facilitates them of the transgene-free plants, but it does not improve the ratio of transgene-free plants. The self-eliminating and Transgene Killer CRISPR system proposed by He et al. (2018) and He and Zhao (2020) may accelerate identification of transgene-free and CRISPR-edited plants. For that they have modified the CRISPR/Cas9 construct with the bacterial BARNASE gene under the control of the rice REG2 promoter to express the suicide transgene during early embryo development to kill all of the CRISPR/Cas9-containing pollen and embryos produced by T0 rice plants that can be used for tuber crops in perspective.

One of the main limiting factors is the low embryogenic competency of local crops to tissue culture. It would be relevant to use the CRISPR/Cas technology to control endogenous plant hormones controlling and triggering plant morphogenesis, including somatic embryogenesis and organogenesis. Another major limiting factor is the size of Cas9, which prevents the use of plant viral vectors to deliver CRISPR/Cas components into a plant genome. Further research in this direction would widespread use the CRISPR/Cas technology to improve agro-important traits of local crop cultivars.

The studies reported in this review indicate that genome improvement in existing potato and other tuber crops is possible with this technology. The use of CRISPR/Cas for gene knockouts and base-editing systems for expression regulation of any gene of interest will facilitate development of non-transgenic crops. Recent developments in CRISPR-based genome editing technology for analyzed crops allow not only targeting of single genes in the genome, but also modification of multiple genes at once. One of the problems was observed that is utilization of genome-editing technology for model cultivars, due to the low regeneration efficiency of local cultivars in tissue culture. By considering the use of CRISPR/Cas technology as reported in this review, scientists may be able to design efficient vectors and transformation systems for employing CRISPR/Cas technology in tuber crops.

AUTHOR CONTRIBUTIONS

TD and SM wrote the manuscript together. Both authors contributed to the article and approved the submitted version.

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C-to-G Base Editing Enhances Oleic Acid Production by Generating Novel Alleles of *FATTY ACID DESATURASE 2* in Plants

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The demand for vegetable oil, which is mainly used for dietary purposes and cooking, is steadily increasing worldwide. It is often desirable to reduce unsaturation levels of fatty acids in order to increase storage stability and reduce *trans*-fat generation during cooking. Functional disruption of FATTY ACID DESATURASE 2 (FAD2) prevents the conversion of monounsaturated oleic acid to polyunsaturated linoleic acid, thereby enhancing the production of the desirable oleic acid. However, *FAD2* null alleles, due to growth defects under stress conditions, are impractical for agronomical purposes. Here, we aimed to attenuate FAD2 activity *in planta* while avoiding adverse growth effects by introducing amino-acid substitutions using CRISPR base editors. In *Arabidopsis*, we applied the adenine base editor (ABE) and cytosine base editor (CBE) to induce semi-random base substitutions within several selected *FAD2* coding regions. Isolation of base-edited *fad2* alleles with higher oleic acid revealed that the CBE application induced C-to-T and/or C-to-G base substitutions within the targeted sequences, resulting in an alteration of the FAD2 enzyme activities; for example, *fad2-144* with multiple C-to-G base substitutions showed less growth defects but with a significant increase in oleic acids by 3-fold higher than wild type. Our "proof-of-concept" approach suggests that equivalent alleles may be generated in vegetable oil crops *via* precision genome editing for practical cultivation. Our targeted semi-random strategy may serve as a new complementary platform for planta engineering of useful agronomic traits.

Keywords: base editing, CRISPR, cytosine base editor, FAD2, oleic acid, vegetable oil

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INTRODUCTION

Vegetable oils contain three types of saturated fatty acids, palmitic acid (16:0), stearic acid (18:0), and arachidic acid (20:0) and five types of unsaturated fatty acids, oleic acid ($18:1^{\Delta 9}$), linoleic acid ($18:2^{\Delta 9,12}$), linolenic acid ($18:3^{\Delta 9,12,15}$), 11-eicosenoic acid ($20:1^{\Delta 11}$), and erucic acid ($22:1^{\Delta 13}$). They can be used in industrial applications or as edible oils depending on their fatty acid composition. It is highly desirable to reduce the polyunsaturated fatty acid content and to increase the oleic acid ($18:1^{\Delta 9}$), which is less vulnerable to oxidation (Holman and Elmer, 1947; Liu and White, 1992). FATTY ACID DESATURASE 2 (FAD2), which synthesizes $18:2^{\Delta 9,12}$ fatty acid from

$18{:}1^{\Delta 9}$ fatty acid, was first identified as an endoplasmic reticulum (ER) membrane-bound protein (Okuley et al., 1994). The activity of FAD2 in plants is related to an increase in the content of dienoic fatty acids, hence increasing the resistance toward cold and salt stress, and is also known to affect development through salicylic acid (SA), abscisic acid, and jasmonic acid (JA) pathways (Martinez-Rivas et al., 2000; Kachroo et al., 2003; Regente et al., 2008).

Several attempts have been made to eliminate FAD2 function in plants in order to enhance desirable oleic acid production. In peanut and soybean, the *FAD2* was knocked out using transcription activator-like effector nucleases (TALENs; Haun et al., 2014; Wen et al., 2018). Likewise, disruption of the FAD2 function in peanut, soybean, and camelina has been demonstrated *via* clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9; Jiang et al., 2017; Do et al., 2019; Yuan et al., 2019). However, *FAD2* null alleles are agronomically impractical because the loss of FAD2 function has adverse growth effects, resulting in a trade-off between reduction in yields and/or susceptibility to pathogens and increased oleic acid contents (Taylor et al., 2002; Anai et al., 2008; Pham et al., 2010). Of note, *fad2* null mutations in *Arabidopsis* have been reported to cause dwarf phenotypes at a lower ambient temperature (Miquel et al., 1993) whereas an EMS allele *fad2-1* appears to be more practical in terms of its growth phenotype (Lemieux et al., 1990). Targeting Induced Local Lesions in Genomes (TILLING) has been proposed as a useful approach for isolating point-mutant alleles for those genes, including *FAD2*, that lead to desirable agronomic traits and development as well (Lakhssassi et al., 2017).

Recently, derivatives of CRISPR/Cas9 systems have been developed to broaden the spectrum of CRISPR applications. One class of such derivatives is base editors (BEs). Nickase Cas9 (nCas9) fused to cytosine deaminases and to a laboratory-evolved adenine deaminase, referred to as a cytosine base editor (CBE; conversion of C to T) and an adenine base editor (ABE; conversion of A to G). These have been used to target deaminase domains to edit specific loci, without the need to generate double-strand breaks (DSBs), converting their respective nucleotides into other DNA bases (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017). BEs can induce base substitutions leading to amino-acid change or splicing modifications. Two CBE systems, base editor 3 (BE3), and activation-induced cytidine deaminase (AID) adapt rAPOBEC1 from rat and PmCDA1 from sea lamprey as a cytidine deaminase domain, respectively (Komor et al., 2016; Nishida et al., 2016). CBEs often generate C-to-G substitutions as well *via* the BER (base excision repair) pathway, which served as a basis for the recent development of C-to-G base editors (Molla et al., 2020; Kurt et al., 2021; Zhao et al., 2021). Base editing has been applied to plants: The *acetyl CoA carboxylase* (*ACCase*) gene was modified in *A. thaliana* and *Oryza sativa* using enhanced ABE (Liu et al., 2020); Soybean flowering has been manipulated *via* amino-acid change within FLOWERING LOCUS T (FT), resulting from cytosine base editing (Cai et al., 2020); Watermelon and *Brassica napus* have been engineered to harbor herbicide resistance from the cytosine base editing in the *ACETOLACTATE SYNTHASE* (*ALS*; Tian et al., 2018; Wu et al., 2020).

In this study, we sought to generate *FAD2* missense alleles by implementing ABE or CBE within several target regions of the *FAD2* coding sequences in *Arabidopsis*. We selected those BE target sites for guide RNA (gRNA) design, based on the prediction of FAD2 protein structure, in order to generate genomic variations with base substitutions that are predicted to confer structural instability (Chavez Zobel et al., 2005; Li et al., 2020). For *Agrobacterium*-mediated plant transformation, we adapted the ABE binary vector, which has been reported in a previous study (Kang et al., 2018), and, for a CBE binary vector, we constructed a new pJY-RpAID vector based on plant-codon-optimized PmCDA fused to nCas9 under the control of *RPS5A* promoter. To isolate desirable *FAD2* alleles with increased oleic acid, we carefully investigated 26 T_1 transformants by measuring the oleic acid contents through massive gas chromatography (GC) analyses on their corresponding T_2 seeds. As a result, we obtained novel *FAD2* alleles showing significantly reduced desaturase activity, which led to enhanced oleic acid content. Genomic analyses confirmed that CBE implementation generated the desired novel missense alleles with multiple amino acid changes often resulting from the C-to-G rather than C-to-T substitutions. Our results also imply that our semi-random BE application to modify genes controlling important agronomic traits is a viable approach to produce novel designer alleles with new SNPs.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All experiments were conducted using *Arabidopsis thaliana* Columbia-0 (Col-0) and *fad2-1* (Miquel and Browse, 1992) as an experimental control. *Arabidopsis* seeds were sterilized with 70% EtOH and 0.5% NaOCl and then were washed 7∼8 times with distilled water before sowing. The seeds were subject to stratification at 4°C for 3 days. They were then grown in 1/2 MS media containing 1% sucrose in a culture chamber at 23°C with a 16-h-light/8-h-dark cycle. About 14-day-grown seedlings in MS media were transferred to soil for further growth.

Plasmid Construction

For basal CBE binary vector pJY-RpAID, plant (*A. thaliana*) codon-optimized coding sequences of PmCDA1 were synthesized by Integrated DNA Technology (Iowa, United States). First, pKI1.1R (Addgene #85808) (Shimada et al., 2010) was used to generate a pJY-RpEmpty vector, in which the original Cas9 sequences were removed, and two enzyme sites *Xma*JI and *Xho*I were inserted instead. Nickase Cas9 (D10A) and the codon-optimized AID were PCR amplified to generate pJY-RpAID binary vectors by the infusion cloning method (Takara, Japan) onto an *Xma*JI/*Xho*I-treated pJY-RpEmpty vector. The pJY-RpAID vector contains the following elements instead of the original Cas9 expression cassette from pKI1.1R: RPS5A promoter-SV40 NLS-nCas9 (D10A)-SV40 NLS-67aa linker-3X FLAG-PmCDA-SV40 NLS-UGI-heat shock protein

18.2 terminator (**Figure 1B** and **Supplementary Figure 1**). *Aar*I-mediated gRNA cloning was performed for plant transformation vectors as previously described (Kim et al., 2016). For base editing the *Arabidopsis FAD2* gene, six different 20-mer guide sequences (**Figure 1E**) were designed using the CRISPR RGEN Tool[1] and cloned into *Aar*I-treated pJY-RpAID or a pJY-RpABE vector.

Arabidopsis Transformation and Transgenic Plant Selection

Agrobacterium strain GV3101 was used to transform *A. thaliana via* the floral dipping method (Clough and Bent, 1998). The resulting T_1 seeds were germinated and grown in 1/2 MS media containing 1% sucrose and 50 ng·μl^{-1} hygromycin. Only surviving plants in the media were selected and transferred to soil for further analyses. Leaf genomic DNA was extracted (Edwards et al., 1991) for Sanger sequencing and was analyzed whether base editing had occurred within the *FAD2* gene.

Sanger Sequencing

The PCR was performed on extracted genomic DNA as a template using Ex Taq (Takara) to generate *FAD2* amplicons for subsequent sequencing analyses. A forward primer 5′-GCATTGTTTCAAACGCTCAA and a reverse primer 5′-TCATAACTTATTGTTGTACCAGTAC were used for 30-cycle PCR as following conditions: pre-denaturation (95°C for 5 min); denaturation (95°C for 30 s); annealing (52°C for 30 s); extension (72°C for 1 min); and post-extension (72°C for 10 min). A purification kit (Cosmo Genetech) was used to purify the PCR products. The quality and the quantity of the products had been determined using Nanodrop (Denovix) before the samples were deposited to a sequencing company (Bioneer).

Fatty Acid Analysis

The 100 seeds were used for the gas chromatography (GC) analysis. About 500 μl of 5% sulfuric acid solution was dissolved in methanol, and toluene was added to each sample. To quantify the fatty acid contents, 15:0 fatty acid was dissolved in sulfuric acid. Each sample immersed with the solution was subject to the reaction in a water bath at 85°C for 2 h. Each sample was then added by 1 ml of 0.9% NaCl. After that, 1 ml of hexane was added to the solution three times and then was centrifuged at 330 × *g* for 2 min. The supernatant was aspirated, transferred to a 6-ml tube, purified fatty acid methyl ester (FAME) using a nitrogen concentrator (Eyela). The extracted FAME was dissolved in 200-μl hexane and then inserted into a GC vial. A DB-23 column (30 m × 0.25 mm, 0.25-μm film, Agilent) was used, and the extracted FAME was analyzed by GC-2030 (Shimadzu). The GC oven temperature was raised from 190 to 230°C at 3°C min^{-1}.

Germination Rate

The germination tests were conducted on the seeds that had been stratified for 3 days at 4°C. The germination events were scored when the roots started to appear in 1/2 MS media, 1/2 MS with 150-mM NaCl, and 1/2 MS with 300-mM Mannitol. All 1/2 MS used in the experiment contained 1% sucrose. The germination tests were carried out every 12 h for 4 days and replicated three times with 40 seeds per each line.

Measurement of Root Growth

Col-0 and *fad2* alleles including *fad2-1* were grown under normal or stress conditions to monitor root development. For stress conditions, 4 DAG (days after germination) seedlings were transferred to 1/2 MS media, containing 75-mM NaCl or 200-mM Mannitol. The measurement of the root length was performed four times each for five individuals. The root length was measured on the 5th day of growing the seedling plant perpendicular to the light. The length measurement was carried out using the Image J program. Relative root length (%) was calculated as the average root length in stress MS media/average root length in MS media × 100.

Statistical Analysis of the Data

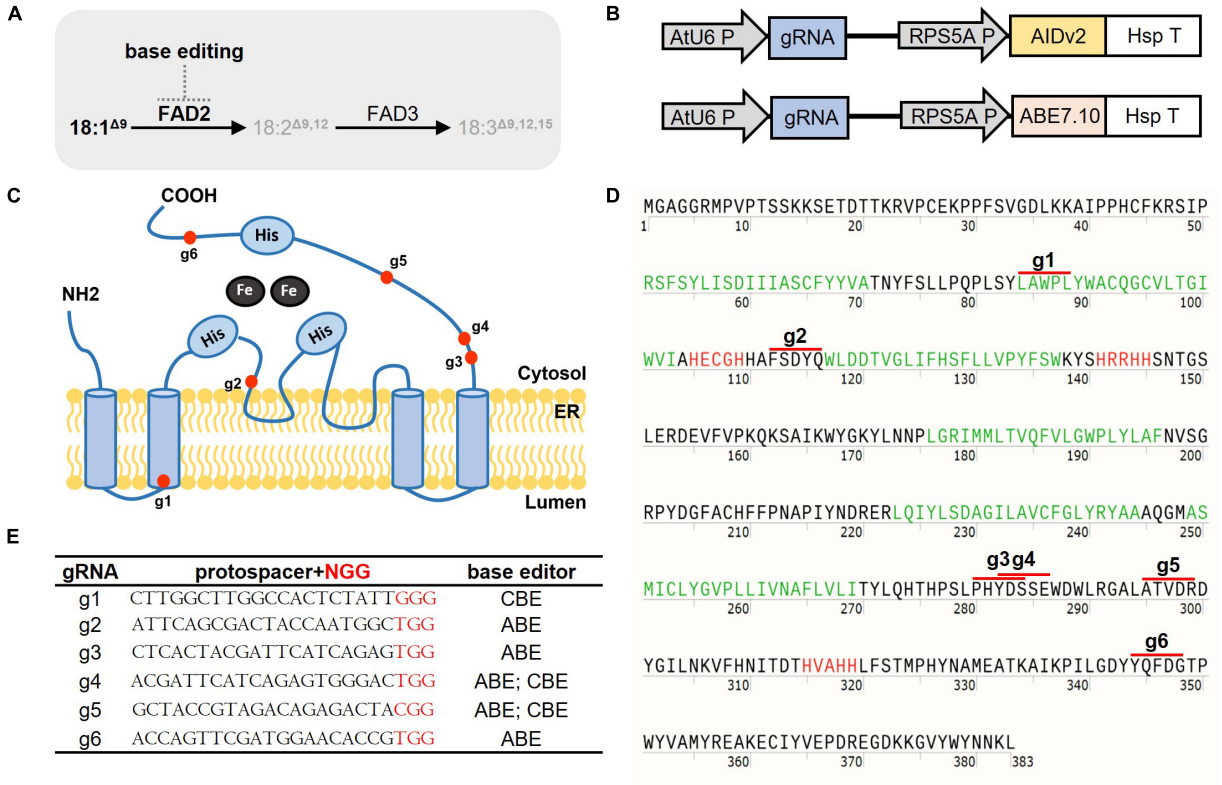
The differences between the wild-type (Col-0) plants, *fad2-1* and the CBE lines, were identified by performing a one-way ANOVA using GraphPad Prism. Asterisks indicate significant differences compared to the wild-type plants ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

RESULTS

Application of the Base Editors *in planta* to Generate "Attenuated" *fad2* Alleles

We aimed to generate potential "attenuated" *fad2* alleles with increased oleic acid at the cost of linoleic acid and linolenic acid, while minimizing adverse "trade-off" effects. We reasoned that certain C-to-T and/or A-to-G base substitutions, achieved by *in planta* CBE or ABE, within the coding regions of *FAD2* might attenuate, rather than abolish, their function (**Figure 1A**). To test this, we introduced random C-to-T and/or A-to-G base substitutions within several coding regions of *FAD2* locus *via in planta* CBE or ABE implementation. We first constructed a basal CBE binary vector pJY-RpAID in which AIDv2 is under the control of the RPS5A promoter, and the gRNA cloning cassette is under the control of the *Arabidopsis* U6 promoter (see section "Materials and Methods" for details; **Figure 1B** and **Supplementary Figure 1**). For the ABE binary vector, we adapted our pJY-RpABE vector reported previously (**Figure 1B**; Kang et al., 2018). Six distinct coding regions within the *FAD2* locus were selected as ABE and/or CBE targets based on the structural properties of the ER-membrane-bound FAD2 protein, which consists of six transmembrane domains and three histidine box motifs (Okuley et al., 1994; Shanklin et al., 1994; **Figures 1C,D**). We designed six independent gRNAs denoted as "g1–6" (**Figure 1E**) for BEs to target the selected coding regions, which correspond to the N-terminal transmembrane domain, tandem His-motif, a membrane-proximal cytosolic domain, cytosolic stretches, and C-terminal domains of the FAD2 protein (**Figures 1C,D**). Five ABE and three CBE constructs (**Figure 1E**) were created and transformed into *Arabidopsis* Col-0. Upon

[1]http://www.rgenome.net



E

gRNA	protospacer+NGG	base editor
g1	CTTGGCTTGGCCACTCTATTGGG	CBE
g2	ATTCAGCGACTACCAATGGCTGG	ABE
g3	CTCACTACGATTCATCAGAGTGG	ABE
g4	ACGATTCATCAGAGTGGGACTGG	ABE; CBE
g5	GCTACCGTAGACAGAGACTACGG	ABE; CBE
g6	ACCAGTTCGATGGAACACCGTGG	ABE

FIGURE 1 | "Semi-random" base editing strategy for selected *FAD2* coding regions. **(A)** The function of FAD2 in desaturation of fatty acids and potential BE action in *Arabidopsis* seeds. FAD2 desaturates 18:1$^{\Delta 9}$ to 18:2$^{\Delta 9,12}$. FAD2 is subjected to base editing to alter its function. **(B)** Core structures of the CRISPR part in T-DNA from binary vectors harboring CBE and ABE in this study. gRNA expression is controlled by the U6 promoter, and AIDv2-dependent CBE and ABE7.10 are under the control of RPS5A promoters. **(C)** A schematic diagram of the FAD2 protein structure adapted from Zhang et al. (2012) ER-located FAD2 has six transmembrane domains and three histidine box motifs (His). Red dots denote BE target regions. **(D)** The FAD2 amino-acid sequence. *Arabidopsis* FAD2 is composed of 383 amino acids. Green-colored sequences indicate transmembrane domains, red-colored sequences indicate His motifs, and red lines indicate the potential amino acids that might be affected by BE targeting *via* corresponding gRNAs. **(E)** A list of selected gRNAs used for CBE and/or ABE with a protospacer sequence for *FAD2* editing in this study.

culture on selective media, 26 T_1 plants were collected for analysis of BE transgenes and transferred to soil for later GC analyses of T_2 seeds.

Individual pools of T_2 seeds harvested from each T_1 plant were subjected to parallel GC analyses to quantify their lipid compositions. Four independent T_1 plants (named as g5CBE1-4) from the CBE constructs with g5CBE guide RNA showed increased oleic acid content in their T_2 seeds (**Supplementary Table 1**). T_2 seeds from g5CBE1, g5CBE2, g5CBE3, and g5CBE4 exhibited increased oleic acid contents up to 36.5, 36.2, 48.9, and 21.6%, respectively, while wild-type Col-0 and other transgenic lines (g1, g4CBEs and g2, g3, g4, g5, g6ABEs) oleic acid contents were no more than 20% (**Supplementary Table 1**; values with asterisks). Furthermore, g5CBE plants displayed decreased polyunsaturated fatty acid levels and increased eicosenoic acid (20:1$^{\Delta 11}$) (**Supplementary Table 1**), implying that the desaturase activities of FAD2 protein had been compromised in those transgenic plants. We progressed to the next segregating T_2 generation with g5CBE1 and g5CBE3, which had the highest amounts of oleic acid accumulation at their T_2 seeds, in order to test whether *bona fide* cytosine base editing, which might be responsible for those increases, had occurred within the targeted *FAD2* region in those transgenic plants. Notably, other gRNAs transformants did not show any significant changes in fatty acids in the T1 generation, which may be due to low levels of either base editing or oleic acid changes. We decided to focus on g5CBE lines for the following experiments.

Analyses of the Base-Editing Patterns and Lipid Contents From the *fad2* Lineages

Seven (g5CBE11-17) and five T_2 progenies (g5CBE31-35) from the g5CBE1 and the g5CBE3 T_1 lineages, respectively, were analyzed for *FAD2* genotypes (**Figure 2A** and **Supplementary Figure 2**). Genomic DNA extracted from leaves of the T_2 plants was subjected to Sanger sequencing covering the CBE target region of the *FAD2* gene (**Figure 2A** and **Supplementary Figure 2**). Interestingly, segregating C-to-G substitutions were observed at positions C2 and C12 in g5CBE11-16. Given that the protospacer adjacent motif (PAM) NGG sequences are regarded as the positions 21–23, these C-to-G substitutions are

expected to result in the amino acid changes A295G and D298E, respectively (**Figure 2A** and **Supplementary Figure 2**). Among T_2 plants, g5CBE12 and g5CBE14 appeared to have discrete C-to-G substitution chromatogram signals at the corresponding positions, indicating exclusive (A295G; D298E)-type amino acid changes had occurred (**Figure 2A** and **Supplementary Figure 2**). g5CBE11, g5CBE13, g5CBE15, and g5CBE16 showed mixed signals of C-to-G and C-to-T substitutions at the C2 position, and mixed signals of C and C-to-G at the C12 position (**Figure 2A** and **Supplementary Figure 2**). These results indicate that the edited bi-alleles at the C2 position and heterozygous C-to-G alleles at the C12 position had been transmitted to T_2 plants. Of note, g5CBE17 showed all wild-type signals without any evidence of base editing events (**Figure 2A** and **Supplementary Figure 2**). Among g5CBE3 lineage, g5CBE31 plants also showed the A295G pattern from C-to-G conversion at the C2 position, accompanying with C-to-T conversion at C6 that would, anyway, result in synonymous mutation (**Figure 2A** and **Supplementary Figure 2**). In the g5CBE32, exclusive C-to-T substitutions occurred at the C2 position, resulting in A295V, and C-to-T and C-to-G at the C5 and C6 positions, respectively, resulting in T296M (**Figure 2A** and **Supplementary Figure 2**). In the g5CBE33 plant, heterozygous or mosaic C-to-T substitution at C2 was observed (**Figure 2A** and **Supplementary Figure 2**). The sequencing results for the g5CBE34 and g5CBE35 plants showed a mixed pool of sequencing traces, suggesting that larger genomic changes, such as indels, rather than point mutations might have occurred (**Figure 2A** and **Supplementary Figure 2**).

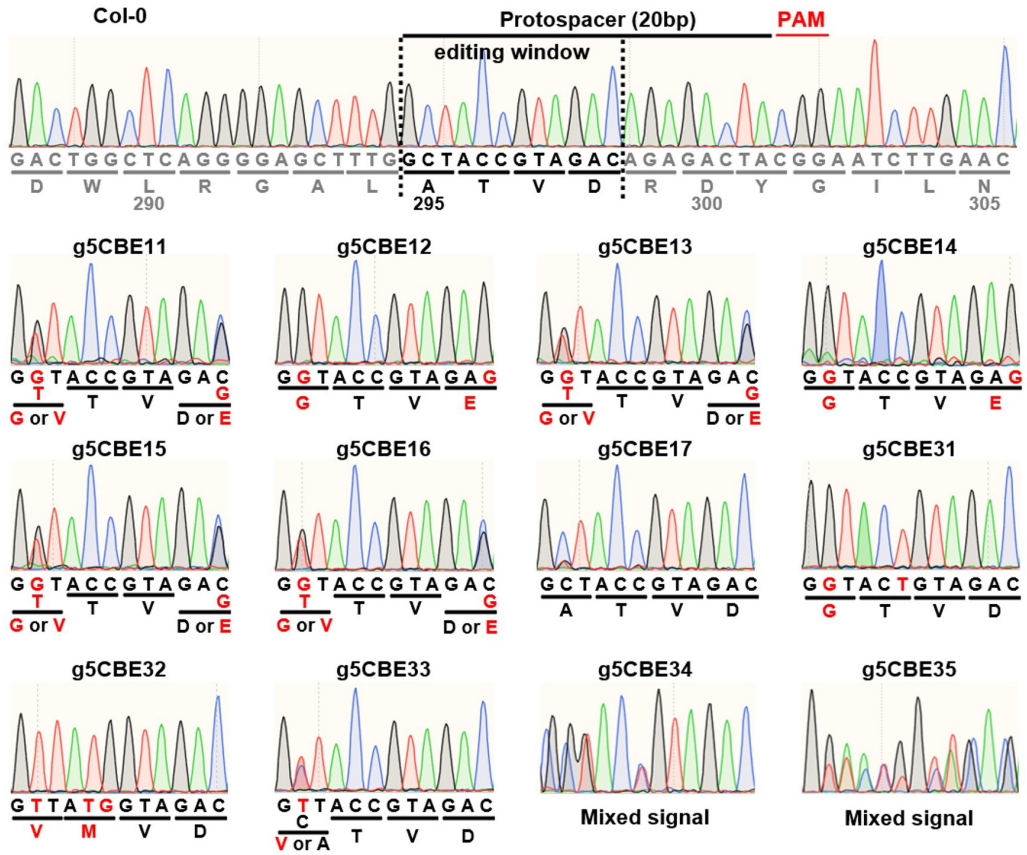
Gas chromatography analyses were then carried out on the corresponding T_3 seeds from each T_2 plant to investigate how the lipid composition had changed according to allelic differences with amino-acid changes. The oleic acid contents increased the most to 64.9, 63.9, and 69.2% in T_3 seeds from g5CBE12, g5CBE14, and g5CBE32 plants, respectively, at the expense of the polyunsaturated fatty acids (**Figure 2B**; red-colored values). These lines are characterized by two concurrent amino acid changes at different residues resulting from two independent cytosine base-edited alleles (**Figure 2A**). The oleic acid content in T_3 seeds from g5CBE11, g5CBE13, g5CBE15, and g5CBE16 showed moderate increases of oleic acid ranging from 42.6 to 45.8% (**Figure 2A**). Segregating C-to-T alleles at the C2 position and/or WT alleles at the C12 position might be responsible for those moderate increases in T_3 seed oleic acid contents as compared to the maximum level increases from g5CBE12 and g5CBE14 (**Figure 2B**). g5CBE31 also showed an intermediate level of increase in oleic acid, indicating that A295G results in the partial suppression of FAD2 activity. g5CBE33, which contains heterozygous A295V, showed only slight increases in the oleic acid content to 25.7%, suggesting that A295V might affect FAD2 activity in a dosage-dependent manner (**Figure 2B**). g5CBE34 and g5CBE35 showed modest increases in oleic acid content up to 54% and 49.5%, respectively, but not to the maximum levels observed in g5CBE12 and g5CBE14 (**Figures 2A,B**).

Isolation of the Base-Edited *fad2* Alleles

We proceeded with g5CBE11, g5CBE14, g5CBE31, g5CBE32, g5CBE33, and g5CBE34 to the next T_3 generation in order to isolate homozygous alleles and to verify whether the changes in fatty acid composition depend on possible T_3 segregations (**Figures 2A,B**; the line numbers with asterisks). g5CBE11 was selected for a representative among the T_2 group containing biallelic A295G/A295V along with heterozygous D298E mutations. g5CBE14 and g5CBE32 were for A295G; D298E and A295V; and T296M allelic groups, respectively. g5CBE31 had the A295G mutation and a synonymous C-to-T mutation. g5CBE33 was chosen for the heterozygous A295V allele. g5CBE34 was also included in this progression group in anticipation of the isolation of an indel allele in their T3 generation. Sanger sequencing analyses were performed in the T_3 progenies of each allelic group. As a result, we successfully isolated all possible base-edited T_3 homozygous alleles for each genotype represented by g5CBE114 (A295G; D298E), g5CBE144 (A295G; D298E), g5CBE316 (A295G), g5CBE321 (A295V; T296M), and g5CBE337 (A295V), hereafter referred to as *fad2-114*, *fad2-144*, *fad2-316*, *fad2-321*, and *fad2-337*, respectively (**Figures 3A,B** and **Supplementary Figure 3**). For the T_3 progenies of g5CBE34, we isolated a 4-bp insertional mutation allele, named *fad2-349* (frameshift mutation), presumably resulting in FAD2 knockout *via* a premature stop codon (**Figures 3A,B** and **Supplementary Figure 3**). Interestingly, segregating T_3 progenies of g5CBE34 also present base-edited alleles (A295G), indicating that our CBE application had generated both base substitution and insertion mutations in a T_2 g5CBE34 plant (**Figure 3A** and **Supplementary Figure 3**). The presence of a transgene was analyzed by PCR amplification of the nCas9 part for the T2 and T3 generations of g5CBE1 and g5CBE3 lines. The results indicate that the transgene had been segregated out from T3 in the g5CBE11 line and T2 in the g5CBE3 line (**Supplementary Figure 4**). Therefore, *de novo* additional base editing in the T3 generation was unlikely to have occurred.

Gas chromatography analyses carried out on all T_3 genotypes revealed that all defined homozygous base-edited alleles showed significant increases in their oleic acid contents at their T_4 seeds compared to wild-type Col-0 (**Figures 3A,C** and **Supplementary Table 2**). *fad2-321* (A295V; T296M) showed the highest increase, in oleic acid content, up to 64.7 ± 5.7%, among the base-edited *fad2* alleles (**Figures 3A,C** and **Supplementary Table 2**), which is consistent with observations in its parental T_2 generation (**Figure 2B**). This increase is even higher than that of the previously reported *fad2-1* (A104T) (Lemieux et al., 1990) (55.9 ± 1.9% in our analyses, **Figure 3C** and **Supplementary Table 2**). *fad2-114* and *fad2-144* (A295G; D298E) showed enhanced oleic acid production, up to 57.9 ± 5.4% and 56.5 ± 2.3%, respectively (**Figures 3A,C** and **Supplementary Table 2**), more than their segregating siblings (38 ± 2%; **Figure 3A** and **Supplementary Table 2**). The increases are likely due to the homozygous changes in two amino acids at the same time, which had been segregating and thus having shown moderate increases in the previous generation g5CBE11 as well (**Figure 2B**). Particularly, the linoleic acid (18:2) and the linolenic acid (18:3) contents were dramatically decreased to below 2.4% and to below 2%, respectively, in both *fad2-321* and *fad2-114*, while those contents were 2.4 ± 0.2 and 6.2 ± 0.5% in *fad2-1* (**Figure 3C** and **Supplementary Table 2**), supporting the notion

A



B

Fatty acid	Control	GC analysis on the T_3 seeds from T_2 lines (n=T_2 line number)											
		g5CBE1n							g5CBE3n				
	Col-0	n=1*	2	3	4*	5	6	7	1*	2*	3*	4*	5
16:0	8.7	7.5	5.9	7.3	5.8	7.6	7.7	7.7	7.1	5.6	8.5	6.3	6.7
18:0	3.4	3.0	2.4	3.0	2.4	3.0	3.1	3.4	3.2	2.4	3.4	3.0	3.0
18:1	18.1	45.8	64.9	44.7	63.9	42.6	43.9	38.7	44.0	69.2	25.7	54.0	49.5
18:2	29.5	8.7	0.8	8.7	0.3	9.2	9.2	12.2	11.5	0.3	26.0	6.1	9.1
18:3	17.6	10.5	2.4	10.9	1.9	11.7	10.6	14.0	13.1	1.7	16.7	8.9	9.7
20:0	2.1	1.6	1.1	1.6	1.2	1.6	1.7	1.9	1.6	1.1	2.1	1.5	1.6
20:1	17.2	22.9	22.5	23.8	24.5	24.2	23.8	22.1	19.5	19.7	17.5	20.2	20.5

FIGURE 2 | Characterization of the FAD2-targeting CBE-transgenic lines. **(A)** Chromatogram results from Sanger sequencing for *FAD2* genotyping within editing windows in the T_2 plants. The editing window is specified in the upper Col-0 panel. Red letters indicate altered DNA and amino-acid sequences. **(B)** Analysis of fatty acid compositions of transgenic lineages. Red-colored numbers denote the three most increased oleic acid contents observed among T_2 plants analyzed. Asterisks (*) indicate the representative T_2 transgenic lines that were progressed to the next T_3 generation for further characterization.

that the increases are due to "attenuated" desaturase activities of FAD2 in those alleles. Notably, the oleic acid content of the attenuated allele, such as *fad2-321*, was comparable to that of a *fad2-349* knockout allele in which the contents were measured as 59.5 ± 10.4% (**Figures 3A,C** and **Supplementary Table 2**).

Characterization of the Growth Responses of *fad2* Alleles

We hypothesized that certain "attenuated" *fad2* alleles in our study might not have as severe growth defects as the *fad2* knockout allele, and possibly even less severe than the previously reported *fad2-1* allele while maintaining increased oleic acid levels. In this regard, we decided to characterize our newly generated *fad2* alleles for phenotypical differences in terms of their growth responses. We analyzed two early growth responses, i.e., germination and root growth rate, for each allele under normal and stress conditions. We used experimental stress conditions of 150-mM NaCl and 300-mM Mannitol, and 75-mM NaCl and 200-mM Mannitol on top of 1/2 MS media for the germination and root growth test, respectively, with the intention of mimicking salt or water-deficit stress as has been

A

T_2 parents	T_3 genotypes (frequencies)		Predicted amino acid changes	New allele names	Oleic acid contents (T_4 seeds)
Col-0	GCTTTGGCTACCGTAGACAGAGACTACGGAATCTT		wt	n/a	18.5±0.3%
g5CBE11	GCTTTGGGTACCGTAGAGAGAGACTACGGAATCTT	(4/11)	A295G; D298E	*fad2-114*	57.9±5.4%
	GCTTTGG(G/T)TACCGTAGA(G/C)AGAGACTACGGAATCTT	(7/11)	Segregating	n/a	38±2.0%
g5CBE14	GCTTTGGGTACCGTAGAGAGAGACTACGGAATCTT	(6/6)	A295G; D298E	*fad2-144*	56.5±2.3%
g5CBE31	GCTTTGGGTACTGTAGACAGAGACTACGGAATCTT	(6/6)	A295G	*fad2-316*	34.6±2.3%
g5CBE32	GCTTTGGTTATGGTAGACAGAGACTACGGAATCTT	(6/6)	A295V; T296M	*fad2-321*	64.7±5.7%
g5CBE33	GCTTTGG(T/C)TACCGTAGACAGAGACTACGGAATCTT	(4/7)	Segregating	n/a	21.6±0.2%
	GCTTTGGCTACCGTAGACAGAGACTACGGAATCTT	(2/7)	wt	n/a	16.2±0.2%
	GCTTTGGTTACCGTAGACAGAGACTACGGAATCTT	(1/7)	A295V	*fad2-337*	30.6%
g5CBE34	mixed signal	(8/12)	n/a	n/a	45.7±3.2%
	GCTTTGGGTACTGTAGACAGAGACTACGGAATCTT	(2/12)	A295G	n/a	29.6±0.9%
	GCTTTGGCTACCGACCGTAGACAGAGACTACGGAA	(2/12)	frameshift	*fad2-349*	59.5±10.4%

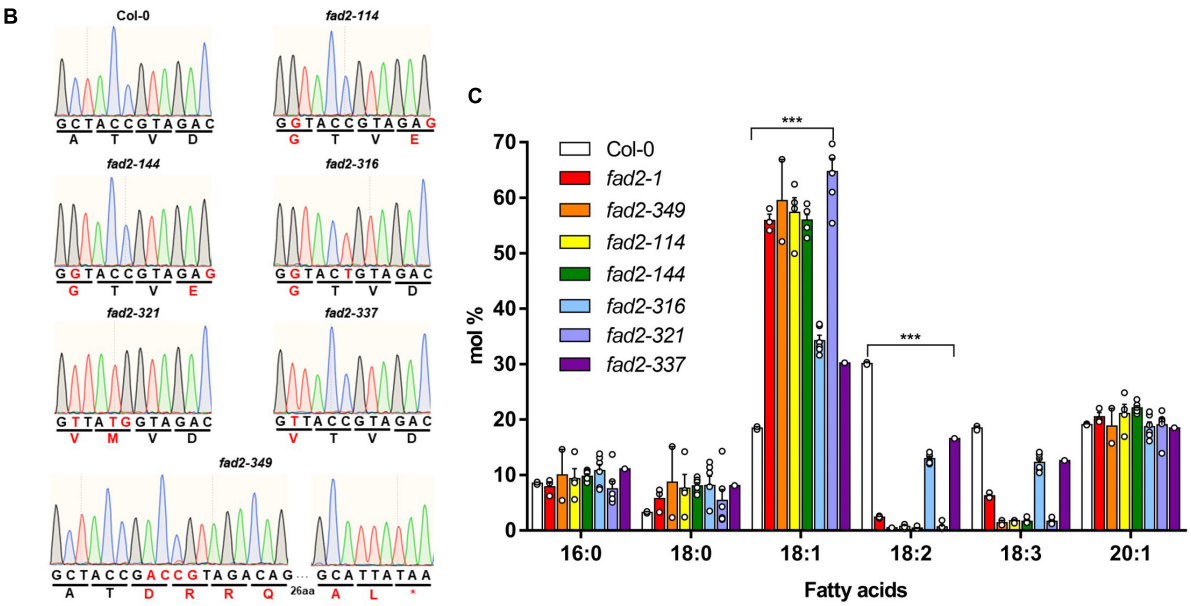


FIGURE 3 | Characterization of the base-edited *fad2 alleles*. **(A)** A summary of T_3 segregation patterns and analyses of oleic acid contents depending on each genotype. Green and gray letterboxes denote protospacer sequence and PAM sequence, respectively. Red letters indicate altered DNA sequences. According to fixed genotypes, novel *fad2* alleles were defined with predicted amino-acid changes. **(B)** Chromatogram results from Sanger sequencing for *FAD2* genotyping within editing windows in the defined *fad2* alleles. Red letters indicate altered DNA and amino-acid sequences. **(C)** Comparison of fatty acid compositions between T_4 seeds from novel *fad2* alleles, *fad2-1*, and Col-0. A one-way ANOVA statistical analysis was used to identify differences between the Col-0 and *fad2* alleles (***$p < 0.001$). Values represent mean ± SD.

described in previous studies (Zhang et al., 2012; Yu et al., 2017). For each of the alleles, germination tests were performed by scoring seed germination events at 12-h intervals during the initial 4 days after planting to assess whether increases in oleic acid content influence germination (**Figures 4A–C**). Under normal conditions, the germination frequencies of all tested alleles reached similar levels after 4 days, showing >95% germination success. Of note, *fad2-349*, *fad2-321*, and *fad2-144* alleles did show slightly reduced germination rates (∼72 h; **Figure 4A**), which coincide with relatively higher oleic acid contents observed in those alleles compared with others (**Figure 3C**). Upon both experimental stress conditions, *fad2-349* and *fad2-321* germination rates deteriorated sharply along with *fad2-1*, which showed significantly lower germination rates at all time points tested (**Figures 4B,C**). However, *fad2-144*, *fad2-316*, and *fad2-337* maintained germination rates comparable to that of WT (**Figures 4B,C**). These results suggest that those *fad2* alleles with increased oleic acid content also have enhanced resistance to stress susceptibility during germination in contrast to the case for *fad2-1*, *fad2-349*, and *fad2-321* (**Figures 4B,C**). Notably, *fad2-144*, which showed one of the highest levels of oleic acid content and contains two concurrent amino acid

changes (A295G; D298E, **Figure 3**), was fairly resistant to the stress conditions tested for germination relative to *fad2-1* (**Figures 4B,C**).

Next, we assessed root growth under normal or stress conditions by measuring root lengths of 4-day-old seedlings from each of the *fad2* alleles (**Figures 4D–F**). Under normal growth conditions, all *fad2* alleles tested showed a range of the root length reduction compared to wild-type Col-0 (**Figures 4D,E**). For example, *fad2-144* and *fad2-321* showed considerable reduction similar to the reduction observed in *fad2-1* and knockout *fad2-349* (**Figures 4D,E**), suggesting that the corresponding allelic changes impede normal root development. On the other hand, *fad2-316* and *fad2-337* appeared to have insignificant levels of root reduction (**Figures 4D,E**), indicating that their allelic variation does not alter normal growth processes. Stress treatments caused a range of reduction rates of root lengths on the seedlings from each allele tested (**Figures 4D,E**). Under the salt-stress condition, the knockout allele *fad2-349* showed a reduction of root length to 55.8% of its length under the normal unstressed condition (**Figure 4F**), which is consistent with the reported growth defects in *FAD2* knockout mutations under salt stress. On the other hand, Col-0 and *fad2-1* showed 66% and 63.6% of their normal growth lengths, respectively (**Figure 4F**). Notably, *fad2-316* (A295G) and *fad2-337* (A295V), which display an intermediate increase of oleic acids from single amino-acid substitutions (**Figure 3**), showed less reduction in root length to 82.9% and 79.7%, respectively (**Figure 4F**). The result suggests that their growth might be even less affected by the salt stress condition than WT. *fad2-144* (A295G; D298E) and *fad2-321* (A295V; T296M), which were regarded as higher oleic acid-producing alleles with two concurrent amino-acid substitutions (**Figure 3C**), displayed 67.8 and 69.2% of relative root length under salt stress, similar to the reduction level observed in Col-0 (**Figure 4F**). The growth defects seemed more obvious in the water-deficit stress condition with 200-mM Mannitol, as the root length of *fad2-349* was reduced to 32.6% while Col-0 was to 45.5% (**Figure 4F**). Again, *fad2-316* and *fad2-337* presented 49.1 and 54% of relative root length, respectively, indicating significantly increased tolerance to water-deficit stress compared with knockout *fad2-349* (32.6%) or previously reported *fad2-1* (35.5%) (**Figure 4F**). The relative root lengths of *fad2-144* and *fad2-321* were 40.4 and 41.1%, respectively (**Figure 4F**). The result suggests that they are still more tolerant than *fad2-349* and *fad2-1* but have increased susceptibility to stress conditions comparable to Col-0.

Seed weight and fatty acid content of *fad2* alleles were measured (**Supplementary Figure 5**). Compared to wild-type Col-0, the weight of *fad2-1* was decreased, while the new *fad2* alleles showed an increase. The fatty acid content of the seeds showed some differences between the new *fad2* alleles. The fatty acid content per seed of *fad2-1* was not significantly different from that of wild type. The allele produced in this experiment had an increase in seed weight compared to the wild type, and there was no difference or a significant increase in the fatty acid content per seed. These results suggest that mutations in the *FAD2* gene can affect seed weight and fatty acid content. The correlation between *FAD2* gene mutation and fatty acid content needs further study.

DISCUSSION

In our study, five different *fad2* alleles were isolated for increased oleic acid *via* semi-random targeting of BEs to six different genomic regions of the *FAD2* locus. All those higher oleic-acid alleles, interestingly, turned out to arise within the g5 gRNA targeting region, which represents the cytosolic stretch of ER-membrane-bound FAD2 protein. In principle, our laboratory-scale "pre-screen" may expedite the next process of recapitulating equivalent alleles in oil crops because one might focus on the corresponding g5 region to create such high oleic-acid variants *via* precision CBE targeting. Of note, the amino-acid residues harboring the allelic changes for increased oleic-acid content we have observed in our study are highly conserved in *FAD2* loci across major oil crops, implying direct value in applications (**Figure 5**). The A295V change observed in *fad2-321* and *fad2-337* has been reported to be one of the four combinatorial mutations conferring the competence in hydroxyl fatty acid production by manipulating the FAD2 function into FAH12-like property (Broun et al., 1998), supporting the notion that the corresponding residue may be pivotal to FAD2 activity. We reasonably anticipate that recapitulating suitable alleles such as a *fad2-144* equivalent in oil crops might be able to provide a clue to "agronomically applicable" genetic variants to mitigate adverse effects on plant growth and stress sensitivity that occurs in *FAD2* knockouts under certain growth conditions. Even intensive base editing within corresponding g5 regions in oil crops again with ABE and CBE might provide desired results with much less effort than traditional approaches.

We proposed and demonstrated that our targeted "semi-random mutagenesis" approach adapting CRISPR/BEs as a mutagenic source might be an effective alternative to generate novel *FAD2* alleles with desirable traits. All the reported *FAD2* mutations thus far were generated using conventional EMS, microRNA, and T-DNA approaches (Lemieux et al., 1990; Okuley et al., 1994; Song et al., 2010; Belide et al., 2012). The recent development of CRISPR/Cas9 facilitated the effective elimination of the FAD2 function in a targeted manner (Jiang et al., 2017; Do et al., 2019; Yuan et al., 2019; Bahariah et al., 2021). However, CRISPR/Cas9 induces DSBs and relies on the endogenous DNA repair process, i.e., NHEJ, to generate indels, which most frequently yield *FAD2* KO alleles, and thus make them unsuitable to field cultivation due to their growth defects. In this study, we aimed to combine the advantage of using CRISPR/BEs with a traditional "forward genetics" approach by randomly applying BEs to select genomic regions to induce missense mutations and then screen for candidate alleles with desirable phenotypes. Our "proof-of-concept" study reveals several advantages over conventional approaches, such as TILLING as follows: (1) much smaller size of screening population is required due to strategic targeting of mutagenic BEs within only select genomic regions; (2) a broader variety of point mutations can be induced, including not only base transitions, such as C-to-T and A-to-G but also

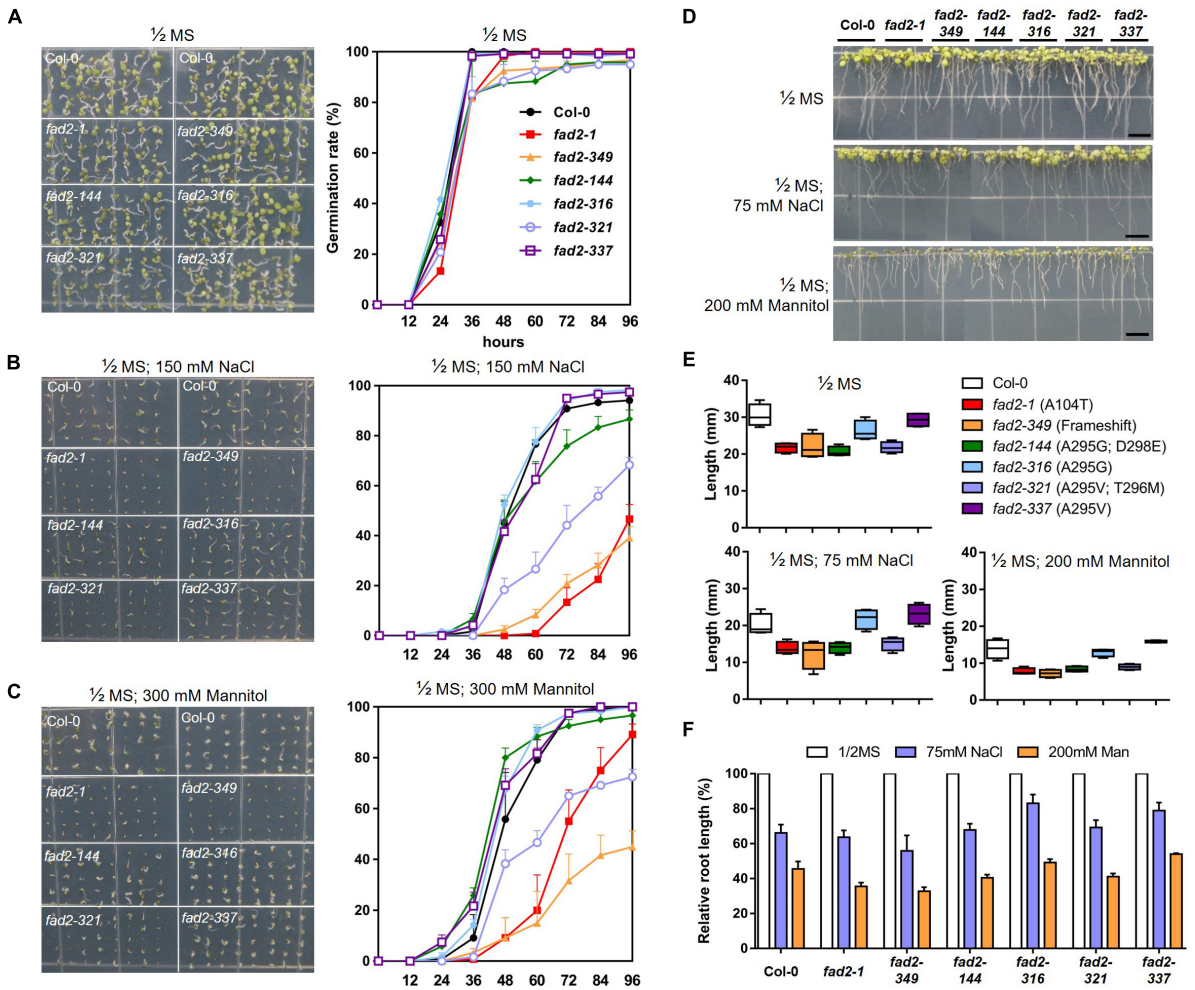


FIGURE 4 | Analyses of physiological responses of "attenuated" *fad2* alleles. **(A–C)** Germination tests for *fad2* alleles conducted in **(A)**, 1/2 MS, **(B)**, 1/2 MS; 150-mM NaCl, and **(C)**, 1/2 MS; 300-mM Mannitol condition to test whether increases in oleic acid content influence germination under both normal and stress conditions. The pictures were taken after 96 h of sowing. Germination rates were measured every 12 h in triplicate. **(D–F)** Analyses of root growth rates of *fad2* alleles, whose seedlings had been grown for 4 days in 1/2 MS and then were transferred to both normal and stress conditions to allow root development for 5 days. The data presented in **(D)**, pictures taken to identify root lengths of 5-day-grown seedling under experimental conditions, **(E)**, graphs indicating the root lengths in different conditions, and **(F)**, a graph showing relative root lengths in 75-mM NaCl and 200-mM Mannitol when the root length in 1/2 MS was set to 100%. All values represent mean ± SD.

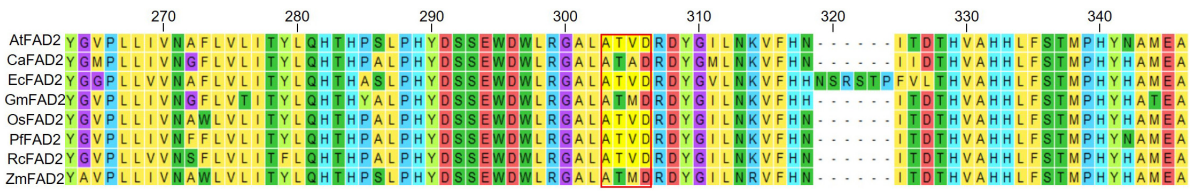


FIGURE 5 | Alignment of FAD2 amino-acid sequences among *Arabidopsis* and various oil crops. Protein alignment was conducted by ClustalW in the MEGA7 program focusing on the g5 region in this study. The red box indicates the base editing window in this study. At, *Arabidopsis thaliana*; Ca, *Corylus avellana*; Ec, *Eucalyptus camaldulensis*; Gm, *Glycine max*; Os, *Oryza sativa*; Pf, *Physaria fendleri*; Rc, *Ricinus communis*; Zm, *Zea* mays. Different colors of amino acid are based on the polarity of the R group.

C-to-G base transversions, some of which are not feasible using EMS mutagenesis; and (3) combinatorial *cis* mutations within a narrowly defined region can be obtained, as seen in *fad2-144* (A295G; D298E) and *fad2-321* (A295V; T296M). Furthermore, the results imply that our proposed "semi-random" mutagenic approach, particularly when combined with high-throughput

gRNA library screening methods (Jae-Young et al., 2019), may offer a novel complementary platform for plant molecular breeding.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

M-EP, J-YY, and HUK performed the experiments, analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.748529/full#supplementary-material

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An Efficient Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-Associated Protein 9 Mutagenesis System for Oil Palm (*Elaeis guineensis*)

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The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system has emerged as a powerful tool for the precise editing of plant genomes for crop improvement. Rapid *in vitro* methods for the determination of guide RNA (gRNA) cleavage efficiency and an efficient DNA delivery system is essential for gene editing. However, we lack an efficient gene-editing system for palm species. In this study, we described the development of a transient oil palm protoplast assay to rapidly evaluate the cleavage efficiency of CRISPR/Cas9 mutagenesis and the generation of stable transformed oil palms using biolistic particle bombardment in immature embryos. Using the *phytoene desaturase* (*EgPDS*) gene, we found cleavage frequency of up to 25.49% in electro-transfected protoplast, which enables the production of transgenic oil palm shoots exhibiting chimeric albino phenotypes as a result of DNA insertions, deletions (InDels), and nucleotide substitutions, with a mutation efficiency of 62.5–83.33%. We further validated the mutagenesis efficiency and specificity of the CRISPR/Cas9 system in oil palm by targeting the *brassinosteroid-insensitive 1* (*EgBRI1*) gene, which resulted in nucleotide substitutions in *EgBRI1* with premature necrosis phenotype in oil palm transgenic shoots and stunted phenotype resulting from DNA InDels. Taken together, our results showed that effective and efficient editing of genes using the CRISPR/Cas9 system can be achieved in oil palm by optimizing the selection of efficient gRNA and DNA delivery methods. This newly designed strategy will enable new routes for the genetic improvement in oil palm and related species.

Keywords: genome editing, CRISPR/Cas9, oil palm, *EgPDS*, *EgBRI1*

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INTRODUCTION

Genome editing technology is emerging as a powerful tool to introduce accurately targeted mutations for plant gene function studies and provide new avenues for crop improvement due to its simplicity, flexibility, consistency, accuracy, and high efficiency (Lozano-Juste and Cutler, 2014; Voytas and Gao, 2014; Bortesi and Fischer, 2015). It has been made simpler with clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system due to its versatility, effectiveness, and efficiency (Feng et al., 2013; Ma et al., 2015; Jiang and Doudna, 2017). The CRISPR/Cas9 system has been successfully established and widely applied in various economically important crops, including wheat, rice, maize, brassica, sweet potato, banana, and grapevine, to enhance crop productivity, grain quality, nutritional value, disease tolerance, resilience to climate change, and herbicide resistance (Shi et al., 2017; Macovei et al., 2018; Okuzaki et al., 2018; Zhang et al., 2018; Wang et al., 2019; Kaur et al., 2020; Li et al., 2020).

Clustered regularly interspaced short palindromic repeat/Cas9 mediates precise genome modification by gene knock in, knock out, base editing, transcriptional activation or repression, epigenetic modification, and RNA editing (Feng et al., 2013; Konermann et al., 2015; Zong et al., 2017; Mao et al., 2018; Papikian et al., 2019). The CRISPR/Cas9 endonuclease protein is directed to a specific chromosomal DNA site by a short sequence-specific single guide RNA (gRNA) to induce DNA double-stranded breaks (DSBs) and enable precise editing of the target DNA sequence with mutation events, including insertions, deletions (InDels), and nucleotide substitutions induced by the cell repair mechanisms, non-homologous end-joining (NHEJ), or homology-directed repair (HDR) (Jiang and Doudna, 2017). In plants, the efficiency of CRISPR/Cas9-mediated mutagenesis is affected by the editing components (Cas9, promoter, gRNA design, and specificity), the transformation or delivery method into plant cells, the ability of plants to regenerate, and the sensitivity of the mutation detection method (Ma et al., 2015; Liu et al., 2016; Thyme et al., 2016; Wang et al., 2018; Montecillo et al., 2020). The CRISPR/Cas9 system can be delivered into plant cells using several methods, including plant transformation of CRISPR/Cas9 and gRNA expression cassettes, virus-mediated gRNA delivery, DNA-free CRISPR–Cas9 ribonucleoprotein (RNP) delivery to protoplasts, and *de novo* meristem induction bypassing tissue culture process (Xu et al., 2014; Ali et al., 2015; Woo et al., 2015; Wang et al., 2018; Montecillo et al., 2020). The efficiency of CRISPR/Cas9 is variable in different plant species and requires fine-tuning of the conditions to achieve successful and highly efficient genome editing.

Global food demand is expected to increase by 60% in 2050, driven by the rapid expansion of the human population reaching approximately 9–11 billion and global dietary shifts intensifying wellness and value addition (Fróna et al., 2019). Oil palm is well recognized as the most efficient oil crop that contributes to 35% of global vegetable oil; thus, it can offer a critical solution for global food insecurity to feed the growing global population (Mielke, 2017). Despite the yield and oil quality improvement achieved through conventional breeding and marker-assisted breeding in the past decades (Soh et al., 2017), the annual growth in palm oil production is likely to slow down in the coming years due to a deceleration in land expansion, yield stagnation, climate change, declining labor force, increase in production costs, and pest and disease issues. Thus, the application of genome editing technology can provide opportunities to accelerate and enhance breeding programs with high precision in oil palm to achieve food security and sustainable agriculture.

Limitations in an efficient genetic transformation system, a plant regeneration process, and an *in vitro* testing system hindered the establishment of the CRISPR/Cas9 system in oil palm. Here, we report an establishment of the CRISPR/Cas9 system that enables efficient genome editing in oil palm. We investigated the efficacy and efficiency of a codon-optimized CRISPR/Cas9 by targeting oil palm *phytoene desaturase* (*EgPDS*) gene as a phenotypic marker to facilitate rapid screening of mutant lines as mutation of the *PDS* gene disrupts the carotenoid pathway and results in albino and dwarf phenotypes (Odipio et al., 2017). We first established a rapid *in vitro* detection method for testing gRNA efficiency in oil palm using protoplast electro-transfection. Subsequently, we demonstrated the delivery of CRISPR/Cas9 and gRNA cassettes into oil palm immature embryos using biolistic particle bombardment-mediated transformation and validated the mutagenesis effects of CRISPR/Cas9, both genotype and phenotype in regenerated oil palms. We further validated the mutagenesis efficiency and specificity of the CRISPR/Cas9 system in oil palm by targeting oil palm *brassinosteroid-insensitive 1* (*EgBRI1*). We showed that mutations in the kinase domain of BRI1 are associated with palms displaying stunted phenotype and necrosis at the leaf apex.

MATERIALS AND METHODS

Plant Materials

Oil palm *dura* (Deli *dura*) immature embryos extracted from the fresh oil palm fruits at 12 weeks after pollination (WAP) were supplied by Oil Palm Breeding, Sime Darby Plantation Research Sdn. Bhd., Banting, Malaysia, and used as the starting material for this study. Before particle bombardment with plasmids at 200 replicates/constructs, the immature embryos were cultured on Murashige and Skoog (1962) media with sucrose and vitamins. Unopened oil palm spear leaf was used for protoplast isolation.

Construction of Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-Associated Protein 9 and Guide RNA Vector

The *Streptococcus pyogenes Cas9* nuclease gene (Jinek et al., 2012) was codon optimized (**Supplementary Data Sheet 1**) by replacing the tandem rare codons in native gene to codon usage bias in oil palm using the OptimumGene^{TM} codon optimization analysis (GenScript USA Inc., Nanjing, China) to enhance the efficiencies in *Cas9* gene expression and translation in oil palm. The codon-optimized *Cas9* was synthesized together with the Gateway attP1 site and nuclear localization signal (NLS) fused

to the 5′ of *SpCas9* and Gateway attP2 site fused to the 3′ of the gene (GenScript USA Inc., Nanjing, China). The synthesized gene construct was shuttled into the pXHb7SNFI-UBIL vector (Vlaams Instituut voor Biotechnologie, Ghent, Belgium) fused to the *Ubiquitin* promoter and β-*glucuronidase* (*GUS*) reporter using Gateway® BP Clonase™ II enzyme mix (Thermo Fisher Scientific, Waltham, MA, United States). The resulting construct is known as pUbil-Cas9 (**Supplementary Figure 1**). The potential target sequences of gRNAs for oil palms *EgPDS* (accession no. XP_010937221) and *EgBRI1* (accession no. XP_010927763) were analyzed and designed using Benchling[1] and CCTop[2] (Stemmer et al., 2015). These gRNAs were selected based on their position in the gene, the predicted efficacy score, and the potential for off-target mutation. These gRNAs (**Supplementary Table 1**) designed to target different exon regions of the *EgPDS* gene (**Figure 1A**) were cloned into a modified pCAMBIA1201 vector, fused to rice *OsU3* promoter and gRNA scaffold cassette (**Supplementary Figure 1**).

Protoplast Electroporation Transformation and Detection of Guide RNA Efficiency

The oil palm mesophyll protoplasts were isolated from the unopened spear leaf as described previously (Masani et al., 2013). These protoplasts were co-transformed with 2 μg plasmids of pUbil-Cas9 and individual gRNA constructed for *EgPDS* and *EgBRI1* genes using electroporation. A single vector of pUbil-Cas9 or gRNA was transformed as controls. The protoplast and plasmid mixture in HEPES-buffered saline solution was electroporated using Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA, United States) with a square wave pulse program: 550 V/cm (220 V setting/0.4 cm width), 10 ms for two times, and a 20 ms interval for poring pulse. The electroporated protoplasts were incubated in HEPES-buffered saline solution under dark conditions at 25°C with gentle swirling for 72 h. The protoplasts were washed using a washing solution, and the genomic DNA of transformed protoplasts were extracted using QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI, United States) according to the instructions of the manufacturer. The electro-transfection efficiency was determined using absolute quantitative real-time PCR. The DNA copies of the *GUS* reporter gene transformed into protoplast were compared against the total DNA copies of the endogenous gene, *Cyclophilin 2* in protoplast, both DNA copies were determined from plasmid dilution standard curve. According to the instructions of the manufacturer, the target site region, flanking the gRNAs of *EgPDS* and *EgBRI1* genes, was amplified, respectively, from the transformed and wild-type protoplasts DNA using region-specific primers (**Supplementary Table 2**) and iProof™ High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Hercules, CA, United States). The mutations were detected by comparing amplified fragments from the transformed and wild-type protoplasts (50:50 ratio) using the AccuCleave™ T7CE Kit and detection using CRISPR Discovery Gel Kit and Fragment Analyzer™ Automated CE System (Agilent Technologies, Palo Alto, CA, United States). The mutation frequency of the protoplast population was determined based on the percentage of cleavage efficiency calculated using the PROsize Data Analysis Software (Agilent Technologies, Palo Alto, CA, United States).

Biolistic Transformation and Plant Regeneration

The pUbil-Cas9 and two efficient gRNA constructs for *EgPDS* and *EgBRI1* (2 μg each) were coated on the surface of 3 mg gold particles in 50% glycerol and mixed with 1M $CaCl_2$ and 16 mM spermidine. The mixture was washed with absolute ethanol three times and was resuspended in 60 μl absolute ethanol. For each bombardment, 10 μl of DNA-microcarrier mixture was placed on the microcarrier and bombarded two times into oil palm immature embryos using the PDS-1000/Hepta Biolistic Particle Delivery System (Bio-Rad Laboratories, Hercules, CA, United States) with 1,100 psi rupture disks, vacuum at 27 mmHg pressure, and 9 cm distance from the tissue. Individual pUbil-Cas9, gRNA, and the combinations of both constructs were bombarded or co-bombarded into 200 replicates of immature embryos. The pUbil-Cas9 and individual gRNAs were used as controls. The transformed immature embryos were maintained in MS agar with sucrose and vitamins (Sigma-Aldrich, St. Louis, MO, United States) supplemented with 50 μg/ml of hygromycin B. Regenerated plants were transferred into MS agar without antibiotic or herbicide and cultured under 16-h light/8-h dark photoperiod for 6–8 months.

Identification of Transgene and Detection of Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-Associated Protein 9 Mutations

Transgenic plants were identified by their resistance to hygromycin at the early phase of tissue culture. Three transformed embryos were randomly selected from the controls and confirmed using β-glucuronidase staining after 2 months on selection media. Putative transgenic plants were confirmed using the Phire Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA, United States) and specific primers targeting *Cas9* and *OsU3* promoter sequences (**Supplementary Table 3**). Five transgenic oil palm mutants with altered phenotypes were randomly selected for sequence analysis. Their genomic DNA was extracted using QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI, United States). The target site of *EgPDS* and *EgBRI1* was amplified using region-specific primers (**Supplementary Table 2**) and iProof™ High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Hercules, CA, United States). Amplicons were purified and analyzed using Sanger sequencing (Applied Biosystems, Foster City, CA, United States) for the detection of specific InDels and substitution. Mutation efficiency for each gRNA was evaluated based on the number of T0 transgenic plants with mutation compared to the number of genotyped T0 transgenic plants. The mutation event and

[1]https://benchling.com/

[2]https://cctop.cos.uni-heidelberg.de:8043/

frequency were analyzed and detected using TIDE[3] (Brinkman et al., 2014). These amplified fragments were cloned into pJET1.2 (Thermo Fisher Scientific, Waltham, MA, United States), and 20 colonies were randomly selected for single-colony plasmid sequencing for chimeric mutation assessment. The sequences were analyzed by aligning to wild-type sequences as reference.

RESULTS

Development of a Highly Efficient System for Genome Editing in Oil Palm

To establish a highly efficient CRISPR/Cas9 system in oil palm, we targeted *EgPDS* as genetic lesions will result in visible phenotypes. Five gRNAs were designed to target mutagenesis at different exon regions of the *EgPDS* gene (**Figure 1A**). First, we examined the functionality of codon-optimized SpCas9 and the effectiveness of the five gRNAs to identify efficient gRNA for further phenotypic validation in the transgenic oil palm, using a fast electroporation-mediated protoplast transient expression system. The protoplast electro-transfection efficiencies ranged from 17 to 26%. Agglutination among protoplasts was observed after electroporation with target constructs without damages (**Figure 1B**). In *EgPDS* mutagenesis, mutations were detected in protoplasts transformed with Cas9/gPDS4 and Cas9/gPDS5 with cleavage frequencies of 6.49 ± 0.7 and 25.49 ± 4.11%, respectively (**Figure 1C**). In contrast, we found minimal mutagenesis activity for gPDS1, gPDS2, and gPDS3 gRNAs, and no mutation activity was detected in the control protoplast pool. These results suggest that the codon-optimized Cas9 and gRNAs are an efficient system for mutagenesis in oil palm and that their efficiency can be easily determined by protoplast electro-transfection.

To examine the efficiency of CRISPR/Cas9 in oil palm, we generated stable transgenic plants by the transformation of pUbil-Cas9 and highly efficient gRNAs for *EgPDS* (gPDS4 and gPDS5) using the biolistic particle bombardment method. Putatively transformed embryos were selected from non-transformants based on their survival on a hygromycin selection medium. A total of 14 putative transformants were generated using pUbil-Cas9 and gPDS4 transformation, whereas 13 putative transformants were generated using pUil-Cas9 and gPDS5 transformation, with a transformation efficiency of 7 and 6.5%, respectively (**Table 1**). The transformation of a single vector cassette for Cas9 or individual gRNA into immature embryos resulted in a relatively higher transformation efficiency of up to 19.5%. We randomly selected the putative transformed immature embryos for GUS histochemical assay (**Figure 2A**). The hygromycin-resistant embryos were later transferred to negative selection media to induce shoot regeneration. Notably, we found that the regeneration efficiencies for *EgPDS*-edited plants ranged from 44 to 77%.

Alteration of *PDS* gene function is predicted to result in albino phenotypes due to defects in carotenoid biosynthesis (Mann et al., 1994). We found that the shoots of regenerated oil palms transformed with pUbil-Cas9/gPDS4 and pUbil-Cas9/gPDS5 displayed albino sectors indicating mosaicism (**Figure 2B**). These transgenic plantlets were scored based on their albino phenotype to provide a preliminary mutation efficiency for each gRNA module. Mutagenesis mediated by pUbil-Cas9/gPDS4 resulted in 83.3% mutation with chimeric albinism phenotype (**Figure 2C**) observed in 8 out of 9 regenerated oil palm shoots (**Table 1**). A total of 7 out of 10 regenerated oil palm shoots exhibited a similar chimeric albinism phenotype with Cas9/gPDS5 mutagenesis. Control plantlets transformed with a single construct of pUbil-Cas9/gRNA did not display signs of albinism. To investigate the effect of multiple mutageneses on plant regeneration and phenotype, we generated mutants transformed with pUbil-Cas9 and both gRNA (gPDS4 and gPDS5) constructs. Notably, only eight hygromycin-resistant immature embryos with gRNA double mutations regenerated into shoots. The EgPDS double mutants showed delayed shoot regeneration compared with the single gRNA mutants and non-edited control equivalents, nevertheless, the albino phenotype was noticeable in the shoots of the three mutants (**Table 1** and **Figure 2B**). All regenerated shoots with albino phenotypes were tested positive for the presence of both Cas9 and gRNA using PCR analysis. To confirm the gene-editing events, we performed PCR amplification and Sanger sequencing analysis using shoots exhibiting a clear EgPDS-edited albino phenotype (**Figures 3A–C**). These sequence traces were first analyzed using the TIDE algorithm (Brinkman et al., 2014) to identify the occurrence of InDels from the DSBs (**Supplementary Figure 2**). The sequence data indicated multiple mutations including InDels and single base substitutions in the EgPDS gene of the genotyped regenerated shoots, and the mutated sequences were validated using gene cloning. Mutations by Cas9/gPDS4 induced independent events with 71% occurrence of deletions (**Figure 3D**) ranging from −1 to −24 bp and 29% of nucleotide substitutions upstream of the protospacer adjacent motif sequence (PAM) region of the target site. The largest deletion (−24 bp) was detected at the boundary of intron 3, and exon 4 of the EgPDS gene may cause disruption in the splice-regulatory sequences and modulate exon skipping or alter splicing of pre-mRNA. Insertion (+1 bp), deletion (−1, −2, and −9 bp), and substitution (1–3 bp) were detected in Cas9/gPDS5 mutants at an occurrence frequency of 29, 43, and 29%, respectively. The nucleotide indels led to disruption in amino acid sequence and exon skipping in one of the mutants, whereas amino acid substitutions resulted from the nucleotide substitutions. In double mutation shoots, deletion (−2 and −16 bp) (75% occurrence) and insertion (+8 bp) (25% occurrence) were detected at the gPDS4 and gPDS5 target regions. These mutations led to amino acid frameshifts and the introduction of multiple stop codons near target regions. Collectively, our data suggest that the optimization of vectors for efficient Cas9 and gRNA activity combined with a rapid transformation system is a critical factor for an efficient gene-editing system in oil palm.

[3]https://tide.nki.nl/

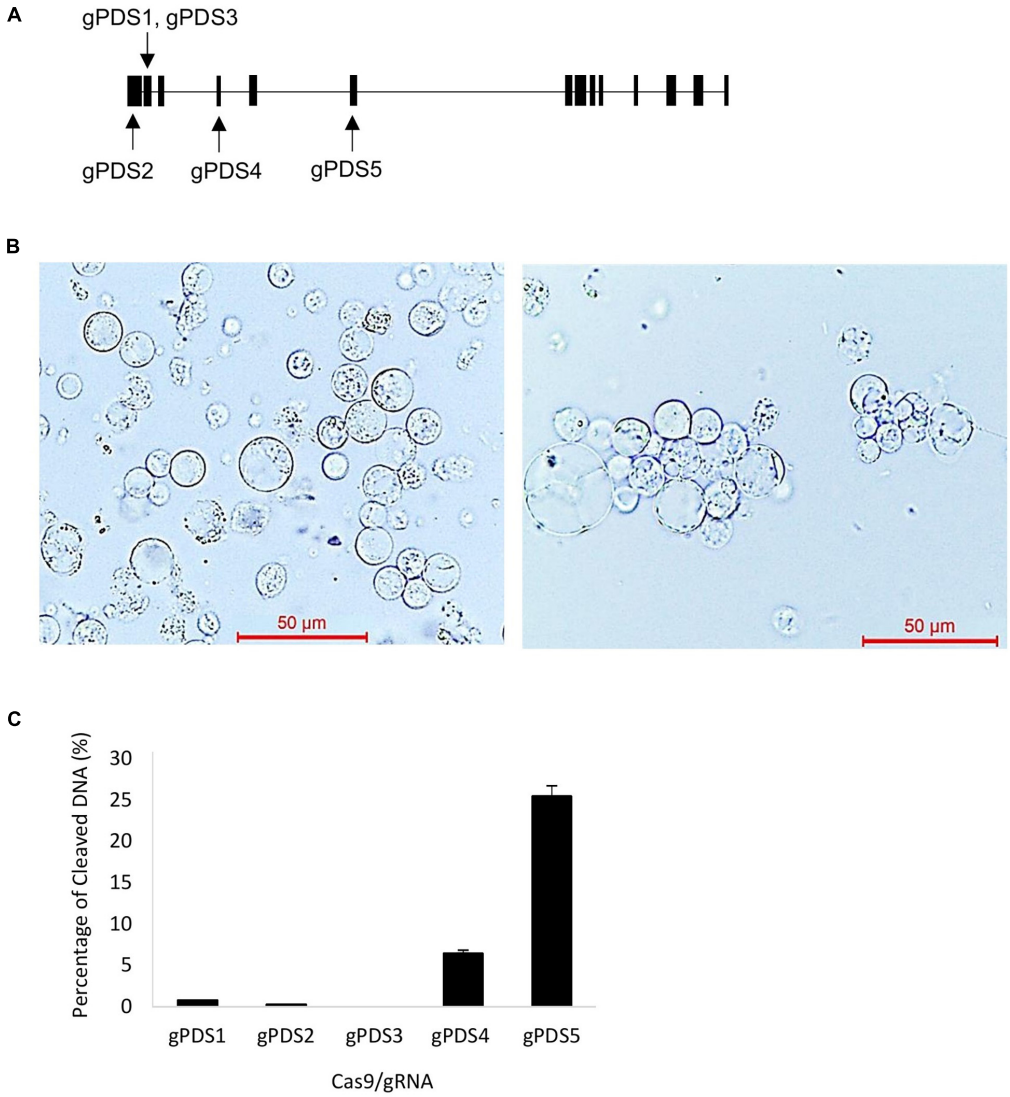


FIGURE 1 | Efficiency testing of clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) and guide RNA (gRNA) activity using electro-transfection in oil palm protoplasts. **(A)** Schematic map of gRNA target site in *phytoene desaturase* (*EgPDS*) gene. **(B)** Oil palm protoplast before (left) and protoplast agglutination after (right) electro-transfection with Cas9/gRNA. **(C)** Cleavage efficiency of Cas9/gRNA in oil palm protoplast following electro-transfection. Data are average values ± SE from four independent experiments.

Targeted Mutagenesis of *Brassinosteroid-Insensitive 1* to Manipulate Brassinosteroid Responses in Oil Palm

BRI1 gene encodes a brassinosteroid receptor and defective in *BRI1* results in dwarf phenotype in *Arabidopsis*, rice, and maize (Clouse et al., 1996; Noguchi et al., 1999; Yamamuro et al., 2000; Kir et al., 2015). Hence, we selected dwarfism as another visual phenotypic marker for the validation of the genome editing system in oil palm. To manipulate the brassinosteroid responses, we selected *EgBRI1* (accession no. XP_010927763) for targeted mutagenesis. The *EgBRI1* genomic DNA contains one intronless open reading frame of 3390 bp, and gRNAs were designed based on PAM sites (**Figure 4A**). The activity of the CRISPR/Cas9 system and the efficiencies of gRNAs targeting the *EgBRI1* gene were first verified using *in vitro* electro-transfection system in protoplast. Mutagenesis of *EgBRI1* gene mediated by Cas9 with gBRI1-1, gBRI1-2, and gBRI1-5 showed cleavage frequencies of 9.8 ± 1.21, 18.32 ± 1.4, and 5.1 ± 0.53%, respectively, in protoplast with electro-transfection efficiencies ranging from 12 to 17% (**Figure 4B**). However, cleavage was not detectable with the remaining gRNAs tested in the protoplast system. The two most efficient gRNAs, gBRI1-1 and gBRI1-2, were selected for transformation in the oil palm. The transformation with pUbil-Cas9 and gBRI1-1 resulted in 24 putative transformants (with a 12% transformation efficiency), while the transformation with pUbil-Cas9 and gBRI1-2 resulted

TABLE 1 | The efficiency of clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) and guide RNA (gRNA) transformation targeting *phytoene desaturase* (*EgPDS)* gene in oil palm and the phenotypes of the transgenic plants.

Gene	Guide RNA	Bombarded immature embryo	Putative transformant	Regenerated T0 shoots	T0 with mutation/genotyped T0 plants	Mutation rate	Transformants with altered phenotype
Control	*Cas9*	200	22 (11%)	17 (77%)	0/5	0	0
	gPDS4	200	39 (19.5%)	29 (74%)	0/5	0	0
	gPDS5	200	15 (7.5%)	10 (67%)	0/5	0	0
EgPDS	*Cas9/gPDS4*	200	14 (7%)	9 (64%)	5/6	83.33%	8 chimeric albino (89%)
	Cas9/gPDS5	200	13 (6.5%)	10 (77%)	5/8	62.50%	7 chimeric albino (70%)
	Cas9/gPDS4 + gPDS5	200	18 (9%)	8 (44%)	3/7	42.86%	3 chimeric albino (37.5%)

in 23 putative transformants (with an 11.5% transformation efficiency) (**Table 2**). Overall, the mutagenesis efficiency for *EgBRI1* ranged between 58.82 and 100%. Targeted mutagenesis of the *EgBRI1* resulted in two distinct phenotypes found in most of the regenerated plants (75–87% regeneration efficiency), reduced plant elongation (stunted), and apex necrosis (**Figures 4C,D**) with mutation efficiency of 58.82 and 66.67% using pUbil-Cas9/gBRI1-1 and pUbil-Cas9/gBRI1-2, respectively. Sequence analysis of the targeted region detected a wide range of mutations in regenerated transgenic oil palm shoots. Those that arose from pUbil-Cas9/gBRI1-1 and pUbil-Cas9/gBRI1-2 had multiple nucleotide insertions (+1, +8 bp), nucleotide deletions (−1 to −25 bp), and nucleotide substitutions at the sequence targeted at an occurrence frequency of 18–20, 30–36, and 45–50%, respectively (**Figures 5A,B**). These nonsense mutations may cause the frameshift and aberrant amino acid sequences with premature stop codon introduction and lead to protein truncation. Likewise, the impaired growth phenotypes in mutants generated using paired-gRNAs resulted primarily in deletions (−1, −2, −18, and −39 bp) and insertions (+4 bp) (**Figure 5C**). Nucleotide substitution mutations are predicted to affect the activity of the leucine-rich repeat receptor-like protein kinase domain of BRI1 (**Figure 5D**) and are responsible for the apex necrosis phenotype observed in these plants.

DISCUSSION

Genome editing provides opportunities for the improvement of the trait in crops, yet its implementation requires an efficient genetic transformation and regeneration system. In oil palm, limitations in the DNA delivery method, inefficient clonal propagation, and regeneration processes constrain rapid progress in genetic engineering. In this study, we developed an efficient system for CRISPR/Cas9 genome editing in oil palm by targeting *EgPDS* and *EgBRI1* genes. Mutation rates mediated by CRISPR/Cas9 varied depending on the specificity and effectiveness of gRNAs to target sequences, delivery methods, and sensitivity of the detection method. We first established an *in vitro* protoplast electro-transfection method to identify mutagenic and efficient gRNAs. Electroporation of Cas9 and gRNA constructs using our optimized conditions resulted in cleavage frequencies of up to 25.49% in viable oil palm protoplasts, suggesting that the codon-optimized Cas9 was effective in inducing cleavage. Several gRNAs failed to elicit cleavage despite high GC-content has been associated with higher Cas9 editing efficiency (Liu et al., 2016). This may be contributed by the sequence composition or secondary structure of several target gRNAs that potentially cause failure to recognize target sequence or formation of functional Cas9-gRNA complex in protoplast (Thyme et al., 2016). In cabbage, neon electro-transfection at 1,000 V was 1.4 times more efficient than PEG-mediated transfection in delivering CRISPR-Cas9 RNP into protoplast and resulted in 3.4% InDels mutation frequency in the *PDS1* gene (Lee et al., 2020). Despite a previously reported PEG-mediated transfection method for protoplast, the protoplast viability and reproducibility are significantly reduced due to toxicity effects of PEG (Masani et al., 2014). Our method for electro-transfection of genome editing tools in oil palm protoplasts provides an effective alternative strategy for gRNA cleavage activity and potential opportunity to develop DNA-free genome-edited palms. However, limitations with oil palm protoplast regeneration require further studies for improvement to enable deployment of the protoplast system for DNA-free gene editing.

High transformation efficiency gene editing has been reported in monocot plant species including 57% in maize, 80% in wheat, 64% in rice, and 14.3% in sorghum (Xu et al., 2014; Char et al., 2017, 2020; Macovei et al., 2018; Zhang et al., 2018). Previous studies in oil palm achieved 0.7–1.5% transformation efficiencies using *Agrobacterium*-mediated and microprojectile bombardment transformation methods in embryogenic callus and regeneration by indirect somatic embryogenesis (Parveez et al., 1998; Masli et al., 2012; Dayang Izawati et al., 2015). In this study, we obtained 5–19.5% transformation efficiencies using particle bombardment into zygotic embryos, and up to 87% of embryos regenerated into shoots through direct embryogenesis. Our results suggest that the optimization of transformation protocol, selection of explant, and regeneration methods are effective in improving oil palm transformation efficiency. Furthermore, a direct embryogenesis approach was employed to enable rapid regeneration with minimized risk

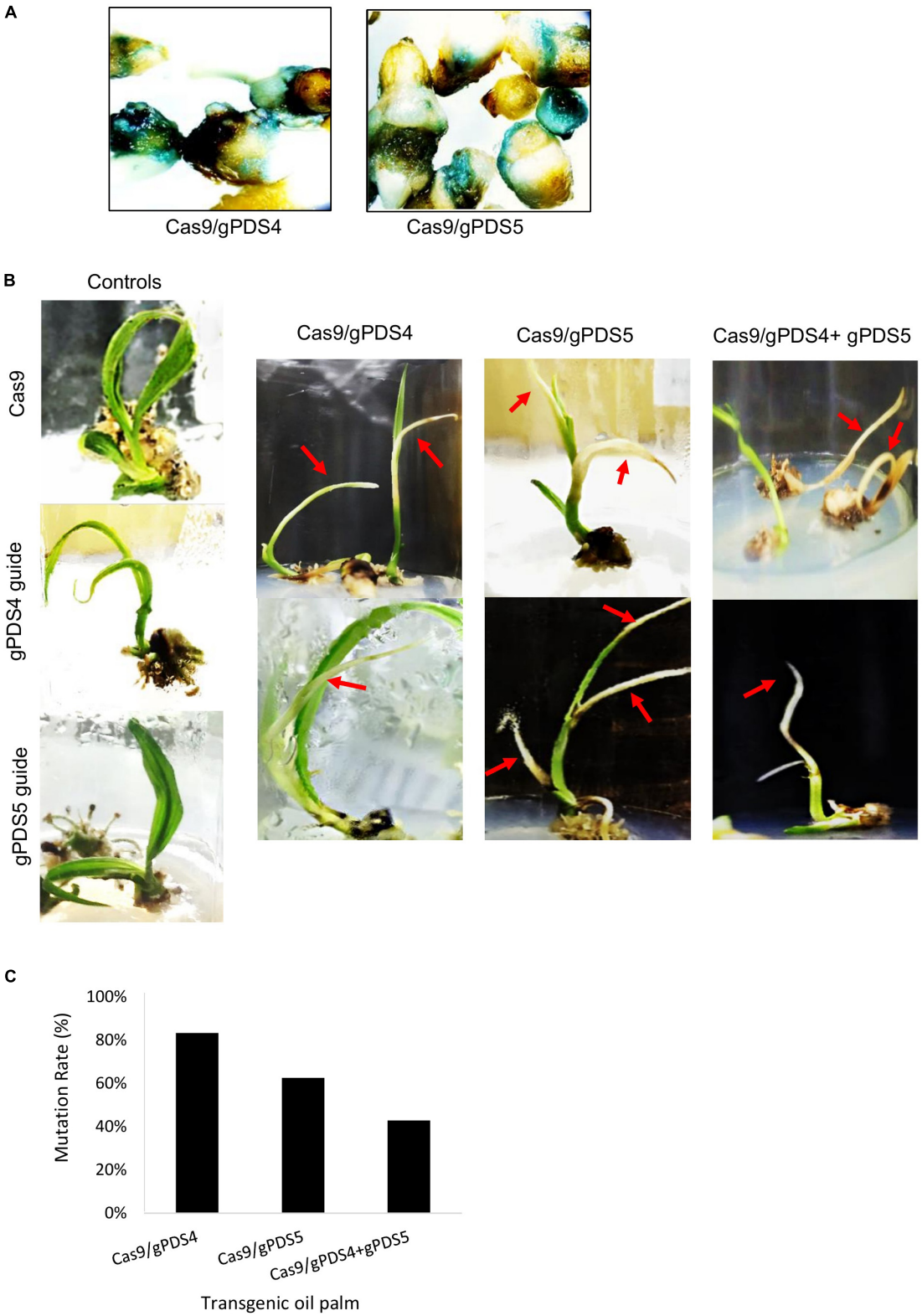


FIGURE 2 | Mutagenesis mediated by CRISPR/Cas9 and gRNA of *EgPDS* in *in vitro* transgenic oil palm. **(A)** Confirmation of genetic transformation by β-*glucuronidase* (GUS) assay in immature embryos transformed with Cas9/gPDS4 and Cas9/gPDS5. **(B)** Phenotypes of the Cas9/gPDS4 or gPDS5-mediated mutagenesis and control transgenic *in vitro* oil palms. Arrow indicating shoots with albino phenotypes. **(C)** Mutation frequencies of CRISPR/Cas9 mutagenesis in *in vitro* transgenic oil palms.

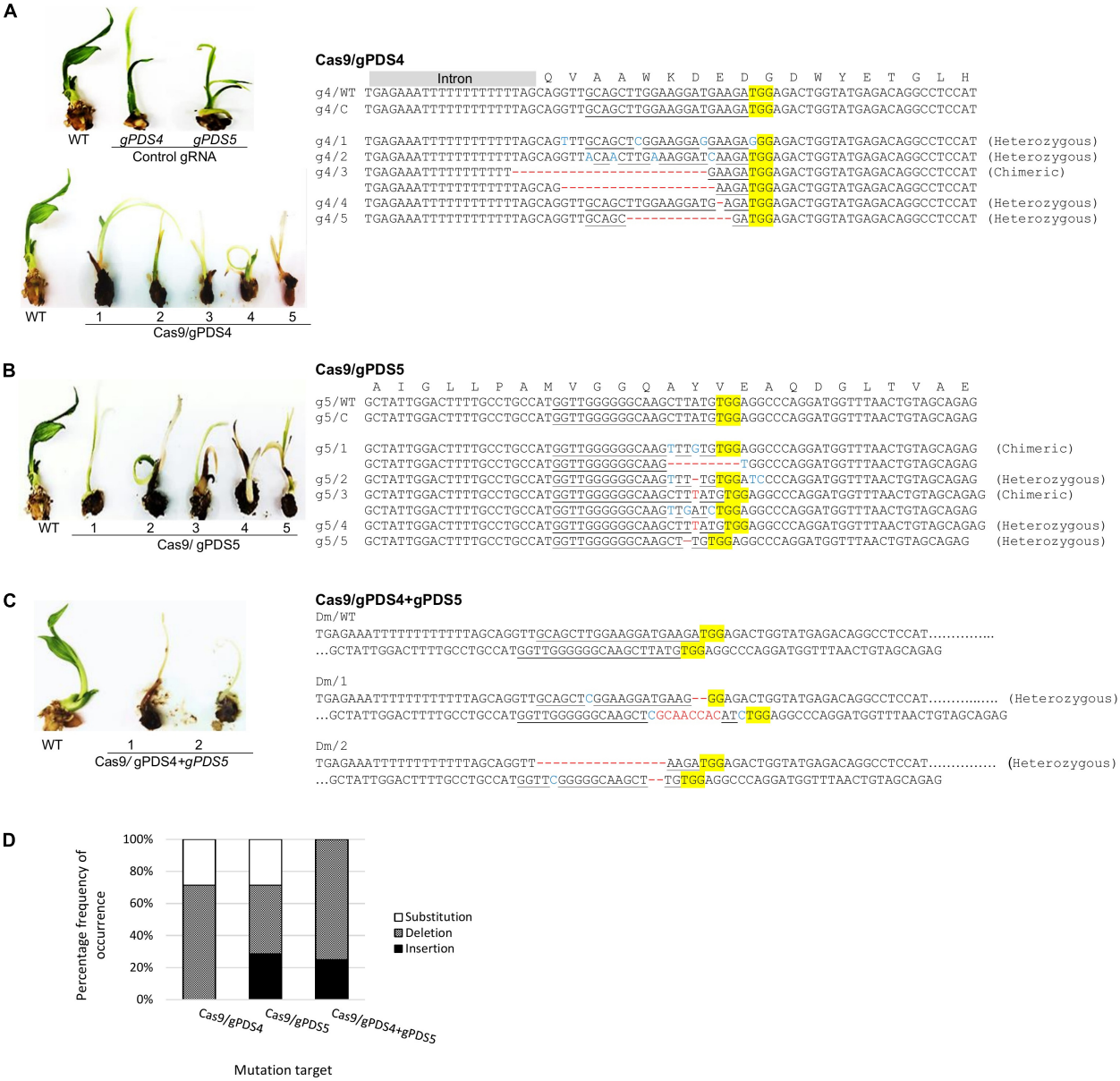


FIGURE 3 | Mutation events of targeted CRISPR/Cas9 mutagenesis of *EgPDS* gene in *in vitro* transgenic oil palm. Mutation events detected in the transgenic oil palms exhibiting the chimeric albino phenotypes using sequencing-based detection at the target sites of **(A)** gPDS4, **(B)** gPDS5, and **(C)** both gPDS4 and gPDS5 in the *EgPDS* gene and **(D)** the percentage frequency of occurrence for each type of mutation event. Protospacer adjacent motif sequence (PAM) is highlighted in yellow, InDels sequences of the target site are shown in red, and nucleotide substitution are marked in blue. g4, gPDS4; WT, wild-type; C, control; g5, gPDS5; Dm, double mutant; 1–5, plantlet number.

of culture-induced genetic variation that commonly occurs during callusing phase (Weckx et al., 2019). Although chimeric albinism phenotype was observed in the transgenic *EgPDS*-edited palms using our method, nevertheless, homozygous mutants and transgene removal can be obtained by genetic segregation and backcrossing.

We achieved high editing efficiency (42.86–100%) at target sites of *EgPDS* and *EgBRI1* genes in transgenic oil palms with our CRISPR/Cas9 system. In rice, high mutagenesis efficiency of 87–100% (di-allelic edits) was achieved using rice codon-optimized SpCas9 compared with the optimized SpCas9 from bacterial, human, and *Chlamydomonas* in T0 transgenic plants (Zhou et al., 2014). High editing efficiency in our system may be attributed to the oil palm codon-optimized *Cas9* gene driven by ubiquitin, an active promoter in monocot (Schledzewski and Mendel, 1994), efficient gRNAs, and delivery method for Cas9/gRNA. Our transgenic plants showed insertions (18–40%), deletions (30–75%), and high levels of nucleotide substitutions

TABLE 2 | The efficiency of CRISPR/Cas9 and gRNA transformation targeting *brassinosteroid-insensitive 1 (EgBRI1)* gene in oil palm and the phenotypes of the transgenic plants.

Gene	Guide RNA	Bombarded immature embryo	Putative transformant	Regenerated T0 shoots	T0 with mutation/genotyped T0 plants	Mutation rate	Transformants with altered phenotype
Control	*Cas9*	200	22 (11%)	17 (77%)	0/5	0	0
	gBRI1-1	200	17 (8.5%)	13 (76.5%)	0/5	0	0
	gBRI1-2	200	10 (5%)	8 (80%)	0/5	0	0
EgBRI1	*Cas9/gBRI1-1*	200	24 (12%)	18 (75%)	10/17	58.82%	6 stunted (33%) 5 leaf necrosis (28%)
	Cas9/gBRI1-2	200	23 (11.5%)	20 (87%)	10/15	66.67%	5 stunted (25%) 10 leaf necrosis (50%)
	Cas9/gBRI1-1 + gBRI1-2	200	36 (18%)	30 (83%)	7/7	100%	30 stunted (100%)

(29–50%) in *EgPDS* and *EgBRI1* genes. Although InDels are the most common mutation induced by error-prone NHEJ repair mechanism that involves direct rejoining of DSB ends regardless of sequence homology (Rodgers and McVey, 2016), high frequency of NHEJ-induced base substitutions are also prevalent in plants and substantial evidence have been reported in rice, cassava, citrus, soybean, and melon (Sun et al., 2015; Odipio et al., 2017; Macovei et al., 2018; Hooghvorst et al., 2019; Dutt et al., 2020). For instance, a high frequency of base substitutions has been reported in melon (91%) and rice (25–45%) (Macovei et al., 2018; Hooghvorst et al., 2019). In our study, InDels in the *EgBRI1* gene resulted in reduced plant elongation phenotype, while leaf apex necrosis was generated from base substitution events in transgenic palms. Early studies in *Arabidopsis* and rice reported dwarf and shortened internode phenotypes in the *bri1* loss-of-function mutant plants (Clouse et al., 1996; Noguchi et al., 1999; Yamamuro et al., 2000). Choe et al. (1999) further reported that mutation in *Arabidopsis bri1* results in a retarded leaf senescence phenotype. Phenotypes of our *EgBRI1* transgenic mutant palms are substantially affected by the types of NHEJ-induced mutations, and the specificity of the repair mechanism for DSBs is critical to driving accurate genomic changes without errors. Interestingly, we observed a low frequency of nucleotide substitutions using pUbil-Cas9/paired-gRNAs in both genes. Blunt end cleavage site generated using single gRNA is commonly repaired by error-prone NHEJ mechanism, but precise end-joining repairs have been reported in human cells through simultaneous DNA DSBs by Cas9/paired gRNAs, suggesting a direct ligation without end processing (Zheng et al., 2014). Moreover, previous studies in mammalian cells reported that the accuracy of the NHEJ repair mechanism is reduced when the distance between two collinear DSBs is beyond 1 kb (Boubakour-Azzouz and Ricchetti, 2008; Guirouilh-Barbat et al., 2016; Guo et al., 2018). Simultaneous DSBs by Cas9/paired-gRNAs at short distance may improve the specificity of the repair mechanism; however, the current understanding of this precise mechanism remains to be determined. Hence, the dynamic interaction between Cas9 cleavage and the endogenous DNA repair mechanism needs further study to enhance the efficiency and specificity of CRISPR/Cas9 system applications in plants.

Furthermore, we adapted the paired-gRNA using multi-cassette (monocistronic) transformation to increase the mutation frequency and chromosomal fragment deletion in the targeted gene. The assayed pUbil-Cas9/paired-gRNAs were mutagenic and active in inducing InDels in two target genes; however, no large chromosomal fragment deletion was detected. Chromosomal fragment deletion has been reported in kiwifruit with 755 and 271 bp deletions using polycistronic tRNA-gRNA (PTG)/Cas9 system, which is 10-fold higher than conventional CRISPR/Cas9 expression cassette (Wang et al., 2018). The PTG/Cas9 system enables a simultaneous expression of multiple gRNAs up to 31 times higher than monocistronic cassette and improves mutagenesis of multiple chromosomal fragment deletions in rice protoplast (Xie et al., 2015). Moreover, large chromosomal segments of more than 100 kb deletions between two genomic loci were achieved in rice protoplasts using the multiplex gRNA expression system (Zhou et al., 2014). Chromosomal fragment deletion is a potential strategy for chromosomal engineering to remove undesired loci with detrimental traits in crops and could be achieved by multiplex gRNA expression or PTG/Cas9 systems using gRNAs with similar efficiency at both target sites.

Our study demonstrated that the CRISPR/Cas9 genome editing system is effective and efficient in editing oil palm genes. In this study, the established *in vitro* electro-transfection assay provides a rapid assessment and evaluation of gRNA efficiency in oil palm protoplast to reduce the time and cost for transformation and regeneration in oil palm using inefficient gRNAs. An efficient transformation system in oil palm or methods to generate non-transgenic gene-edited mutants are highly desired to enhance the occurrence of targeted mutagenesis with the CRISPR/Cas9 system. This enabling platform for genome editing may accelerate the exploration of gene function for trait improvement in oil palm, in particular, the agronomic traits that address challenges in oil palm cultivation including basal stem rot or *Ganoderma* disease, abiotic stress tolerance to reduce losses due to climate change, and ease of harvesting traits such as dwarf, long stalk,

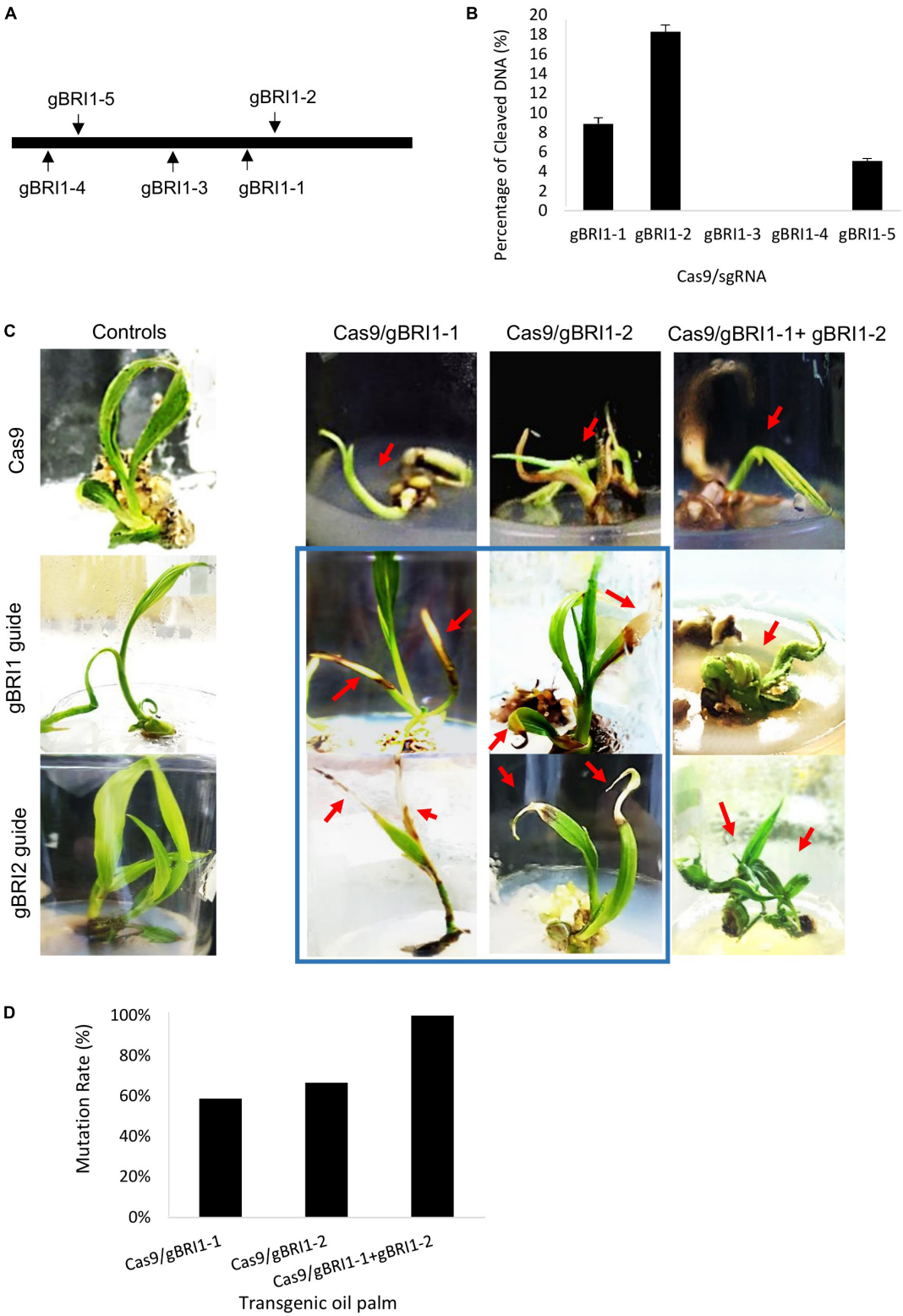


FIGURE 4 | Clustered regularly interspaced short palindromic repeat/Cas9-targeted mutagenesis of *brassinosteroid-insensitive 1(EgBRI1)* gene in *in vitro* oil palm plantlets. **(A)** Schema of gRNA targeting intronless *EgBRI1* gene in oil palm. **(B)** Cleavage efficiency of Cas9/gRNA targeting *EgBRI1* gene in oil palm protoplast. Data are average values ± SE from four independent experiments. **(C)** CRISPR/Cas9-induced *EgBRI1* mutations at different gRNA target sites showing stunted growth and leaf apex necrosis phenotypes (boxed in blue) in transgenic oil palm shootlets and controls transformed with either Cas9 or gRNA alone. **(D)** Mutation frequencies of CRISPR/Cas9 mutagenesis based on the occurrence of stunted or necrotic leaf apex phenotypes in transgenic oil palm.

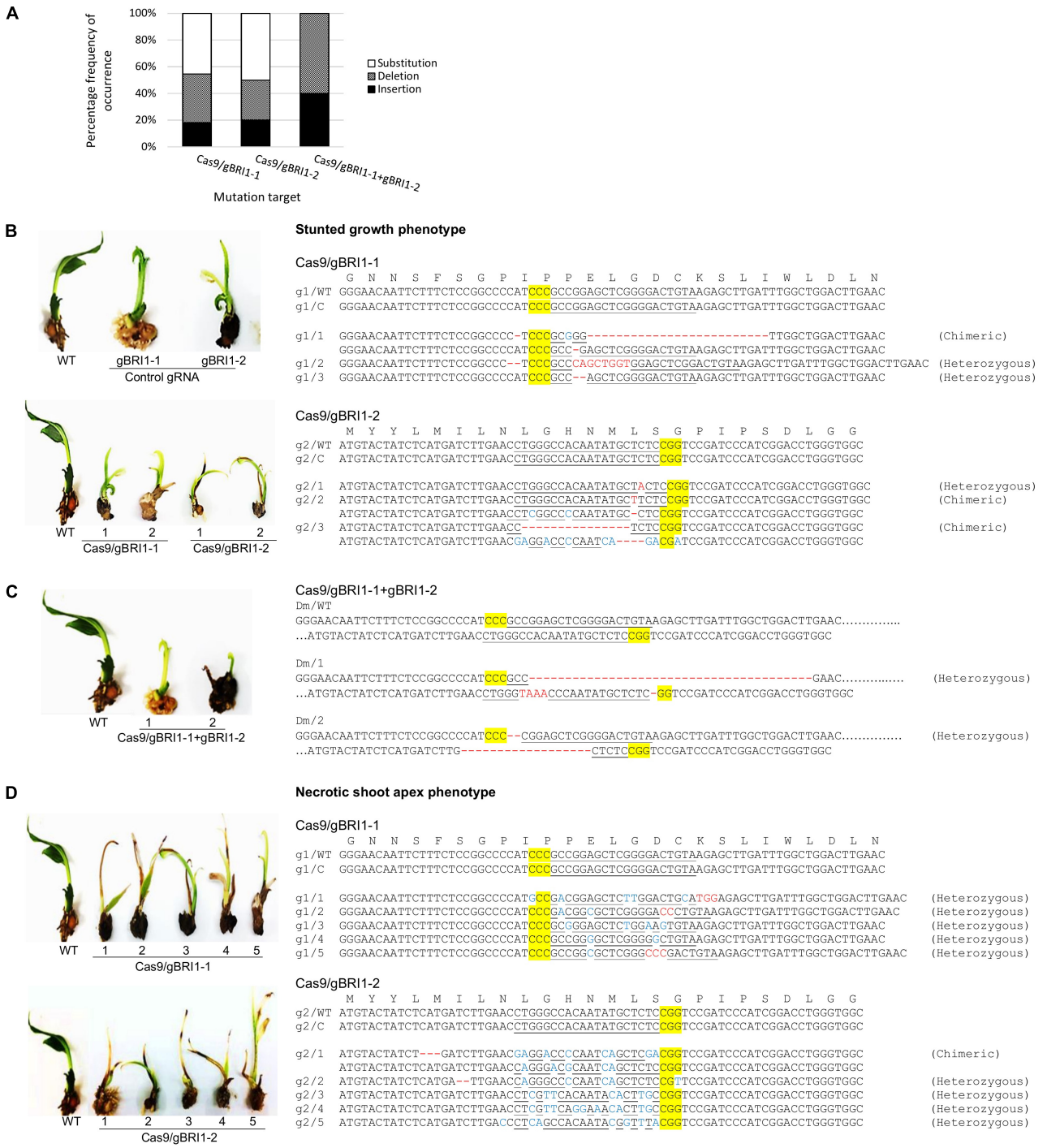


FIGURE 5 | Clustered regularly interspaced short palindromic repeat/Cas9-mediated mutation events and phenotypes of *EgBRI1* mutant transgenic oil palm. **(A)** The overall percentage frequency of occurrence for each type of mutation event detected at gBRI1-1 and gBRI1-2 targeted sites. The mutation events detected in the transgenic oil palms exhibiting **(B)** stunted growth phenotype induced by CRISPR/Cas9 and gBRI1-1, gBRI1-2, or **(C)** dual gRNAs mutation induction in the *EgBRI1* gene, and **(D)** necrotic shoot apex phenotype. PAM is highlighted in yellow, InDels sequences of the target site are shown in red, and nucleotide substitution is marked in blue. g1, gBRI1-1; g2, gBRI1-2; WT, wild-type; C, control; Dm, double mutant; 1–5, plantlet number.

and virescens. In addition, our study offers new insights to other related palm species including date palm and coconut that shared similar limitations in the DNA delivery method, low efficacy of genetic transformation, and inefficient tissue culture propagation and regeneration processes. The availability of full genome sequence opened a new opportunity for genetic improvement

in date palm and coconut through the CRISPR/Cas9 genome editing approach. This approach will be helpful to develop date palm and coconut resistance against biotic and abiotic stresses.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

W-CY, DRA, and HK conceived the experiments. W-CY constructed the vectors, performed oil palm protoplast experiments, analyzed the data, and wrote the manuscript. NCMK, NJ, and MRM conducted the oil palm transformation and tissue culture and regenerated transgenic oil palm. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.773656/full#supplementary-material

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The CRISPR/Cas9-Mediated Modulation of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* in Alfalfa Leads to Distinct Phenotypic Outcomes

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Alfalfa (*Medicago sativa* L.) is the most widely grown perennial leguminous forage and is an essential component of the livestock industry. Previously, the RNAi-mediated down-regulation of alfalfa *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* (*MsSPL8*) was found to lead to increased branching, regrowth and biomass, as well as enhanced drought tolerance. In this study, we aimed to further characterize the function of *MsSPL8* in alfalfa using CRISPR/Cas9-induced mutations in this gene. We successfully generated alfalfa genotypes with small insertions/deletions (indels) at the target site in up to three of four *MsSPL8* alleles in the first generation. The efficiency of editing appeared to be tightly linked to the particular gRNA used. The resulting genotypes displayed consistent morphological alterations, even with the presence of up to two wild-type *MsSPL8* alleles, including reduced leaf size and early flowering. Other phenotypic effects appeared to be dependent upon mutational dosage, with those plants with the highest number of mutated *MsSPL8* alleles also exhibiting significant decreases in internode length, plant height, shoot and root biomass, and root length. Furthermore, *MsSPL8* mutants displayed improvements in their ability to withstand water-deficit compared to empty vector control genotypes. Taken together, our findings suggest that allelic mutational dosage can elicit phenotypic gradients in alfalfa, and discrepancies may exist in terms of *MsSPL8* function between alfalfa genotypes, growth conditions, or specific alleles. In addition, our results provide the foundation for further research exploring drought tolerance mechanisms in a forage crop.

Keywords: crop improvement, drought tolerance, forage, genome editing, *Medicago sativa*, precision breeding

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INTRODUCTION

The livestock industry depends critically on our ability to grow forage crops in a highly productive manner. Of the perennial leguminous forages, alfalfa (*Medicago sativa* L.) is the most widely grown, with an estimated global cropping area of approximately 30 million hectares (Singer et al., 2018a,b). The prominence of this crop derives from its many beneficial characteristics, including

its perennial nature, relatively high yield, quality and palatability (Radović et al., 2009). Alfalfa's symbiotic relationship with *Rhizobium* spp., which allows for nitrogen fixation, also enhances soil nutrition and reduces fertilization needs. Since the global demand for livestock-derived products is predicted to escalate in line with population growth and rising affluence, and the land base for forage production is decreasing (Kingston-Smith et al., 2013), there is a vital need for the provision of new alfalfa cultivars with superior productivity in the face of future climate change scenarios in a relatively short timeframe (Singer et al., 2018a). While conventional breeding approaches have been used to enhance various traits in alfalfa (Smith et al., 2000; Acharya and Steppuhn, 2012; Acharya, 2013), it can be challenging in this species due to its typically allogamous reproductive behavior, high levels of genetic variation and environmental interactions. Therefore, the time required for cultivar development using these approaches can be prohibitive (Annicchiarico et al., 2015) and thus achieving expeditious improvements in certain traits will undoubtedly necessitate the use of modern biotechnological approaches as a complementary platform in order to meet future demand.

SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) proteins are a family of plant-specific transcription factors that possess a highly conserved zinc-containing DNA binding region termed the SBP-domain (Yamasaki et al., 2004; Birkenbihl et al., 2005) and function in a partially overlapping manner in the regulation of an exceptionally diverse set of processes such as vegetative growth, response to abiotic stress and yield (Yamasaki et al., 2009; Xing et al., 2013; Xu et al., 2016; Gao et al., 2018a; Hu et al., 2021). Many *SPL* genes are targeted by microRNA156 (miR156), which is an abundant and highly conserved miRNA in plants that regulates the expression of its target genes through transcript cleavage and translational inhibition (Gandikota et al., 2007; Gao et al., 2016). While this group of transcription factors tends to be well-conserved across monocot and eudicot plant species (Wang et al., 2009), family members may have distinct functions between species in certain cases (Preston et al., 2016; Gou et al., 2019; Cao et al., 2021).

In alfalfa, a total of sixteen *SPL* genes have been identified to date, ten of which are directly silenced by miR156 (Gao et al., 2016; Feyissa et al., 2021). Intriguingly, while *SPL8* does not appear to be a target of miR156 in *Arabidopsis thaliana* (Xing et al., 2010), it is cleaved by miR156 in alfalfa (Feyissa et al., 2021). This, along with the fact that there is considerable functional disparity among *SPL8* homologs in different plant species (Unte et al., 2003; Zhang et al., 2007; Xing et al., 2013; Gou et al., 2018, 2019), suggests that it may have evolved in a distinct manner at some point following the divergence of particular lineages. In alfalfa, plants with RNAi-mediated down-regulation of *MsSPL8* have been found to exhibit many similarities to miR156 over-expression genotypes, including increased forage biomass production through enhanced branching and accelerated regrowth, as well as superior feed value and tolerance to drought and salinity (Gou et al., 2018). Unfortunately, despite the potential of such genotypes for economic benefit, their implementation will almost certainly be hindered by their transgenic nature. Since crops developed using RNAi technology are considered to be "genetically modified" ("GM"), they would raise public concern and be subject to prohibitively costly and lengthy regulatory processes (Singer et al., 2021a).

Genome editing technology based on CRISPR/Cas (Jinek et al., 2012) is a relatively new breeding platform that in its simplest form involves the endogenous non-homologous end-joining (NHEJ)-mediated repair pathway, which typically leads to the directed production of a small insertion or deletion (indel) at the selected target site. This platform requires a Cas nuclease, which produces a double-stranded DNA break, and a single guide RNA (sgRNA), which includes a short user-defined sequence that guides the Cas nuclease to a highly specific chromosomal locus of choice immediately upstream of a protospacer-adjacent motif (PAM). Due to the unlinked nature of the resulting edit and the introduced transgene, this technology allows for the rapid production of non-transgenic germplasm bearing mutations that are identical in nature to those achieved spontaneously or through conventional breeding approaches such as chemical mutagenesis (Subedi et al., 2020a,b). While the regulatory status of crop varieties derived from genome editing is still uncertain in some countries, many others, including the United States, have concluded that in the absence of foreign DNA they are not "GM," and will therefore not be subjected to costly and burdensome regulatory processes (Schmidt et al., 2020; Singer et al., 2021a).

CRISPR/Cas has proven to be very effective in a range of polyploid plant species (Morineau et al., 2017; Liu et al., 2018; Huang et al., 2020), including alfalfa (Chen et al., 2020; Wolabu et al., 2020), due to its ability to simultaneously mutate multiple alleles of a target gene in a single generation. As such, our aim was to assess the effectiveness of CRISPR/Cas9 technology in alfalfa in terms of eliciting phenotypic alterations along a scale of mutational dosages by targeting the *MsSPL8* gene, and also to gain a further understanding of the functionality of this gene in this species. In addition, the successful exploitation of this technology in this context could potentially allow for the downstream generation of transgene-free germplasm exhibiting improvements in traits such as adaptability to water-deficit, which would provide a novel source of germplasm for alfalfa breeding endeavors in the future.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The highly regenerable N4.4.2 alfalfa genotype (Badhan et al., 2014) was utilized for transformation experiments throughout this study, while the cultivar AC Blue J (provided by Dr. Surya Acharya, AAFC Lethbridge) was used for crossing. Plants were grown in square pots (10.5 cm across and 12.5 cm in height) filled with Cornell soilless potting mix (Boodley and Sheldrake, 1972) in a greenhouse with supplemental lighting providing a 16 h/8 h photoperiod, a temperature of approximately 20°C/15°C day/night, and a light intensity of approximately 150 $\mu mol/m^2/s$. Unless otherwise specified, plants were watered daily (without fertilizer) from the bottom for 15 min, after which time any excess water was drained off. Due to alfalfa's obligatory outcrossing nature, the N4.4.2 background genotype, as well as all transgenic

plants generated in this study, were propagated vegetatively through rooted stem cuttings to preserve particular genotypes throughout the experiment and generate "pools" of individual genotypes for downstream investigations. In every case, rooted cuttings were transferred to pots at the same time point, and were re-grown after cutting back to approximately 5 cm at least twice prior to assessments. During all trials, pots were rotated randomly every other day to minimize variations due to microclimate effects.

Identification of *MsSPL8* Homologs and Expression Analysis

BLAST searches were performed against the *M. sativa* cultivated alfalfa at the diploid level (CADL; v0.95 and v1.0) genome database[1,2], the Alfalfa Gene Index and Expression Atlas database[3] and the tetraploid alfalfa genome sequence (Chen et al., 2020; NCBI database project PRJNA540215) using the *Medicago truncatula* Medtr8g005960 sequence, which encodes the *SPL8* homolog in this species (Gou et al., 2018). Nucleotide and amino acid alignments were carried out on identified homologs using Geneious Prime software (Biomatters Inc., San Diego, CA, United States), while domain searches were conducted using SMART[4]. Expression of alfalfa *MsSPL8* homologs was screened using the gene expression profiling tool in LegumeIP V3 (Dai et al., 2021) for *M. sativa* (CADL; v1.0) in different tissues.

Generation of Binary CRISPR Vectors

Three separate guide RNAs (gRNAs; 20-nt) were designed immediately upstream of 5′—NGG—3′ PAM sequences (Doench et al., 2014) within a conserved region of the first exon of the four highly similar *MsSPL8* alleles (chr 8.2 83866861:83868757, chr 8.3 81917086:81918958; chr 8.4 80839466:80841286 and 80871132:80872948; **Figure 1A**). All three gRNAs exhibited 100% complementarity with all four *MsSPL8* alleles (as did all primers and probes utilized in this study). The specificity and/or potential for off-target effects of each gRNA was evaluated using the Cas-OFFinder algorithm (Bae et al., 2014). Each gRNA was inserted into the binary pKSE401 vector (Xing et al., 2014; Addgene plasmid #62202). This background vector includes a cassette in which the expression of the sgRNA is driven by the Arabidopsis U626 polymerase III promoter and another in which the expression of a *Zea mays* codon-optimized *Cas9* coding region (derived from *Streptococcus pyogenes* and flanked on either side by nuclear localization signals) is driven by a constitutive partially duplicated CaMV 35S promoter. This vector is designed to elicit NHEJ-mediated edits within the target gene.

In each case, 20-nt oligonucleotides corresponding to each gRNA in both orientations were designed and then synthesized by a third-party [Integrated DNA Technologies (IDT) Inc., Coralville, IA, United States]. Each oligonucleotide possessed 4-nt additions engineered at their 5′ termini to facilitate cloning into a *Bsa*I site within pKSE401 (5′—ATTG—3′ for forward oligonucleotides and 5′—AAAC—3′ for reverse oligonucleotides; Xing et al., 2014). Equimolar ratios of each oligonucleotide pair were then annealed by incubating at 65°C for 5 min, followed by cooling at room temperature. Each double-stranded gRNA was then ligated into pKSE401 that had been linearized with *Bsa*I (**Figure 1B**). All three of the resulting vectors were subjected to sequencing to confirm their identities.

Alfalfa Transformation

The three CRISPR/Cas9 binary vectors targeting three distinct regions of *MsSPL8* alleles, as well as the empty vector (pKSE401), were introduced into *Agrobacterium tumefaciens* LBA4404 using electroporation and transferred separately into the alfalfa N4.4.2 genotype through *Agrobacterium*-mediated transformation of leaf explants as described previously (Badhan et al., 2014). Selection was carried out using 50 mg/L kanamycin for 10 days followed by 75 mg/L kanamycin for the remainder of tissue culture. Kanamycin-resistant genotypes were transferred to water in 15 mL polypropylene tubes for 3–4 days for acclimation, and subsequently transplanted to pots under greenhouse conditions with supplemental light. To confirm the presence of the transgenic cassette in kanamycin-resistant genotypes, genomic DNA was extracted from leaf tissues using the BioSprint 96 DNA Plant Kit (Qiagen Inc., Toronto, ON, United States) and the presence of each transgene was verified by PCR using Invitrogen Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) and primers Cas9F1 and Cas9R1 (see **Supplementary Table 1** for primer sequences; 621-bp amplicon). These primers anneal to the *Cas9* coding region within the pKSE401 transgenic cassette, and allow the differentiation of genotypes based on the presence/absence of the transgene. Thermal parameters included an initial denaturation step of 98°C for 30 s, 35 cycles of 98°C for 10 s, 57°C for 10 s, and 72°C for 30 s, and a final extension step of 72°C for 1 min. Positive control PCRs were conducted using GoTaq Green Master Mix (Promega Corp., Madison, WI, United States) and primers WD40F2 and WD40R2 (see **Supplementary Table 1** for primer sequences; 865-bp amplicon) to ensure DNA quality. Thermal parameters included an initial denaturation step of 95°C for 2 min, 35 cycles of 95°C for 30 s, 51°C for 30 s and 72°C for 1 min, and a final extension of 72°C for 5 min. Primary transformants were labeled SPL8-gRNA1, SPL8-gRNA2 or SPL8-gRNA3 according to the gRNA used, while genotypes bearing the pKSE401 empty vector were termed EV.

Droplet Digital PCR

Gene editing frequencies (GEF) were assessed using GEF-droplet digital PCR (GEF-ddPCR; Mock et al., 2016), which used two distinct probes that anneal within a single amplicon to quantify wild-type and NHEJ-affected alleles. Primers were designed using the PrimerQuest Tool (IDT). Primers MsSPL8Fwd2 and MsSPL8Rev2 were used to amplify a 375-bp region surrounding the potential gRNA1 and gRNA2 editing sites, while primers MsSPL8Fwd3 and MsSPL8Rev3 were used to amplify a 122-bp region surrounding the potential gRNA3 editing site (see **Supplementary Table 1** for primer sequences). Locked nucleic acid (LNA) probes (Tm = 66°C, 5′ hexachloro-fluorescein [HEX]

[1] www.alfalfatoolbox.org

[2] http://plantgrn.noble.org/LegumeIP

[3] http://plantgrn.noble.org/AGED/

[4] http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1

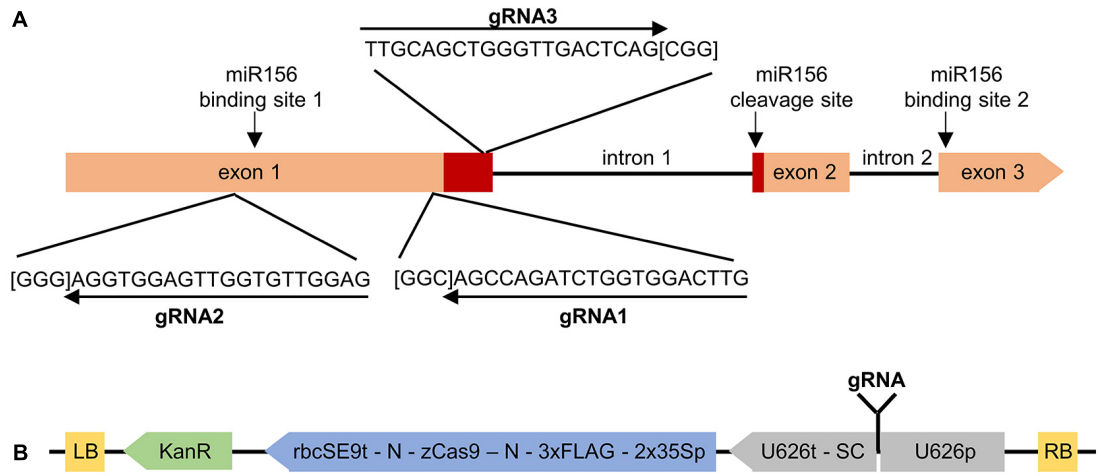


FIGURE 1 | Targeting *MsSPL8* alleles for NHEJ-based editing using CRISPR/Cas9. **(A)** Genomic structure of *MsSPL8* alleles and gRNA target regions. The region encoding the SBP domain is displayed in red and PAM sequences are indicated in square brackets. miR156 binding and cleavage sites are indicated with arrows. **(B)** Three gRNAs targeting different regions of *MsSPL8* alleles were inserted into the pKSE401 background vector to yield SPL8-gRNA1, SPL8-gRNA2 and SPL8-gRNA3 binary vectors. 2x35Sp, partially duplicated CaMV 35S promoter; 3xFLAG, polypeptide tag; KanR, kanamycin resistance cassette; LB, left border; N, nuclear localization signal; RB, right border; rbcSE9t, pea RuBisCO small subunit E9 terminator; SC, sgRNA scaffold; U626p; Arabidopsis U6 polymerase III promoter; U626t; U6 terminator; zCas9, *Zea mays* codon-optimized Cas9.

labeled) were designed to bind to predicted Cas9 cut sites in each of the three gRNA target regions as a means of detecting indels. Control probes (Tm = 68°C, 5′ fluorescein amidite [FAM] labeled) were also designed to bind to a region within each amplicon that would not be impacted by gene editing (see **Supplementary Table 2** for probe sequences). All probes bore a 3′ Iowa Black fluorescent quencher. Mini-genes representing the gRNA3 target region with a single nucleotide insertion (123-bp in length) or deletion (121-bp in length) were synthesized (IDT) and utilized as positive controls to validate the sensitivity and specificity of the method in alfalfa.

Genomic DNA from leaves derived from a single shoot of all confirmed independent T_0 transgenic genotypes, as well as five independent transgenic EV genotypes, was used as template for the initial screening of all three gRNA target sites. Subsequently, genomic DNA from leaves derived from eight separate and randomly chosen primary branches of three independent T_0 SPL8-gRNA1 genotypes (SPL8-gRNA1-1, -2 and -3), as well as an EV control, respectively, was assayed as a means of examining the possibility of mosaicism in genotypes bearing a mutation at the gRNA1 cleavage site.

Assays were performed using ddPCR Supermix for Probes (no dUTP; Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) following the manufacturer's instructions. Briefly, 20 μL reactions containing 900 nM of each primer, 250 nM of the appropriate probe and 40 ng of genomic DNA were utilized for assays, and droplets were generated using the Automated Droplet Generator (Bio-Rad). Thermal cycling was performed using a C1000 Touch Thermal Cycler with 96-deep well reaction module (Bio-Rad) with parameters of 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 1 min, followed by enzyme deactivation at 98°C for 10 min. Droplets were then analyzed with the QX200 Droplet Reader (Bio-Rad). Analysis thresholds were established for each assay and the ratio of NHEJ-sensitive vs. NHEJ-insensitive droplets, which is based on the concentrations of events per μL, was calculated using the QuantaSoft Analysis Pro software V 1.0.596 (Bio-Rad). To quantify the relative number of edited *MsSPL8* alleles compared to total alleles within each sample, GEF was calculated as the percentage of edited droplets. In the case of T_0 SPL8-gRNA1 genotypes, all subsequent experiments were carried out using plants derived from vegetative stem cuttings of the shoot exhibiting the highest level of GEF in the original T_0 genotype.

T7E1 Assay

To provide further confirmation of editing in SPL8-gRNA1 genotypes, T7E1 mismatch cleavage assays were also carried out using genomic DNA isolated from SPL8-gRNA1-1, -2 and -3 genotypes (with GEF of approximately 75, 50, and 25%, respectively), as well as three independent EV controls, as template. Primers MsSPL8F2 and MsSPL8Rev2 were designed to amplify a 255-bp region of *MsSPL8* encompassing the predicted editing location for gRNA1 (see **Supplementary Table 1** for primer sequences). In the presence of an indel at the predicted cut site, and hence cleavage of the heteroduplex by the T7E1 enzyme, fragments of 156-bp and 99-bp in length would be generated. PCR amplification was carried out using Invitrogen Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific) and a thermal program of 98°C for 30 s, 35 cycles of 98°C for 10 s, 55°C for 10 s and 72°C for 30 s, and a final extension at 72° for 5 min. Heteroduplexes of each PCR amplicon were formed by denaturing for 10 min at 95°C, cooling to 85°C at a ramp rate of 2°C/s, and then cooling to 25°C at a ramp rate of 0.3°C/s before digestion with 10 U of T7EI (New England Biolabs, Whitby, ON,

Canada) at 37°C for 15 min. Digested products were separated and visualized on a 2% agarose gel.

Assessment of On- and Off-Target Editing Events in SPL8-gRNA1 Genotypes

T_0 transgenic SPL8-gRNA1-1, -2 and -3 genotypes (exhibiting GEF of approximately 75, 50, and 25%, respectively) were examined to confirm on-target mutations, and assessed for possible off-target mutations, using Sanger sequencing. To determine precise editing events at the gRNA1 target site, a 1.4-kb region of *MsSPL8* was amplified from genomic DNA derived from each genotype (including EV controls) using Invitrogen Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific) and primers MsSPL8F1 and MsSPL8R2 (see **Supplementary Table 1** for primer sequences). The amplicons were subsequently cloned into the pGEM-T-Easy vector (Promega), and clones from 21–33 colonies were subjected to Sanger sequencing (Génome Québec Centre D'Expertise et de Service, Montreal, QC, Canada).

In addition, the three most likely potential off-target sites were also chosen for evaluation, possessing two single nucleotide mismatches and a 2-nt RNA bulge (two sites), or three single nucleotide mismatches and a 1-nt RNA bulge (one site; **Supplementary Figure 1**) based on findings obtained using the Cas-Offinder program. In each case, an approximately 700-bp region spanning the potential off-target site was amplified from the same three transgenic SPL8-gRNA1 genotypes, as well as untransformed N4.4.2 and an EV genotype using primers MsOffTarget1FWD1 and MsOffTtarget1REV1, MsOffTtarget2FWD1 and MsOfTftarget2REV1, and MsOffTarget3FWD1 and MsOffTarget3REV1, respectively (see **Supplementary Table 1** for primer sequences). The resulting amplicons were then cloned as described above and clones from 8–12 colonies from each genotype and for each possible off-target site were sequenced to assess for mutations.

Morphological Evaluations

For evaluation of plant height, branch number and internode length, two separate trials were carried out 30 days (pre-flowering) and 60 days (post-flowering) following the second cut. For the trial carried out 30 days following cutting, five biological replicates of well-watered SPL8-gRNA1-1 (∼75% GEF) and SPL8-gRNA1-2 (∼50% GEF), respectively, as well as five biological replicates for each of three independent EV control genotypes (15 biological replicates total), were analyzed in each case. For the trial carried out 60 days following cutting, ten biological replicates of well-watered SPL8-gRNA1-1 and -2 genotypes, respectively, as well as ten biological replicates for each of three independent EV control genotypes (30 biological replicates total), were assessed. Branch number was determined by counting the total number of branches per plant, while the longest shoot of each plant was utilized to measure plant height and the length of the longest internode.

The onset of flowering was recorded when petals first began to emerge from flower buds following the second cut. Subsequent to flowering (60 days following cutting), all aboveground biomass was removed and weighed to determine fresh weight (FW), and was then dried at 65°C for 1 week and weighed to determine dry weight (DW). Root length was evaluated by measuring the longest primary root, and root DW was resolved following drying at 65°C for 1 week.

Leaf length, width and area were measured using the middle leaflet of the third fully expanded trifoliate leaf from the shoot tip. Measurements were made 30 days following the second cut using five biological replicates (derived from vegetative stem cuttings) of each SPL8-gRNA1 genotype, and five biological replicates from each of three independent EV transformants, with three leaves assessed per plant. Leaf area was determined using the Petiole plant leaf area meter app (version 2.0.1[5]) and leaf dry weight was established after drying at 80°C for 24 h. Specific leaf area (SLA) was calculated by dividing the leaf area by dry weight in each case.

Drought Treatment

Prior to the commencement of drought treatment, a volumetric soil moisture content of 50% was established in each pot using a ML3 ThetaKit soil moisture meter with 6 cm probes (Hoskin Scientific Ltd., Burnaby, BC, Canada), after which time water was withheld and volumetric soil water contents were measured daily until they reached approximately 1% (approximately 2–3 weeks). Assessments of water deficit symptoms were carried out on the basis of soil moisture contents rather than days following the withholding of water to minimize possible effects due to possible variation in plant water uptake. Subsequently, plants were re-watered normally for approximately 2 weeks and survival was assessed. Furthermore, volumetric soil moisture content was noted at the first instance of stress symptoms, which initially included the appearance of wilted or drooping leaves and shoots, in both edited and EV genotypes. Well-watered plants were utilized as controls. Six biological replicates of SPL8-gRNA1-1 (∼75% GEF) and SPL8-gRNA1-2 (∼50% GEF) genotypes, as well as three independent EV control genotypes (18 biological replicates total), were utilized in each experimental set. The withholding of water was initiated 14 days following the second cut.

Measurement of Relative Water Content

Plant water status was estimated by measuring the relative water contents (RWC) of third fully expanded trifoliate leaves from six biological replicates, with two leaves assessed per replicate plant, of SPL8-gRNA1-1 and -2 genotypes, respectively, as well as 18 biological replicates of EV controls (six biological replicates from three independent transformants). Measurements were carried out as described previously (Barrs and Weatherley, 1962) when soil moisture contents reached approximately 50% (well-watered), 12–16% (moderate drought) and 7% (severe drought). In brief, the fresh weight (FW) of trifoliate leaves was documented immediately following detachment, turgid weights (TW) were determined after submersing petioles in water in an enclosed 1.5 mL microcentrifuge tube for 4 h, and dry

[5]https://play.google.com/store/apps/details?id=com.petioleapp.petiole&hl=en_CA&gl=US

weights (DW) were resolved by drying turgid leaves at 80°C overnight. Relative water content (%) was then calculated as [(FW-DW)/(TW-DW)] × 100.

Detached Leaf Water Loss Assays

Detached leaf water loss assays were carried out essentially as described previously (Singer et al., 2021b) using fully expanded trifoliate leaves (third from the shoot tip) from eight biological replicate plants of each SPL8-gRNA1 genotype (two leaves per plant; sixteen replicates total for each) and seventeen biological replicate control plants (derived from three independent transformants with two leaves sampled per plant; thirty-four replicates total) under well-watered conditions. Assays were carried out by weighing leaves immediately upon harvest ($W_{initial}$), placing them on a benchtop, and then weighing every 30 min for 240 min. The rate of water loss was calculated as $(W_{initial} - W_{atparticulartime}/W_{initial}) \times 100$.

Assessment of Photosynthetic Parameters

Light-saturated photosynthetic rate (Asat), stomatal conductance (g_s), and transpiration rate (E) were evaluated using a LI-6800 (LI-COR Inc., Lincoln, NE, United States). The middle leaflet of a third fully expanded trifoliate leaf was used for measurements on four biological replicate plants of each SPL8-gRNA1 genotype, and four biological replicate plants of each of three EV control genotypes (twelve replicates total). All assessments were conducted between 10:30 am and 12:00 pm under greenhouse conditions. Measurements were made on one set of plants grown under well-watered conditions (soil moisture content of approximately 50%) and another subjected to drought treatment (average soil moisture content of approximately 11%). Within the chamber, light intensity was held at 1,500 μmol m^{-2} s^{-1}, relative humidity at 50%, CO_2 level at 410 μmol CO_2/mol air, and heat exchanger temperature was set to 25°C. Leaflets were allowed to stabilize for 1.5 min within the chamber prior to measurement. All measurements were adjusted for leaf area, which was determined using the Petiole Pro plant leaf area meter app (version 1.4.15[6]).

Crossing of Alfalfa Genotypes and Molecular Assessment of F_1 Genotypes

Due to the outcrossing nature of alfalfa, we carried out manual reciprocal crosses between SPL8-gRNA1-1 (∼75% GEF) and the cultivar AC Blue J, as well as between SPL8-gRNA1-2 (∼50% GEF) and AC Blue J, using non-emasculated flowers. In each case, four distinct genotypes of AC Blue J were used along with eight biological replicates of each mutant genotype (derived from vegetative stem cuttings). A total of 32–64 seeds from each cross were sown and grown in the greenhouse as described in a previous section. Genomic DNA was harvested from a single leaf of each resulting F_1 plant and PCR was conducted as described for T_0 plants to screen for the presence of the transgene. T7E1 and ddPCR assays were carried out to assess editing frequencies at the gRNA1 target site using genomic DNA derived from F_1 genotypes lacking a transgene as described previously for T_0 plants.

[6]https://petioleapp.com

Statistical Analyses

Statistical differences between the means of SPL8-gRNA1 genotypes and EV controls were determined using student's *t*-tests assuming unequal variance. Means were considered significantly different at $P \leq 0.05$.

RESULTS

Selection of *MsSPL8*-Specific gRNA Sequences

As a means of eliciting mutations in alfalfa *MsSPL8*, three gRNAs were designed to simultaneously target four *MsSPL8* alleles using CRISPR/Cas9 technology. These four alleles corresponded to MSAD_231217 and MSAD_231229 in the *M. sativa* genome sequence at the diploid level (CADL), or chr 8.2 83866861:83868757, chr 8.3 81917086:81918958; chr 8.4 80839466:80841286 and 80871132:80872948 from a tetraploid reference genome sequence (Chen et al., 2020). These four alleles exhibited 98.0% identity within their coding regions at the nucleotide level and 98.2% identity at the amino acid level. Using the freely available LegumeIP database and the *M. sativa* CADL genome (v1.0), the expression profiles of MSAD_231217 and MSAD_231229 in a selection of tissue types indicated that both transcripts were highly expressed in flowers, and to a lesser extent in elongated stems, while only low levels of expression were observed in roots, nodules and leaves (**Supplementary Figure 2**). The coding regions of the *MsSPL8* alleles possess three exons and two introns, and the predicted proteins incorporate a single SBP domain that is identical among *MsSPL8* alleles and also to that of *M. truncatula* Medtr8g005960. All three gRNAs were designed to target regions of the coding sequence within exon 1, and exhibited 100% complementarity to all four *MsSPL8* alleles. While gRNA1 and gRNA2 targeted regions upstream of the SBP domain, gRNA3 targeted a region within this domain (**Figure 1A**).

Gene Editing Frequencies in SPL8-gRNA Alfalfa Genotypes at Population and Plant Levels

In order to target *MsSPL8* alleles using CRISPR/Cas9 in alfalfa, three pKSE401-based binary vectors bearing distinct gRNAs targeting all four *MsSPL8* alleles (SPL8-gRNA1, SPL8-gRNA2 and SPL8-gRNA3; **Figure 1B**) were generated. Each vector, along with an EV control consisting of pKSE401 without any gRNA sequence, was introduced into alfalfa, and PCR assessment of kanamycin-resistant genotypes provided confirmation for 26, 12, 12 and 17 independent SPL8-gRNA1, SPL8-gRNA2, SPL8-gRNA3 and EV transgenic genotypes, respectively.

In order to quantitatively assess the frequency of editing in our transgenic genotypes, GEF-ddPCR was carried out using genomic DNA from each of the confirmed independent transgenic SPL8-gRNA genotypes, as well as five independent

EV negative control genotypes (**Figures 2A,B**). Of the transgenic genotypes assessed, 10 of 26 (38.5%) SPL8-gRNA1 genotypes, 5 of 12 (41.7%) SPL8-gRNA2 genotypes, and 2 of 12 (16.7%) SPL8-gRNA3 genotypes exhibited GEF $\geq$ 0.5%, which was chosen as the threshold indicating the presence of at least a small number of edits in a sample. None of the five EV controls exhibited a GEF above 0.2%. In the case of SPL8-gRNA1, SPL8-gRNA2 and SPL8-gRNA3 genotypes, GEF of up to 76.5, 9.5, and 1.3%, respectively, were observed (**Figure 2B**). Since gRNA1 appeared to exhibit superior editing efficiency compared to gRNA2 and gRNA3, we selected three independent T_0 transgenic SPL8-gRNA1 genotypes with relatively high GEF [labeled SPL8-gRNA1-1 (76.5% GEF), SPL8-gRNA1-2 (54.7% GEF), and SPL8-gRNA1-3 (26.1% GEF)] for subsequent experiments.

Since there are four *MsSPL8* alleles in alfalfa, a GEF of less than approximately 25% indicates probable mosaicism for editing events within a single plant. To evaluate whether edited genotypes exhibiting a GEF above 25% were also mosaics, genomic DNA was isolated from leaves derived from eight distinct branches of SPL8-gRNA1-1, SPL8-gRNA1-2 and SPL8-gRNA1-3 genotypes, respectively, and subjected to GEF-ddPCR. While SPL8-gRNA1-1 exhibited a rather wide range of GEF among branches (between 24.6% and 76.2%), SPL8-gRNA1-2 and -3 displayed much narrower ranges (between 50.4–57.5% and 25.7–29.2%, respectively) (**Figure 2C**). In an attempt to minimize variation in GEF in the SPL8-gRNA1-1 genotype, all subsequent experiments were carried out using plants derived from vegetative stem cuttings of the branch exhibiting a 76.2% GEF in the original T_0 transformant.

Validation of NHEJ-Derived Mutations and Specificity in SPL8-gRNA1 Alfalfa Genotypes

To provide further confirmation of NHEJ-mediated gene editing in *MsSPL8* alleles within SPL8-gRNA1 genotypes, we carried out T7E1 mismatch cleavage assays on genomic DNA isolated from SPL8-gRNA1-1, -2 and -3 plants, as well as three independent EV controls. Single bands of approximately 250-bp were observed in all EV controls, which indicates that no indels were present in any *MsSPL8* allele within the amplified region. Conversely, all three SPL8-gRNA1 genotypes assessed exhibited multiple bands, including uncut amplicon of approximately 250-bp, as well as fragments consistent with the presence of an indel at the gRNA1 target site (approximately 150-bp and 100-bp; **Figure 3A**), which confirms the presence of edits at the target site in these genotypes.

To identify the precise genetic changes that occurred at the gRNA1 target site in edited alfalfa genotypes, we subsequently cloned and sequenced a region encompassing the gRNA1 target site from SPL8-gRNA1-1 (76.4% GEF), SPL8-gRNA1-2 (57.5% GEF) and SPL8-gRNA1-3 (29.2% GEF) genotypes, as well as untransformed N4.4.2. Clones from between 21 and 33 colonies were sequenced in every case. No clone derived from N4.4.2 exhibited any genetic variation within the *MsSPL8* gRNA1 target region. In contrast, in the case of SPL8-gRNA1-1, we detected three distinct mutations 2- to 4-bp upstream of the gRNA1-associated PAM (a 6-bp deletion, a 9-bp deletion and a 1-bp deletion) along with wild-type sequence. In SPL8-gRNA1-2, we observed two distinct mutations at this same site (a 1-bp insertion and a 5-bp deletion) along with wild-type sequence. In SPL8-gRNA1-3, a single mutation at the predicted gRNA1 cut site (6-bp deletion) and wild-type sequence were observed (**Figure 3B**). While the 6-bp and 9-bp deletions would result in missing SR or RSP amino acid residues just upstream of the SBP domain, the 1-bp deletion, 1-bp insertion and 5-bp deletion would lead to a frameshift and the presence of premature stop codons. All of these stop codons are located either upstream of, or within, the SBP domain (**Figure 3C**).

To assess for possible off-target effects in the SPL8-gRNA1 alfalfa genotypes, we initially screened the *M. sativa* CADL v0.95 genome sequence with that of gRNA1 using the Cas-Offinder program. No matches were identified with a 1-nt mismatch and a 0-, 1- or 2-nt RNA bulge, or with two mismatches and 0- or 1-nt RNA bulge. However, possible off-target sites were identified with two single nucleotide mismatches and a 2-nt RNA bulge, and three single nucleotide mismatches and a 1-nt RNA bulge. To confirm that off-target mutations had not occurred in our three selected SPL8-gRNA1 genotypes, we cloned and sequenced three regions that spanned possible off-target sites for this gRNA (**Supplementary Figure 1**) from SPL8-gRNA1-1, -2 and -3, as well as untransformed N4.4.2 controls. No genetic variation was noted within the three possible off-target regions in any of the 8–12 clones sequenced for each genotype.

Due to a lack of a frameshift mutation in the SPL8-gRNA1–3 genotype, all further experiments focused on SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes.

SPL8-gRNA1 Genotypes Exhibit Morphological Phenotypes That Are Distinct From EV Genotypes

To determine whether SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes differed from EV plants in terms of their morphology, we assessed various aboveground and belowground characteristics under well-watered conditions. Although no changes in the number of branches were observed prior to flowering, both SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes displayed decreases in internode length (35.7% and 27.9% relative decreases, respectively), and SPL8-gRNA1-1 exhibited a significant reduction in plant height (26.1% relative decrease), compared to EV controls (**Figures 4A,B**). Similarly, post-flowering, significant reductions in plant height and internode length (**Figures 4A,C**), shoot fresh weight (FW) and dry weight (DW) (**Figure 4D**), and root length and DW (**Supplementary Figure 3**) were noted in SPL8-gRNA1-1, but not SPL8-gRNA1-2, compared to EV controls. As was the case pre-flowering, no significant differences were observed between either SPL8-gRNA1 genotype and EV controls with respect to branch number post-flowering (**Figure 4C**). Taken together, these results suggest that shoot and root growth phenotypes in SPL8-gRNA1 genotypes may be gene dosage-dependent, with the genotype exhibiting an approximately 75% GEF in *MsSPL8* alleles exhibiting more severe alterations than that with an approximately 50% GEF.

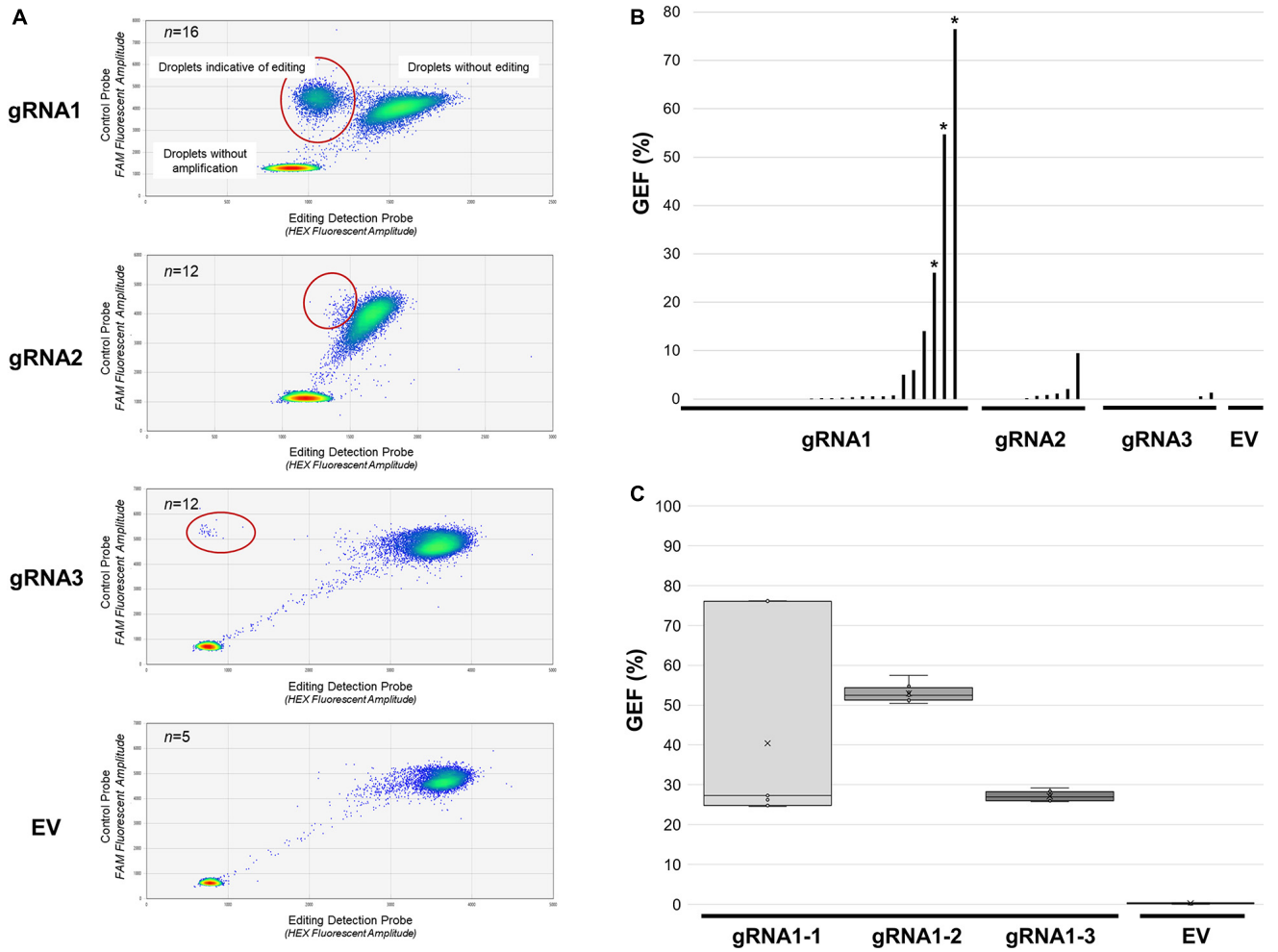


FIGURE 2 | Assessment of *MsSPL8* editing frequencies in transgenic plants using GEF-ddPCR. **(A)** FAM (control probe) and HEX (editing probe) fluorescence following GEF-ddPCR of SPL8-gRNA transgenic genotypes, as well as a selection of EV controls. Heat maps indicate droplets that were not amplified, as well as those that bound both probes (not edited) and those that only bound the control probe (potentially edited, circled in red). **(B)** GEF of each transgenic genotype assessed using GEF-ddPCR. Asterisks denote genotypes that were selected for screening of separate branches [shown in panel **(C)**]. **(C)** Evaluation of GEF in eight distinct branches from three independent edited SPL8-gRNA1 genotypes, as well as EV controls. Blocks represent the upper and lower quartiles of the values obtained, x denotes median values, and bars indicate maximum and minimum values. EV, empty vector controls, gRNA1, SPL8-gRNA1 genotypes, gRNA2, SPL8-gRNA2 genotypes, gRNA3, SPL8-gRNA3 genotypes.

In addition to growth phenotypes, both edited SPL8-gRNA1 genotypes exhibited significant reductions in the time required for flowering onset compared to EV controls, with SPL8-gRNA1-1 and -2 flowering on average 42.6 and 41.6 days after cutting and EV genotypes flowering on average 48.5 days after cutting (**Figures 4A,E**). Furthermore, leaf characteristics were consistently altered in both SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes compared to EV controls (**Figure 5A**). Specifically, SPL8-gRNA1-1 and SPL8-gRNA1-2 exhibited significant reductions in leaf width (27.1% and 25.9% relative decreases, respectively; **Figure 5B**), leaf length (6.5% and 8.8% relative decreases, respectively; **Figure 5C**) and leaf area (29.2% and 29.4% relative decreases, respectively; **Figure 5D**) compared to EV genotypes.

SPL8-gRNA1 Genotypes Exhibit Enhanced Drought Resilience Compared to EV Controls

In order to evaluate whether genotypes with mutations in *MsSPL8* alleles were able to better withstand drought stress than EV genotypes, we withheld water from plants with an initial soil moisture content of approximately 50% and assessed the soil moisture content at which they began to exhibit signs of stress, including wilted or drooping leaves and shoots (**Figure 6A**). Both SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes wilted at a significantly lower soil moisture content (an average of 6.2% and 5.6% volumetric soil moisture content, respectively) than EV controls (an average of 9.1% volumetric soil moisture) (**Figures 6A,B**). In line with this, while leaf RWC did not differ

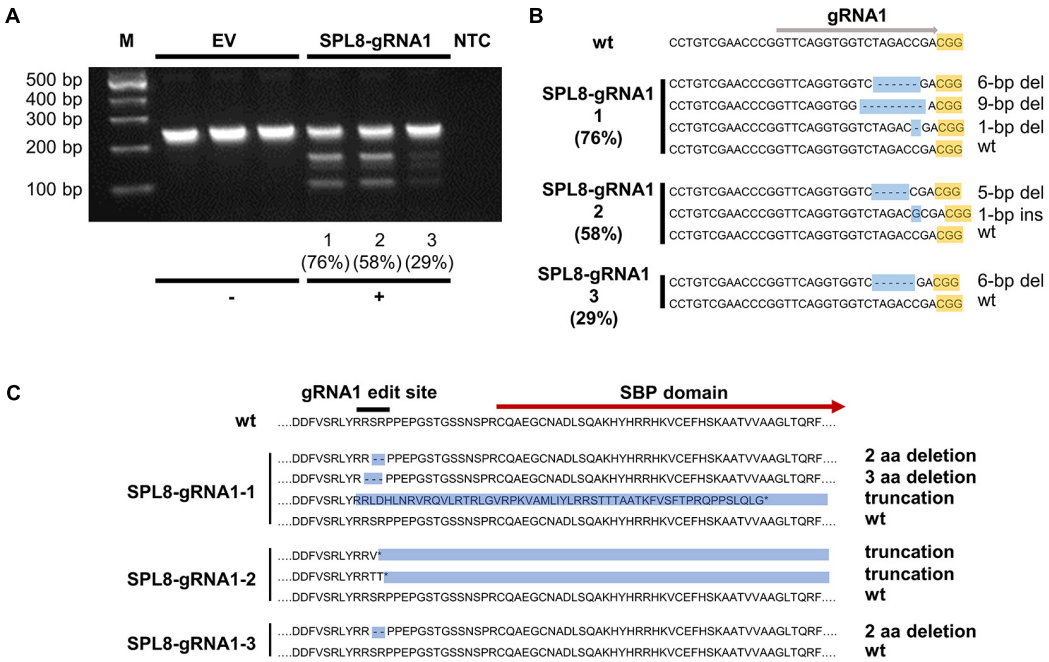


FIGURE 3 | Confirmation of *MsSPL8* editing in SPL8-gRNA1 genotypes. **(A)** T7E1 assay results from three empty vector (EV) control genotypes and SPL8-gRNA1-1, SPL8-gRNA1-2 and SPL8-gRNA1-3 genotypes. While genotypes with no alterations within the *MsSPL8* gRNA1 target region show a single band, those with genetic modifications within the gRNA1 target region exhibit multiple bands. Percentages indicate gene editing frequencies determined *via* GEF-ddPCR. **(B)** Nucleotide sequences surrounding the *MsSPL8* gRNA1 target region from untransformed (wt), SPL8-gRNA1-1, -2 and -3 genotypes as determined by Sanger sequencing. All results are representative of all genetic variants observed within each genotype following the sequencing of 21–33 clones. Percentages indicate gene editing frequencies determined *via* GEF-ddPCR. **(C)** Amino acid sequences of *MsSPL8* gRNA1 target region from untransformed (wt), SPL8-gRNA1-1, -2 and -3 genotypes.

between genotypes under well-watered conditions, both SPL8-gRNA1 genotypes exhibited significant increases in leaf RWC under moderate (approximately 14% volumetric soil moisture content) and severe (approximately 7% volumetric soil moisture content) drought conditions (**Figure 6C**), which indicates that the edited genotypes were better able to retain water in their leaves in the face of water deficit. This was also apparent in the physical appearance of plants at soil moisture levels of approximately 8%, at which point EV control plants consistently displayed obvious water-deficit symptoms, while SPL8-gRNA1 plants remained visibly unaffected (**Figure 6A**). Furthermore, while no significant difference was noted in light-saturated photosynthetic rate between EV and SPL8-gRNA1 genotypes under well-watered conditions, only EV genotypes displayed a significant reduction in light-saturated photosynthetic rate under drought compared to control conditions (**Figure 6D**). This suggests that drought treatment negatively impacted photosynthesis in EV genotypes, but not SPL8-gRNA1 genotypes. Finally, when plants were allowed to reach a soil moisture content of 1% and were then re-watered for approximately 2 weeks, only 50% of EV genotypes survived, while all SPL8-gRNA1 genotypes flourished, exhibiting necrosis in only a small number of leaves (**Figures 6E,F**), which further illustrates the enhanced drought tolerance of the edited genotypes in this study.

To determine whether differences in water use/loss were involved in the superior drought tolerance of SPL8-gRNA1 genotypes, we first assessed the rate at which soil moisture was lost from pots bearing different genotypes, and found no significant differences among genotypes (**Figure 7A**). Similarly, while SPL8-gRNA1-1 plants exhibited a significant decrease in detached leaf water loss rates compared to EV plants at early time points, no significant differences were observed at subsequent time points, or between SPL8-gRNA1-2 and EV plants (**Figure 7B**). Furthermore, while no significant differences were apparent in stomatal conductance or transpiration rate between SPL8-gRNA1 and EV genotypes either under well-watered or drought conditions, EV genotypes, but not SPL8-gRNA1 genotypes, exhibited significant reductions in both stomatal conductance and transpiration rate under drought compared to control conditions (**Figures 7C,D**). Taken together, these results suggest that unlike EV genotypes, SPL8-gRNA1 genotypes did not respond to the same level of drought stress by reducing stomatal conductance and transpiration rate, and that traits related to water use and/or water loss were not solely responsible for the improvement of SPL8-gRNA1 genotypes in terms of their ability to withstand water deficit.

Generation of Non-transgenic Edited F_1 Genotypes

Heritability of the *MsSPL8* edits was evaluated by crossing SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes, respectively, with

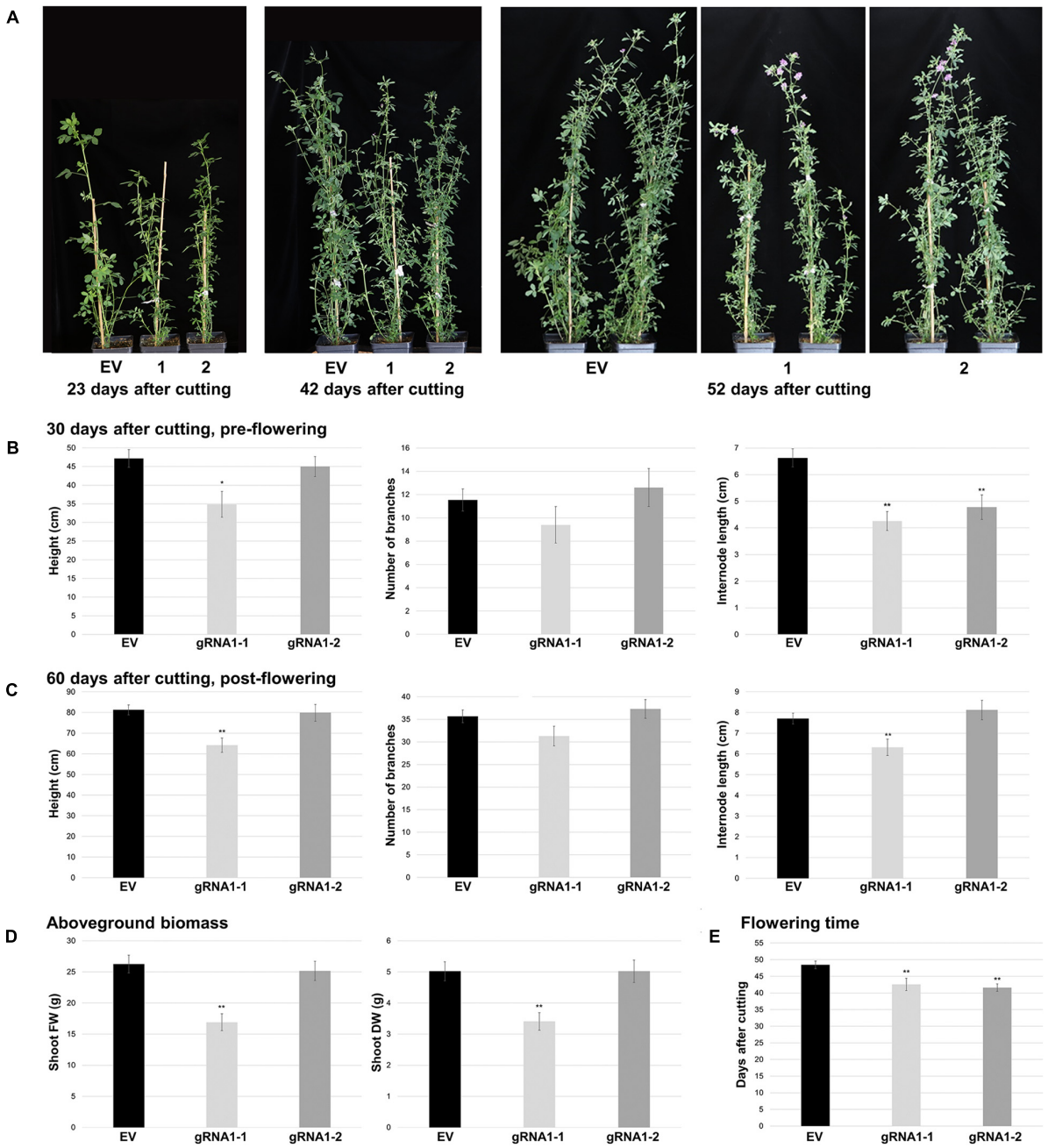


FIGURE 4 | Aboveground growth characteristics of SPL8-gRNA1 and EV control genotypes. **(A)** Representative images are of edited SPL8-gRNA1-1 (1) and SPL8-gRNA1-2 (2) genotypes, as well as EV controls, 23, 42 and 52 days following cutting. **(B,C)** Graphs depict plant height, total number of branches and internode length 30 **(B)** and 60 **(C)** days after cutting. **(D)** Shoot fresh weight (FW) and dry weight (DW) 60 days after cutting when all plants were flowering. **(E)** Number of days following cutting at which plants began to flower. For all graphs, each block represents the mean of five SPL8-gRNA1-1 (gRNA1-1), five SPL8-gRNA1-2 (gRNA1-2) and fifteen EV (five each of three independent transformants) biological replicates **(B)** or ten SPL8-gRNA1-1 (gRNA1-1), ten SPL8-gRNA1-2 (gRNA1-2) and thirty EV (ten each of three independent transformants) **(C–E)** biological replicates derived from stem cuttings, while bars denote standard errors. Asterisks denote means that are significantly different from EV controls as determined by 2-tailed student's *t*-tests assuming unequal variance (*$P \leq 0.05$; **$P \leq 0.01$).

several genotypes of the cultivar AC Blue J. A total of 202 seedlings were established from the crosses, and PCR analysis demonstrated that 23.8% of them lacked a transgene. Furthermore, T7E1 and ddPCR assays indicated that of the transgene-negative plants, 77.1% bore an edit in at least one *MsSPL8* allele (**Table 1**). None of the AC Blue J plants assessed exhibited a transgene or editing of this gene in any case, and GEF of 74.5% and 55.7% were confirmed

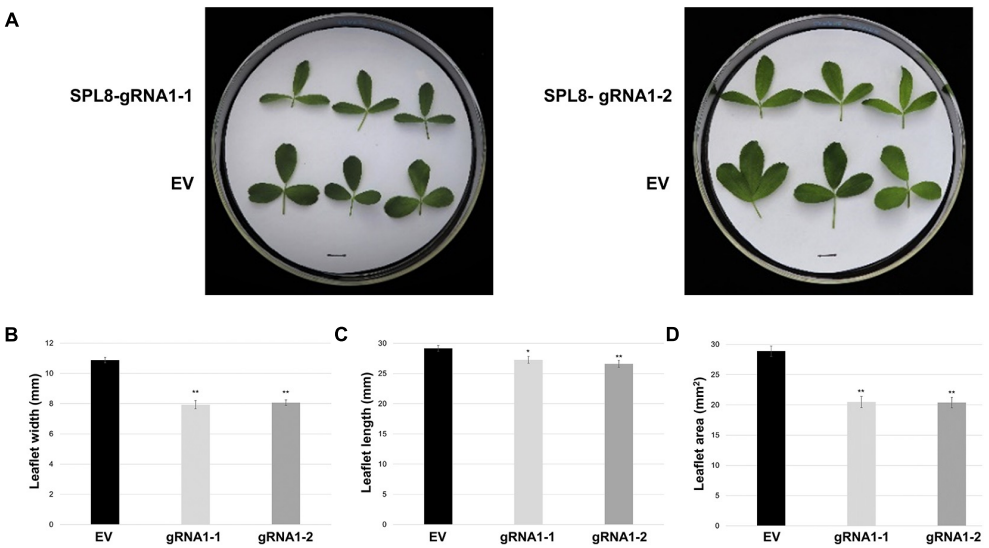


FIGURE 5 | Leaf characteristics of SPL8-gRNA1 and EV control genotypes. **(A)** Representative images of trifoliate leaves (third from the shoot tip) from edited SPL8-gRNA1-1 (mutations in three *MsSPL8* alleles) and SPL8-gRNA1-2 (mutations in two *MsSPL8* alleles) genotypes, as well as empty vector (EV) controls. Scale bars = 1 cm. **(B–D)** Measurements of the width, length and area of the middle leaflet of trifoliate leaves (third from the shoot tip) at 30 days after cutting. Each block represents the mean of three leaflets from five biological replicates of each SPL8-gRNA1 genotype, and five biological replicates from each of three independent EV transformants (fifteen replicates total), while bars denote standard errors. Asterisks denote means that are significantly different from EV controls as determined by 2-tailed student's *t*-tests assuming unequal variance (*$P \leq 0.05$; **$P \leq 0.01$).

for SPL8-gRNA1-1 and SPL8-gRNA1-2 genotypes, respectively (**Table 1** and **Supplementary Figure 4**).

Intriguingly, with the exception of one genotype that exhibited a GEF of 34%, all transgene-negative seedlings fell into one of five GEF classes: 0% (0–0.6% GEF), 25% (24.0–28.7% GEF), 50% (48.7–53.2% GEF), 75% (74.5%) and 100% (99.9%). In both instances where SPL8-gRNA1 genotypes acted as the recipient for AC Blue J pollen, all transgene-negative plants possessed an edit in *MsSPL8*. Conversely, where AC Blue J was utilized as the recipient for SPL8-gRNA1-1 and SPL8-gRNA1-2 pollen, respectively, 40.0% and 30.0% of transgene-negative plants bore no edits (**Table 1** and **Supplementary Figure 4**). Of the transgene-negative edited genotypes overall, 29.7% bore no edit, 59.5% possessed a GEF of approximately 25%, 35.1% possessed a GEF of approximately 50%, 2.7% possessed a GEF of approximately 75% and 2.7% possessed a GEF of 100% (**Table 1** and **Supplementary Figure 4**). The genotypes with GEF of approximately 75% and 100% were derived from crosses where AC Blue J was utilized to pollinate SPL8-gRNA1-1 and SPL8-gRNA1-2, respectively, which suggests that a low level of selfing likely took place in the SPL8-gRNA1 genotypes. Taken together, these results confirm that edited genotypes lacking a transgene can be achieved in the next generation, and that editing was present in a range of allelic combinations.

DISCUSSION

As a means of further understanding the role of *MsSPL8* in plant development and abiotic stress response, and concomitantly generating transgene-free germplasm with reduced MsSPL8 activity, we utilized CRISPR/Cas9 technology to simultaneously edit multiple *MsSPL8* alleles in alfalfa. Although the CRISPR/Cas9 platform has proven extremely effective in many plant species to date, including those with polyploid genomes (Morineau et al., 2017; Liu et al., 2018; Huang et al., 2020), it has only very recently begun to gain traction in alfalfa (Gao et al., 2018b; Chen et al., 2020; Wolabu et al., 2020; Bottero et al., 2021; Curtin et al., 2021). The first attempt at utilizing this technique in this species involved the targeting of another *SPL* gene (*MsSPL9*), and while successful edits at the target site were detected, they occurred with extremely low frequencies of up to approximately 2.2% of alleles within the tissue tested (Gao et al., 2018b). As a result of the high level of mosaicism in these cases, phenotypic alterations were not observed. However, subsequent studies have led to substantially higher editing frequencies and consequent phenotypic alterations in alfalfa, with up to 100% allelic mutation frequency observed in the first generation when targeting the *M. sativa* stay-green (*MsSGR*), phytoene desaturase (*MsPDS*) and *PALM1* genes (Chen et al., 2020). While the use of a multiplex gRNA CRISPR/Cas9 system was required to achieve high editing frequencies in alfalfa in certain instances (Wolabu et al., 2020; Bottero et al., 2021), this has not always been the case (Chen et al., 2020). In the current study, we were able to achieve high frequencies of indels in *MsSPL8* alleles in the first generation (with up to three of four alleles mutated) with a single gRNA (**Figures 2B**, **3B,C**), and in a manner that was homogeneous in at least a proportion of genotypes (**Figure 2C**). In addition, this was achieved in a manner that was highly specific with no off-target mutations observed in the selection of sites analyzed (**Supplementary Figure 1**).

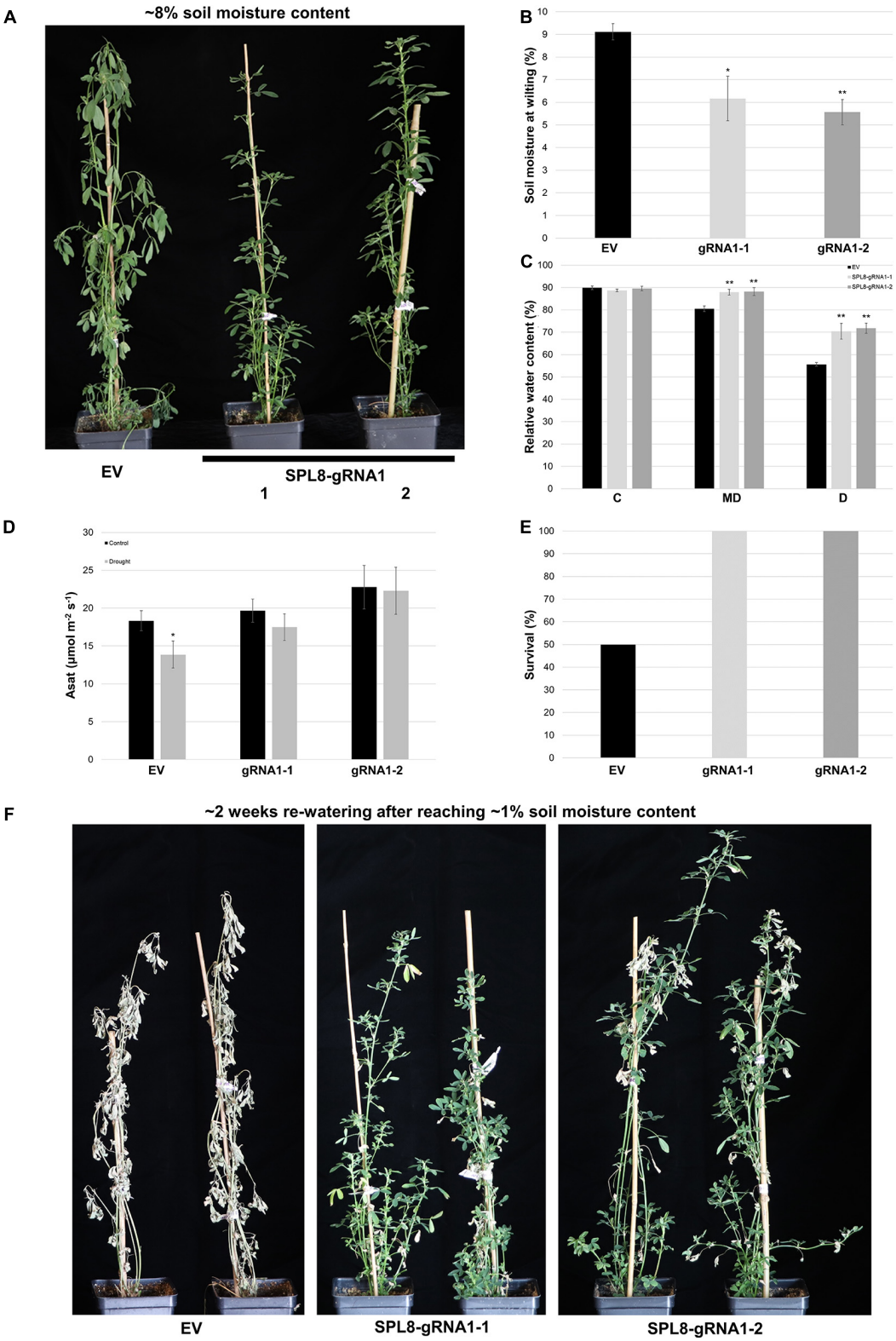


FIGURE 6 | Response of SPL8-gRNA1 and EV genotypes to drought stress. **(A)** Representative images of SPL8-gRNA1-1, SPL8-gRNA1-2 and EV genotypes after withholding water until volumetric soil moisture levels reached approximately 8%, 11 days after initiating the withholding of water. **(B)** Soil moisture level at which SPL8-gRNA1 and EV genotypes began to wilt. **(C)** Relative water content (RWC) of third fully expanded trifoliate leaves assessed when soil moisture contents were

(Continued)

FIGURE 6 | at approximately 50% [control (C)], 14% [moderate drought (MD)] and 7% [drought (D)]. Blocks in each graph consist of the mean value of six SPL8-gRNA1-1 (gRNA1-1), six SPL8-gRNA1-2 (gRNA1-2) and eighteen EV (six each of three independent transformants) biological replicates derived from stem cuttings. **(D)** Light-saturated photosynthetic rate (Asat) of middle leaflets from third fully expanded trifoliate leaves under well-watered (soil moisture content of approximately 50%) and drought (average soil moisture of approximately 11%) conditions. Measurements were adjusted for leaf area. Blocks represent the mean value of leaflets from four SPL8-gRNA1-1, four SPL8-gRNA1-2, and twelve EV (four each of three independent transformants) biological replicate plants derived from stem cuttings. **(E)** Proportion of plants that survived following 2 weeks of re-watering after allowing soil moisture contents to reach 1%. Blocks consist of the mean value of six SPL8-gRNA1-1 (gRNA1-1), six SPL8-gRNA1-2 (gRNA1-2) and eighteen EV (six each of three independent transformants) biological replicates derived from stem cuttings. **(F)** Representative images of SPL8-gRNA1-1, SPL8-gRNA1-2 and EV genotypes following approximately 2 weeks of re-watering once plants reached 1% soil moisture contents. For all graphs, bars denote standard errors and asterisks denote means that are statistically different in SPL8-gRNA1 genotypes compared to EV genotypes **(B,C)** or under drought compared to well-watered conditions **(D)** as determined by 1-tailed **(D)** or 2-tailed **(B,C)** *t*-tests assuming unequal variance (*$P \leq 0.05$; **$P \leq 0.01$).

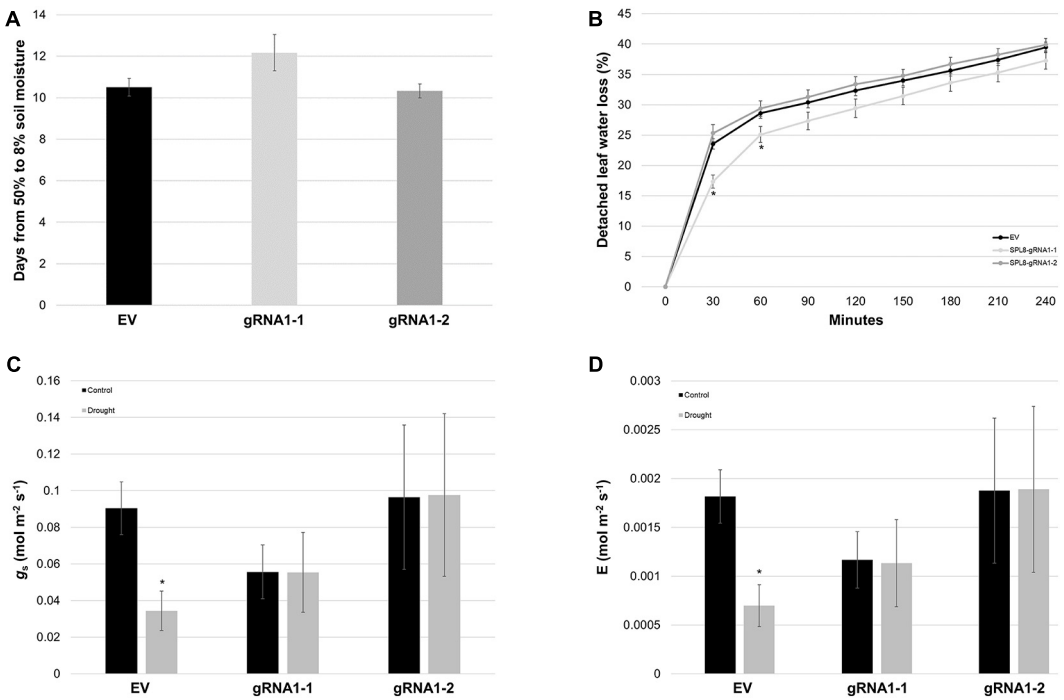


FIGURE 7 | Water loss-related traits in SPL8-gRNA1 and EV genotypes. **(A)** Number of days required for volumetric soil moisture contents to decrease from 50 to 8% following the withholding of water. Blocks denote the mean value of six SPL8-gRNA1-1, six SPL8-gRNA1-2 and eighteen EV (six each of three independent transformants) biological replicates derived from stem cuttings. **(B)** Rates of water loss from detached leaves derived from well-watered plants. Blocks denote the mean value of twelve SPL8-gRNA1-1 (two leaves from six plants), twelve SPL8-gRNA1-2 (two leaves from six plants) and thirty-six EV (twelve leaves from three independent transformants) biological replicates. **(C,D)** Stomatal conductance (g_s) and transpiration rate (E) of middle leaflets from third fully expanded trifoliate leaves under well-watered (soil moisture content of approximately 50%) and drought (average soil moisture content of approximately 11%) conditions. Measurements were adjusted for leaf area and blocks represent the mean value of leaflets from four SPL8-gRNA1-1, four SPL8-gRNA1-2, and twelve EV (four each of three independent transformants) biological replicate plants derived from stem cuttings. For all graphs, bars denote standard errors and asterisks denote means that are statistically different in SPL8-gRNA1 genotypes compared to EV genotypes **(B)** or under drought compared to well-watered conditions **(C,D)** as determined by 1-tailed **(C,D)** or 2-tailed **(B)** *t*-tests assuming unequal variance (*$P \leq 0.05$). gRNA1-1, SPL8-gRNA1-1; gRNA1-2, SPL8-gRNA1-2.

TABLE 1 | Molecular evaluation of F_1 genotypes derived from crosses between SPL8-gRNA1 and AC Blue J genotypes.

Cross	Total plants	Transgene-negative plants	GEF				
			~0%	~25%	~50%	~75%	~100%
SPL8-gRNA1-1 (F) × AC Blue J (M)	60	10	0	4	5	1	0
SPL8-gRNA1-2 (F) × AC Blue J (M)	56	8	0	6	1	0	1
AC Blue J (F) × SPL8-gRNA1-1 (M)	55	20	8	6	6	0	0
AC Blue J (F) × SPL8-gRNA1-2 (M)	31	10	3	6	1	0	0
Total	202	48	11	22	13	1	1

F, female parent; M, male parent.

As has been reported elsewhere in the literature for both alfalfa and other plants species (Bruegmann et al., 2019; Ren et al., 2019; Wolabu et al., 2020; Zhang et al., 2020), we noted a considerable difference in editing efficiencies among gRNAs used in this study (**Figure 1A**), with gRNA1 exhibiting much higher editing frequencies than gRNA2 and gRNA3 (**Figures 2**, **3**). While some progress has been made in terms of identifying gRNA characteristics that are important for cleavage efficiency in animal systems (Doench et al., 2014), a sound understanding of this issue specifically in plants remains largely elusive. Currently existing gRNA ranking prediction algorithms for plants are based on experiments in animals, and it has been shown that there is little correlation among rankings achieved using these prediction tools and effectiveness *in vivo* (Naim et al., 2020). It has been suggested that GC content and the proportion of purines in the last four positions of the gRNA may be important for gRNA efficiency in plants (Bruegmann et al., 2019; Ren et al., 2019); however, these parameters were not responsible for the higher effectiveness of gRNA1 in the current study since all three gRNAs bore a GC content of 55% and gRNA2 possessed the highest number of purines near the 3′ terminus (three of four nucleotides) (**Figure 1A**). Furthermore, although the particular DNA strand targeted by a gRNA has also been proposed to play a role in its activity in certain studies (Bruegmann et al., 2019), this has not always been a factor (Doench et al., 2014) and did not appear to be the case in the current study since both gRNA1 and gRNA2 targeted the same strand (**Figure 1A**).

Highly similar phenotypes were apparent in both of the edited genotypes analyzed (derived from independent transformation events), including reductions in internode length and leaf size, as well as early flowering (**Figures 4**, **5**). Plant height, shoot and root biomass, and root length were also significantly reduced in SPL8-gRNA1-1, but not SPL8-gRNA1-2, compared to EV controls (**Figure 4** and **Supplementary Figure 3**), which reflects the mutational dosage in the two genotypes. SPL8-gRNA1-1 possessed a single *MsSPL8* allele bearing a frameshift mutation, two alleles bearing mutations that would result in two and three amino acid deletions, respectively, and a wild-type allele. Conversely, SPL8-gRNA1-2 bore frameshift mutations in two *MsSPL8* alleles along with two wild-type *MsSPL8* alleles (**Figure 3C**). While it is currently unclear whether the deletions present in the two *MsSPL8* alleles in SPL8-gRNA1-1 would lead to complete inactivation of the SPL8 protein, given the proximity of the cleavage site to the SBP box it is certainly feasible that they could negatively impact activity. As such, it is not entirely surprising that the edited genotypes displayed a gene dosage effect in line with the number of alleles that bore mutations in each case, with the genotype bearing mutations in three *MsSPL8* alleles (SPL8-gRNA1-1) exhibiting phenotypic alterations that were more profound than the genotype bearing mutations in only two *MsSPL8* alleles (SPL8-gRNA1-2). While this type of additive effect has been noted previously following editing in other polyploid plant species such as *Camelina sativa* (Morineau et al., 2017), *Brassica napus* (Huang et al., 2020) and *Triticum aestivum* (Li et al., 2021), this is the first instance in which it has been demonstrated in alfalfa. However, this phenomenon may not occur in every instance, and a previous study where CRISPR/Cas9 was used to successfully target *MsPDS* and *MsPALM1* in alfalfa found that an altered phenotype was only observed when all four alleles had been mutated simultaneously (Chen et al., 2020). This discrepancy may be attributable to the particular gene target and/or the resulting phenotypic change that occurs following editing, although further research will be required to unravel such distinctions in plants fully.

Interestingly, the pleiotropic phenotypes observed in MsSPL8-gRNA1 genotypes did not parallel the expression patterns noted previously for this gene, whereby expression was primarily detected in flowers and elongated stems, with little expression in leaves or roots (**Supplementary Figure 2**). This phenomenon is not specific to alfalfa, and a similar discrepancy has been observed previously in Arabidopsis, whereby root phenotypes were observed in *spl8* mutants despite an apparent lack of expression in this tissue (Zhang et al., 2007). Although similar *SPL8* expression patterns have been observed in Arabidopsis and *Medicago* spp. (Xing et al., 2013; Gou et al., 2018; **Supplementary Figure 2**), there appears to be considerable distinctions between the functions of *SPL8* homologs across species (Unte et al., 2003; Zhang et al., 2007; Xing et al., 2013; Gou et al., 2018). Unlike *MsSPL8* in alfalfa, Arabidopsis *SPL8* appears to be mainly involved in the regulation of male and female fertility, as well as root growth, with very few apparent roles in aboveground vegetative growth (Unte et al., 2003; Zhang et al., 2007; Xing et al., 2013). In addition, while *SPL8* does not appear to be regulated by miR156 in Arabidopsis (Xing et al., 2010), it is a target of this miRNA in alfalfa (Feyissa et al., 2021), which suggests that some level of evolutionary divergence has occurred in this gene among plant species. However, despite these possible functional and regulatory disparities among species, both Arabidopsis and alfalfa *SPL8* have been suggested to function, at least in part, through the transcriptional regulation of genes involved in phytohormone biosynthesis or signaling (Zhang et al., 2007; Xing et al., 2013; Gou et al., 2018), although their precise functions remain to be unraveled in full. In line with this, *M. truncatula spl8* mutant plants were found to accumulate higher GA_4 levels in mature leaves and the shoot apical meristem than the wild-type control (Gou et al., 2018). In the current study, the phenotypes noted in the vegetative tissues of independent SPL8-gRNA1 genotypes were reminiscent of GA-deficient mutants, which tend to be dwarfed in stature with reductions in leaf expansion and stem elongation (Thomas and Sun, 2004; **Figures 4**, **5**). Conversely, we also observed early flowering in our edited genotypes (**Figure 4E**), which is instead typical of mutants with an over-accumulation of GA (Huang et al., 1998). Although GA levels were not evaluated in the present study, Arabidopsis *SPL8* provides opposing roles in inflorescences and seedlings in the context of GA-dependent developmental processes (Zhang et al., 2007), which hints at the possibility that a similar phenomenon may be occurring in alfalfa. However, other mechanisms could also be involved in the observed phenotypes in SPL8-gRNA1 genotypes, and in depth transcriptomic assessments, as well as evaluations of GA levels, GA signaling and GA-related pathways, will be necessary to unravel these differences between species and studies in full.

While the reduced stature and internode length observed in SPL8-gRNA1 genotypes in the current study resembled RNAi

genotypes in which other miR156-regulated *SPL* genes, including *SPL9* and *SPL13*, were down-regulated in alfalfa (Gao et al., 2018a; Hanly et al., 2020), our edited genotypes did not exhibit the increased branching, enhanced biomass or delayed flowering that are often seen in such genotypes (**Figure 4**). Similarly, the SPL8-gRNA1 genotypes in the current study did not closely resemble *MsSPL8* RNAi genotypes or the *M. truncatula spl8* mutant, which displayed increased branching and forage biomass, accelerated regrowth and no apparent change in flowering time or leaf size (Gou et al., 2018). It is not clear why this was the case; however, it is possible that differences in growth conditions (including potting mix, pot size, light intensity or temperature, for example), the background genotype utilized for the study, or the specific alleles targeted in RNAi and CRISPR/Cas9 studies may have played a role in these discrepancies.

A subset of miR156-targeted *SPL* genes in alfalfa have been found previously to lead to an increased ability to withstand abiotic stresses such as drought (*SPL13*, *SPL9*; *SPL8;* Arshad et al., 2017; Gou et al., 2018; Feyissa et al., 2019; Hanly et al., 2020), heat (*SPL13*; Matthews et al., 2019), salinity (*SPL8*; Gou et al., 2018) and/or flooding (*SPL13*; Feyissa et al., 2021) when down-regulated. Correspondingly, both SPL8-gRNA1 genotypes assessed in the present study exhibited improvements in their resilience to water deficit, with lower volumetric soil contents at the first sign of wilting, an improved ability to maintain RWC and photosynthetic rate under drought treatment, and higher survival following extreme drought stress (**Figure 6**). This improvement in drought tolerance across SPL8-gRNA1 genotypes does not appear to be a direct result of decreased water loss from their leaves (**Figure 7**). However, it is possible that reduced leaf size, which is known to be associated with superior drought tolerance in plants (Quan et al., 2016; Singer et al., 2021b), could be contributing to an overall reduction in water loss at the plant level. Intriguingly, enhancements in drought tolerance in RNAi alfalfa genotypes in which other *SPL* genes were down-regulated have been suggested to occur, at least in part, through the up-regulation of anthocyanin biosynthetic genes, thus resulting in an increased accumulation of this secondary metabolite and a consequent enhancement in ROS scavenging capacity (Gou et al., 2018; Feyissa et al., 2019; Hanly et al., 2020). While this may also be the case in our SPL8-gRNA1 genotypes, the precise mechanisms driving enhanced drought resilience remain to be elucidated.

Due to inbreeding depression, there is a need to outcross alfalfa, and as such, we carried out reciprocal crosses between our edited genotypes and a distinct cultivar bearing wild-type *MsSPL8* alleles (AC Blue J) in order to achieve progeny in which the transgenic cassette had been segregated out, but that possessed edits within *MsSPL8* alleles. Approximately 25% of the resulting seedlings assessed were found to lack the transgene, and of the transgene-free F_1 genotypes, upward of 75% possessed an edit in at least one *MsSPL8* allele (**Table 1** and **Supplementary Figure 4**), which confirms the previous finding that mutated alleles are stably inherited in this species (Chen et al., 2020). While the vast majority of genotypes fell into GEF classes of 25% and 50% (one of four and two of four *MsSPL8* alleles edited, respectively; **Table 1** and **Supplementary Figure 4**), two genotypes exhibited GEF of 75% and 100% (three of four and four of four *MsSPL8* alleles edited, respectively). Since SPL8-gRNA1 genotypes were utilized as the female parent in both instances where unexpectedly high GEF were observed in offspring, it is highly likely that some level of selfing occurred in these genotypes. While self-pollination is not the predominant route for seed production in alfalfa typically, they are not self-incompatible, and selfing rates up to approximately 52% have been noted in this species (Riday et al., 2015).

In conclusion, we have successfully utilized CRISPR/Cas9 technology to edit up to three of four *MsSPL8* alleles in alfalfa in the first generation with a single gRNA, and up to 100% of alleles in the second generation. Despite the fact that at least one wild-type copy of *MsSPL8* remained in our first generation plants, we observed distinct morphological alterations in SPL8-gRNA1 genotypes, including small leaf size, reduced internode length and early flowering. These phenotypes differed from those observed previously in alfalfa RNAi genotypes targeting *MsSPL8*, which may be attributable to distinctions in growth conditions, the background genotype, or the specific alleles targeted between studies. The severity of phenotypic changes tended to be dependent on allelic mutation dose, and only genotypes with mutations in three of four *MsSPL8* alleles, but not those with mutations in two of four *MsSPL8* alleles, demonstrated significant reductions in plant height, shoot and root biomass, and root length. This is the first instance of such a phenomenon having been demonstrated in alfalfa. Finally, both SPL8-gRNA1 genotypes evaluated in the current study exhibited a superior ability to survive under water-deficit conditions compared to EV controls, which is an invaluable trait for a future of climate change, and is the first case in which an improvement in abiotic stress tolerance has been elicited in alfalfa using gene editing. Further in-depth examination of these edited genotypes will shed light on functional discrepancies among *SPL8* homologs from different species and/or genotypes, precise mechanisms driving improvements in drought resilience, and could potentially provide the capacity to target genes that function downstream of *MsSPL8* as a means of achieving abiotic stress tolerance without the reductions in biomass that are observed with high allelic mutation rates.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SS, AH, SA, and GC conceived and designed the experiments. SS and GC were responsible for the supervision of students and technicians. SS, KBH, US, and GD conducted molecular and alfalfa transformation experiments. SS, US, and KK carried out morphological and drought tolerance assessments. SS, KBH, and

US wrote the manuscript. All authors were involved in revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.774146/full#supplementary-material

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Generating Novel Male Sterile Tomatoes by Editing Respiratory Burst Oxidase Homolog Genes

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Hybrid breeding of tomatoes (*Solanum lycopersicum*), an important vegetable crop, is an effective way to improve yield and enhance disease and stress resistance. However, the efficiency of tomato hybridization is hindered by self-fertilization, which can be overcome using male sterile lines. It has been reported that reactive oxygen species (ROS) act as a key regulator for anther development, mediated by *RBOH* (*Respiratory Burst Oxidase Homolog*) genes. Here, two tomato anther-expressed genes, *LeRBOH* (Solyc01g099620) and *LeRBOHE* (Solyc07g042460), were selected to cultivate novel tomato male sterile strains. By using a CRISPR/Cas9 system with a two-sgRNA module, the *lerboh*, *lerbohe*, and *lerboh lerbohe* mutant lines were generated, among which the *lerbohe* and *lerboh lerbohe* mutants displayed complete male sterility but could accept wild-type pollens and produce fruits normally. Further analysis uncovered significantly decreased ROS levels and abnormal programmed cell death in *lerboh lerbohe* anthers, indicating a key role of ROS metabolism in tomato pollen development. Taken together, our work demonstrates a successful application of gene editing via CRISPR/Cas9 in generating male sterile tomatoes and afforded helpful information for understanding how *RBOH* genes regulating tomato reproduction process.

Keywords: male sterile, tomato, CRISPR, RBOH, hybridization breeding

INTRODUCTION

Tomato (*Solanum lycopersicum*) is a highly cultivated and consumed vegetable around the world. Improvements in tomato crops are largely dependent on hybrid breeding, which can efficiently increase yields or enhance biotic/abiotic resistance (Tamta and Singh, 2017; Kim and Zhang, 2018). However, because tomato can self-fertilize, hybrid breeding requires excessive time and labor for artificial emasculation. Thus, tomato male sterile germplasms are critical for efficient hybrid breeding (Kim and Zhang, 2018). Nearly 50 male sterile mutants have been reported (Gorman et al., 1997; Sawhney, 2004; Cheema and Dhaliwal, 2005; Gorguet et al., 2009; Zhang et al., 2016; Pucci et al., 2017; Cao et al., 2019; Liu et al., 2019; Yu et al., 2020), but most strains possess undesirable phenotypes. Some sterile lines can still perform some self-crossing as they still produce a few active pollens. In some functional sterile strains, the stigmas are shorter than the anther tube, which

effectively prevents natural self-crossing but makes artificial pollination very difficult (Perez-Prat and Campagne, 2002). In addition, in a strain with completely abortive pollens, the pistils are also anomalous, resulting in a low outcrossing rate (Chen et al., 2004). Thus, novel male sterile germplasms overcoming these disadvantages are still urgently needed.

Among the many factors affecting the tomato reproduction process, just as temperature and light (Li et al., 2006; Fu et al., 2014; Zhu et al., 2015; Shen et al., 2019), ROS (Reactive Oxygen Species) is also proved as a key regulator of anther development and thus pollen fertility (Luo et al., 2013). The transcription factor MADS3 up-regulates *MT-1-4b*, a ROS scavenging protein, and thus negatively regulates ROS content in rice anthers, influencing the maturation of pollens (Hu et al., 2011). As a necessary process for normal pollen maturation, the programmed cell death (PCD) of the tapetum is associated with ROS metabolism (Joanna, 2003). In rice, the mitochondrial protein Wa352 inhibits the ROS scavenging function of COX11, promoting PCD in the tapetum and subsequently leading to pollen abortion (Luo et al., 2013; Wang et al., 2018). Another rice male sterile mutant *dtc1* also exhibits decreased ROS accumulation in the anthers and a delayed PCD in hypertrophic tapetum (Yi et al., 2016). In *Arabidopsis*, reduced ROS in the tapetum results in delayed PCD, resulting in decreased pollen fertility (Xie et al., 2014). Similarly, increasing ROS accumulation caused by Brassinolides (BRs) was found to promote degradation of the tapetum in tomatoes (Yan et al., 2020). Together, these reports confirm the important role of ROS in tapetum development, but the regulatory mechanism of ROS accumulation seems rather complex in general (Ullah and Yi, 2019). Thus, it is necessary to analyze genes relating to ROS metabolism in anthers.

As a membrane protein that converts NADPH to O^{2-} and electrons, RBOH (Respiratory Burst Oxidase Homolog) members are important resources of ROS (Chapman et al., 2019). As described in our previous work, *AtRBOHE* plays a key role in regulating ROS accumulation in the anthers and subsequently mediates development of the tapetum in *Arabidopsis* (Xie et al., 2014). In tomatoes, knock-down of *LeRBOH1* via RNAi (RNA interference) reduces ROS content and influences PCD in the tapetum, resulting in an about 40% decrease in seed number (Yan et al., 2020). However, as a protein family with many homologous members, the function of other tomato RBOHs during anther development is still unclear, and whether a practical male sterile germplasm can be generated by modulating RBOH genes is still uncertain. In tomatoes, there are in total 8 *RBOH* genes (*LeRBOHA*, *LeRBOHB*, *LeRBOHD*, *LeRBOH*, *LeRBOHE*, *LeRBOHH*, *LeRBOH1*, and *LeWif1*), among which *LeRBOHE* and *LeRBOH* show a temporal expression pattern during anther development (Yu et al., 2017). As the qPCR results describing, *LeRBOH* displayed a anther-specific high expression while *LeRBOHE* exhibited the highest expression in anther and stem, indicating they may play a role in regulating anther development. However, whether these two *RBOH* genes regulate pollen fertility remains unknown.

In certain cultivars, mutation of key fertility genes via CRISPR can be an efficient alternative to the occasionally used, time-consuming process of obtaining male sterile lines by screening natural mutants (Du et al., 2020). Here, relying on a double-sgRNA module, we edited *LeRBOHE* and/or *LeRBOH* with SpCas9 in the commercial tomato variety Alisa Craig (AC). We observed that *LeRBOHE* and *LeRBOH* played a predominate and minor roles, respectively, in mediating tomato pollen maturation, concomitant with defects in ROS synthesis. Our work revealed mechanism by which *RBOHs* regulate development of tomato anthers, and successfully established a complete male sterile strain for tomato hybrid breeding.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tomato (*Lycopersicon esculentum*, Alisa Craig) were grown in a climate chamber at 25/20°C (day/night) under long days (16 h day and 8 h night).

Plasmid Construction

LeRBOH (Solyc01g099620) and *LeRBOHE* (Solyc07g042460) gene loci were retrieved from the tomato website.[1] sgRNAs for the CRISPR/Cas9 target sites of *LeRBOH* and *LeRBOHE* were designed with http://skl.scau.edu.cn/targetdesign// (sgRNA1: TAGCTAGCAAGCTCGAAAAG; sgRNA2: TCTAGCAAGTAA TCCGTCTT). Using the tRNA-scaffold as a template, the fragments were amplified with gLeRBOHE-F/R primers respectively, and were transferred into the CRISPR/Cas9 vector after digestion with Bsa I. All primers used in the study are listed in **Supplementary Table 1**.

Mutant Detection

The genomic DNA of the transgenic plants was extracted from leaves via the CTAB method (Porebski et al., 1997). Hyg-F/R, Cas9-F/R, and M13-F/gRNA-R primers were used to detect the hygromycin resistance gene, Cas9 and the sgRNA, respectively. LeRBOH-F/R and LeRBOHE-F/R were used for identification of edited targets.

Characterization of the Mutant Phenotype

The morphology of the plant, pistil, stamen, and fruit were photographed with a digital camera (Nikon, D750). Seed numbers, seed generating ratios, leaf numbers and plant heights of AC lines and mutants were analyzed by Student's *t*-test. The mature pollen was dyed with Alexander (Solarbio, Beijing, China) as described previously (Peng et al., 2013), and photographed with a Leica microscope (DMLB, Germany).

Observation of Pollen Morphology

Anther morphology was observed using a scanning electron microscope (Leica, DM6B, Germany). Open flowers in good growth condition were taken. The petals were removed, and half of the anthers were torn off and gently stuck onto a metal stage with double-sided tape. For pollen observation, wild-type

[1] https://solgenomics.net/

and mutant pollens were soaked in 2.5% glutaraldehyde fixative solution, and fixed at 4°C for 24 h. After rinsing 3 times with phosphate buffer solution, and dehydrating through successive alcohol gradients, the pollens were placed in isoamyl acetate and dried at the critical point of CO_2. Finally, the samples were photographed by scanning electron microscope (HITACHI, TM3030) after vacuum coating.

TUNEL Assay

Paraffin-embedded anthers at different developmental stages were collected for processing. *In situ* nick-end labeling of nuclear DNA fragmentation was performed with the Dead End Fluorometric TUNEL system according to the supplier's instructions (Promega, G3250). Samples were analyzed with a Leica DM6B microscope using a 488-nm/510-nm excitation/emission spectrum for fluorescein and a 530-nm/640-nm excitation/emission spectrum for propidium iodide as previously described (Phan et al., 2011).

Histology of Anthers and Histochemical Assays for Reactive Oxygen Species

Anthers at different developmental stages were fixed and then embedded in paraffin. Sections (8 μm) were cut using a Leica Histocore MULTICUT microtome, stained with ruthenium red, and photographed with a Leica (DM6B, Germany) microscope in bright field. To visualize levels of the superoxide anion as a measure of ROS content, anthers of different developmental stages were stained with 0.5 mM NBT (Sigma, N5514).

DAPI Staining

Pollens of different developmental stages were placed in 1 mg/L DAPI (Aladdin, D106471) solution and placed in a dark, moisturizing environment at 60°C to stain the microspores. The number of nuclei at each stage of microspore development was observed under a fluorescence microscope (DMLB, Leica, Germany).

RESULTS

Mutation of *LeRBOH*/*LeRBOHE* via CRISPR/Cas9

It has been reported that *LeRBOHE* and *LeRBOH* show a temporal expression pattern during anther development (Yu et al., 2017). To analyze the function of *LeRBOH* and *LeRBOHE* during anther development, we generated tomato mutants via the CRISPR/Cas9 system. Two sgRNAs, with spacer sequences specifically targeting the first exon of *LeRBOH* and *LeRBOHE*, respectively, were separated by tRNA and driven by the *Arabidopsis* U6 promoter, while SpCas9 was driven by a CaMV35S promoter (**Figure 1A**). The modules were constructed into the T-DNA containing plasmid and transformed into the AC strain via *Agrobacterium tumefaciens*. Eight T-DNA-free mutants were identified in the T_1 lines (**Supplementary Figure 1**). Among these mutants, there was a homozygous double knockout mutant (*lerboh lerbohe-1*) with an "A" insertion in the *LeRBOH* gene locus (induced an early "TAA"), and a 132-bp deletion (caused a 44-amino-acid deletion in the NADPH-Ox domain of LeRBOHE) in the *LeRBOHE* locus (**Figure 1D** and **Supplementary Figure 2**). As coproducts, two T-DNA-free lines containing the same mutation in either *LeRBOH* (*lerboh-1*) or *LeRBOHE* (*lerbohe-1*) as *lerboh lerbohe-1* were also selected, respectively (**Figures 1B,C**). In addition, we also obtained a mutant line with a "T" insertion in the first exon of *LeRBOH* (*lerboh-2*), generating an early "TAA," a mutant line with a "A" insertion in the first exon of *LeRBOHE* (*lerbohe-2*), resulting in premature termination by an early "TAG," and another double mutant (*lerboh lerbohe-2*) with the same *LeRBOH* and *LeRBOHE* mutations as *lerboh-2* and *lerbohe-2*, respectively (**Figures 1B–D**).

lerbohe and *lerboh lerbohe* Mutants Caused Absolute Male Sterility

To analyze the role of *LeRBOH* and *LeRBOHE* in tomato male fertility, *lerboh-1*, *lerbohe-1*, *lerboh lerbohe-1*, and AC lines were cultivated together at suitable conditions for their life-cycle (**Figure 2**). Amazingly, both *lerbohe-1* and *lerboh lerbohe-1* lines could not fruit at all, while fruits were produced normally in *lerboh-1* (**Figures 2A–D**). And interestingly, there were no obvious visible differences in either vegetative growth or flower development between the mutants and AC. Both *lerbohe-1* and *lerboh lerbohe-1* mutants displayed nearly normal growth morphology as the AC stains, including similar plant heights and numbers of compound leaves on main stem (**Table 1**). After growing under normal condition in greenhouse for 80 days, there were no obvious difference in both flower numbers and flower morphology between mutant and AC lines (**Table 1** and **Figures 2E–G**). Furthermore, as the cross-pollination assay showed, when wild-type pollens were stuck to the stigma, *lerbohe-1* and *lerboh lerbohe-1* could fruit normally (**Figures 2C,D**). The hybrid fruits became ripe with ordinary morphology (**Figures 2H,I**), full of normal seeds (with similar seed numbers as the AC fruits) with a good germination ratio (**Figures 2J,K**). However, no fruits appeared when the mutated pollens were stuck to the stigma of emasculated AC (**Supplementary Figure 3**). In addition, *lerboh-2*, *lerbohe-2,* and *lerboh lerbohe-2* exhibited the same phenotypes as *lerboh-1*, *lerbohe-1,* and *lerboh lerbohe-1*, respectively (**Supplementary Figure 4**). Thus, we acquired near-ideal tomato male sterile strains with no obviously undesirable side effects by mutating two *RBOH* genes, with the effects driven largely by *LeRBOHE*.

lerbohe and *lerboh lerbohe* Mutants Displayed Abnormal Pollen Development

The cross-pollination assays suggested that the *lerbohe* or *lerboh lerbohe* mutants produced abnormal pollens. To confirm this hypothesis, pollens of *lerboh-1*, *lerbohe-1*, *lerboh lerbohe-1*, and AC lines were selected and dyed with Alexander to visualize pollen activity (**Figure 3**). There were much fewer pollens spread from anthers of two male sterile mutant lines (*lerbohe-1*, *lerboh lerbohe-1*) than wild type or *lerboh* ones. And as expected,

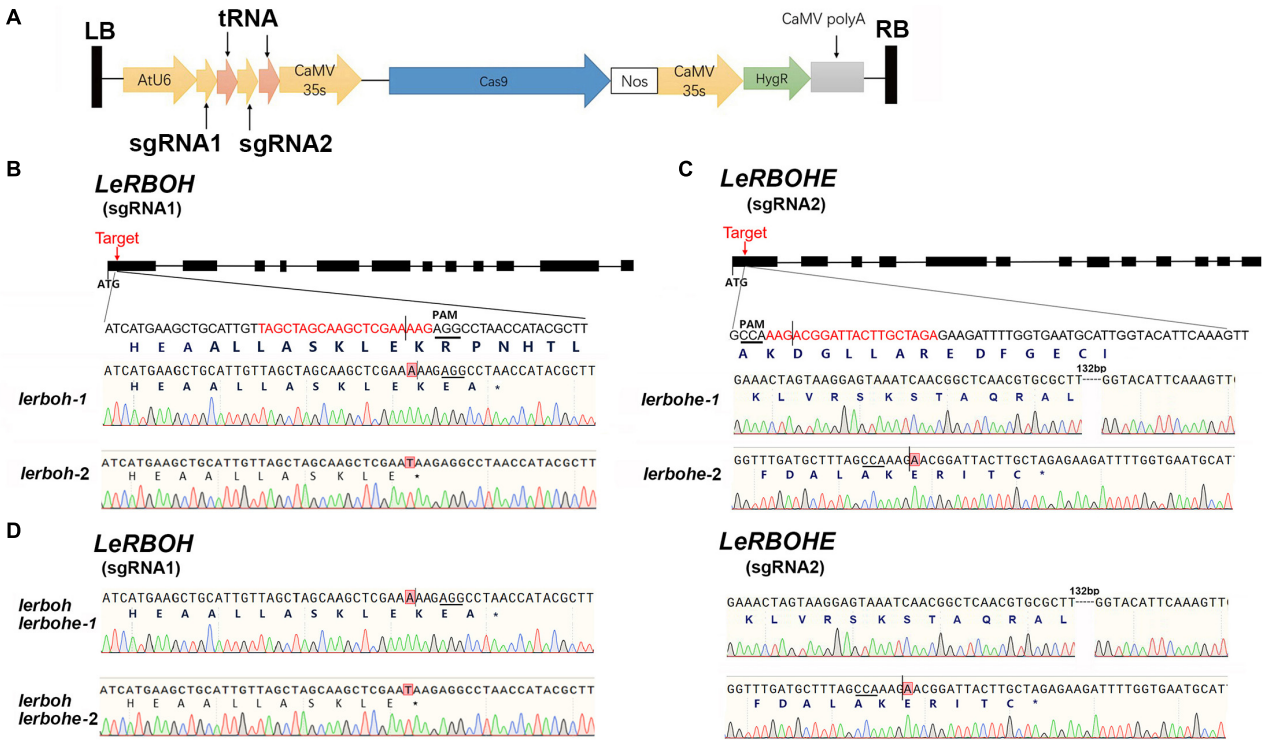


FIGURE 1 | Construction of *lerboh/lerbohe* via CRISPR/Cas9. **(A)** Structure of T-DNA insertion containing double sgRNAs for *LeRBOH* and *LeRBOHE*, Cas9 driven by the CaMV35S promoter, and *HygR* (hygromycin resistance gene) as a resistance marker. **(B–D)** Mutations in the *LeRBOH* or *LeRBOHE* locus in *lerboh* **(B)**, *lerbohe* **(C)**, and *lerboh lerbohe* **(D)** mutants. Black squares indicate exons while line segments indicate introns in the sketch maps of the *LeRBOH/LeRBOHE* genes (top). The protospacer is marked as red while the PAM is underlined. In the Sanger sequencing results, short vertical lines indicate cleavage sites by Cas9 in the *LeRBOH/LeRBOH* genes. Inserted bases are highlighted. The dotted lines represent deletions in the mutants. * means stop condon.

wild-type and *lerboh* pollens were active, appearing strongly stained, round and full, while nearly all *lerbohe-1* and *lerboh lerbohe-1* pollens were inactive, appearing weakly stained, small, and shriveled. When observed by scanning electron microscope (SEM), many normal oval pollens were released from the dehiscent anthers of AC and *lerboh-1* while only a few shrunken pollens adhered around the uncracked anthers of *lerboh lerbohe-1* (**Figure 3**). Significantly, most *lerbohe-1* pollens looked the same as the double mutant, but a few ones exhibited nearly normal morphology. Thus, our data suggested that the male sterility was largely caused by abnormal pollens.

To understand the specific causes for the abortive pollens, four anther developmental stages, including tetrad, microspore, mitosis, and dehiscence stages (Jeong et al., 2014), were observed through paraffin sectioning (**Figure 4**). No significant differences in anther structure or germ-cell morphology were observed between AC and *lerboh lerbohe-1* during the tetrad stage, when the microsporocyte undergoes meiosis to form a tetrad. At the microspore stage, AC microspores gradually vacuolized after releasing from the tetrads and the cell wall of the tapetum began to deform. However, in *lerboh lerbohe-1* anthers, the tapetum was still hypertrophic and the microspores were shrunken. After that in the mitosis stage, the wild-type cytoplasm-thickened pollens underwent mitosis and most of the cytoplasm of the tapetum cells was degraded to provide nutrients for the microspores, whereas in *lerboh lerbohe-1*, deformed pollens shrunk together and the tapetum layer was still kept thick. Finally, during the dehiscence stage, the gradually maturing wild-type pollens became full, round and the tapetum layer was completely degraded, while the mutant pollens completely shrank without accumulation of organotrophy and tapetum degradation was still not complete. Consistent with the trends of the SEM results (**Figure 3**), *lerbohe-1* exhibited a similar developmental alteration as the double mutant, but contained a few pollens with nearly normal morphology during the dehiscence stage. Together, these results suggest the abortive pollens were probably caused by abnormal degradation of the tapetum layer.

Decreased Reactive Oxygen Species Accumulation and Abnormal Programmed Cell Death in *lerboh lerbohe*

As *RBOH* genes are associated with accumulation of ROS (Xie et al., 2014), we analyzed ROS metabolism in the anthers of *lerbohe-1*, *lerboh lerbohe-1*, and AC via staining with NBT (Nitrotetrazole blue chloride), which visualizes superoxide (**Figure 5** and **Supplementary Figure 5**). In AC, NBT staining was visible in the anthers starting at stage 7 and deepened gradually until stage 11, after which it decreased again. However,

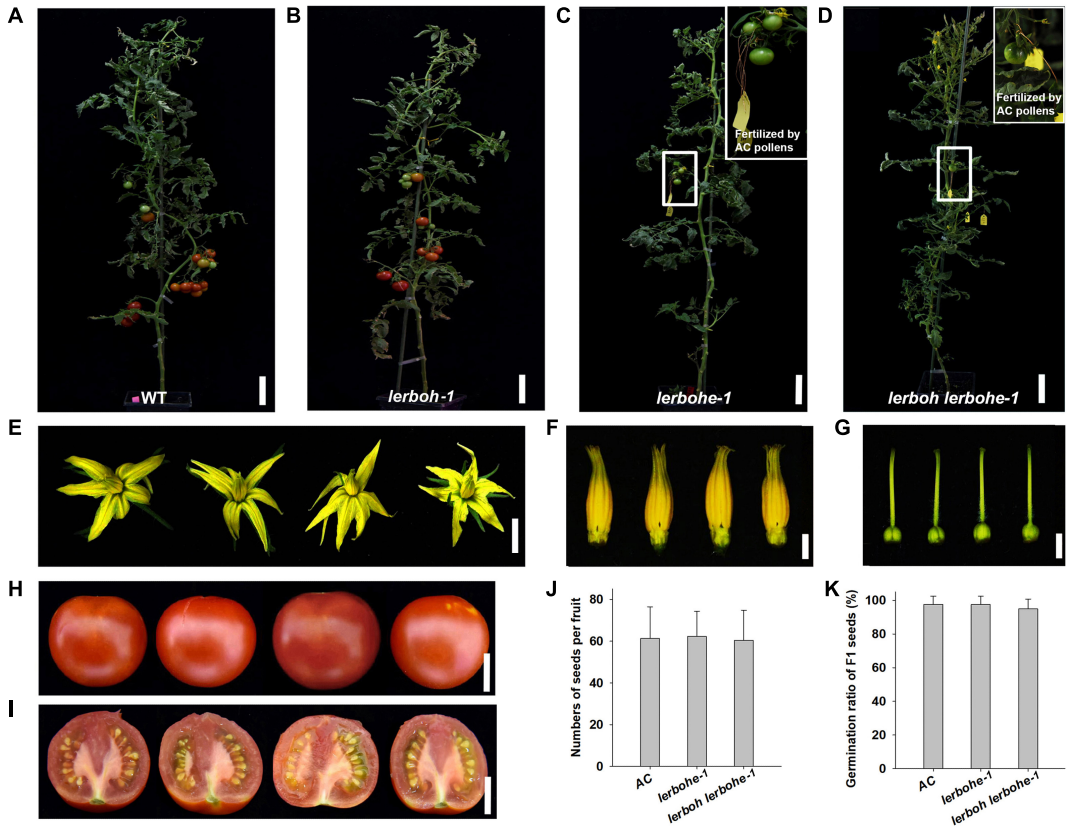


FIGURE 2 | Developmental phenotypic analysis of *lerboh/lerbohe* mutants. **(A–D)** Growth phenotype of WT (AC) **(A)**, *lerboh-1* **(B)**, *lerbohe-1* **(C)**, and *lerboh lerbohe-1* **(D)** plants. The cross fruits fertilized by AC pollens in *lerbohe-1* or *lerboh lerbohe-1* lines are boxed and shown enlarged in the inset. **(E–G)** Phenotypic analysis of flowers **(E)**, stamens **(F)**, and Pistils **(G)** for (from left to right) WT (AC), *lerboh-1*, *lerbohe-1*, and *lerboh lerbohe-1*. **(H,I)** Fruit phenotype of (from left to right) AC, *lerboh-1* (self-fertilized) and *lerbohe-1*, *lerboh lerbohe-1* (fertilized by AC pollens). **(J)** Number of seeds in a fruit of AC (self-fertilized) and *lerbohe-1*, *lerboh lerbohe-1* (fertilized by AC pollens). **(K)** Germination ratio of F_1 seeds. Scale bar: plants, 10 cm; flowers, 1 cm; flower organs, 3 mm; fruits, 2 cm.

TABLE 1 | Growth phenotypic analysis of *lerboh/lerbohe* mutants.

	AC	*lerboh-1*	*lerbohe-1*	*lerboh lerbohe-1*
Number of compound leaves	26 ± 3	26 ± 3	27 ± 4	27 ± 3
Number of flower clusters	8 ± 2	8 ± 2	9 ± 2	9 ± 2
Flower number per cluster	11 ± 3	11 ± 3	11 ± 3	11 ± 3
Plant height (cm)	164.3 ± 7.0	163.7 ± 6.0	169.7 ± 8.5	165.0 ± 7.1

NBT staining and thus ROS content of *lerboh lerbohe* anthers was significantly lower than AC throughout anther development. The *lerbohe* mutant showed clear NBT staining through stage 11, although at reduced levels compared to WT (**Figure 5**). In addition, we observed hardly any differences in NBT staining between *lerboh-1* and AC (**Supplementary Figure 5**). These results indicated a potential role of ROS accumulation in anther development caused by *RBOH* genes.

As the degradation of tapetum layers was usually a result of PCD which was also closely related to ROS metabolism, we evaluated the progression of tapetal PCD via the TUNEL (Terminal-deoxynucleotidyl-transferase-mediated dUTP Nick-End Labeling) assay in the WT and *lerboh lerbohe* lines. Since the temporal changes in ROS accumulation were not completely vanished in *lerbohe-1*, we did not further evaluate it (**Figure 5**). In the WT tapetum, positive TUNEL signals (indicating PCD) were visible in the microspore to dehiscence stages, consistent with the gradual degradation of tapetum cells (Joanna, 2003). In contrast, no obvious TUNEL positive signals appeared in the tapetum of the *lerboh lerbohe* mutant until the mitosis stage, but significant positive signals emerged in genital cells since the microspore stage (**Figure 6A**), consistent with the observed tapetum degradation delay and the inactive pollen grains (**Figure 4**). Furthermore, DAPI staining revealed that there were one (in microspores) or two nuclei (in mitotic or mature pollens) in AC cells. However, in *lerboh lerbohe-1* and *lerbohe-1*, only a few one-nuclei cells (microspores and mitotic pollens) were observed and nearly all mature pollens were enucleated

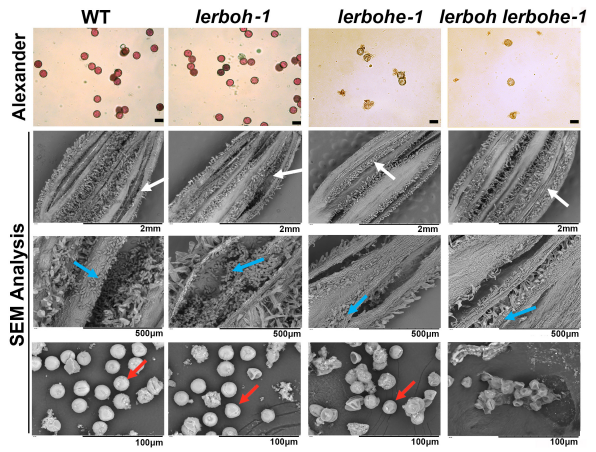


FIGURE 3 | Analysis of pollen morphology and activity in AC, *lerboh-1*, *lerbohe-1*, and *lerboh lerbohe-1* lines. First line, Alexander stain of pollens; second to fourth lines, SEM detection for anthers and pollens. White arrow, anther cracking; blue arrow, pollens; red arrow, pollens with normal morphology. Scale bar in the first line: 20 μm.

(**Figure 6B** and **Supplementary Figure 6**). Together these results demonstrate that our male sterile strain might be caused by abnormal PCD of tapetum cells, which was perhaps associated with the changed ROS accumulation in tomato anthers.

DISCUSSION

lerbohe and *lerboh lerbohe* Mutants Were Useful Male Sterile Strains

Cross-breeding is critical for the tomato industry as it increases fruit yield, stress tolerance and disease resistance. Male sterile lines are invaluable for this process as they significantly promote the efficiency of hybridization (Kim and Zhang, 2018). However, most existing male sterile germplasms still exhibit problematic defects, such as partial self-crossing, difficult artificial pollination, or a low cross-seed setting rate (Perez-Prat and Campagne, 2002; Chen et al., 2004). Here, by mutating two tomato anther-expressed *RBOH* genes via CRISPR/Cas9-mediated gene editing, we obtained male sterile strains with several advantages to existing lines. Firstly, pollens of either *lerbohe* or *lerboh lerbohe* mutants were completely abortive, resulting in no self-crossing (**Figures 2**, **3** and **Supplementary Figure 4**). Secondly, both *lerbohe* and *lerboh lerbohe* flowers had normal pistils (**Figure 2G**) and could bear normal fruits full of active seeds when given normal pollens (**Figures 2H–K**), indicating an easier pollination and a better outcrossing rate in cross-breeding. Thirdly, unlike many weak growing mutants which have fewer flowers, knocking out *LeRBOHE* and/or *LeRBOH* did not noticeably influence the numbers and the reproductive function of flowers, except for the abnormal maturation of anthers (**Figure 2**). In addition, although our mutants were strict male sterile, they could be maintained by hybridization between female parent *lerbohe* or *lerboh lerbohe* and the male parent *LeRBOHE*$^{\pm}$ or *lerboh LeRBOHE*$^{\pm}$, respectively, by which half F_1 plants were identified as male sterile lines after flowering while another half were heterozygote used for the next round of hybridization. Taken together, we consider *lerbohe* and *lerboh lerbohe* mutants as useful male sterile strains for tomato hybrid breeding.

LeRBOHE/*LeRBOH* Regulated Pollen Maturation via Reactive Oxygen Species Accumulation and Tapetum Programmed Cell Death

Abortive pollens are usually caused by abnormal development of the tapetum, which provides nutritional support for the

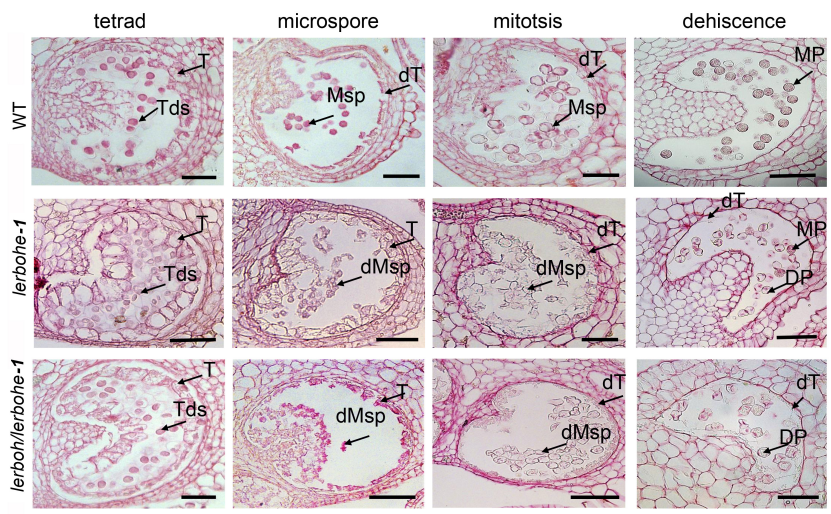


FIGURE 4 | Phenotypic analysis of the tapetum layer in AC, *lerbohe* or *lerboh lerboh* anthers via paraffin sectioning. T, tapetum; dT, degraded tapetum; Tds, tetrad; PMC, pollen mother cell; Msp, microspores; MP, mature pollen; DP, degraded pollen; dMsp, degraded microspores. Scale bar: 100 μm.

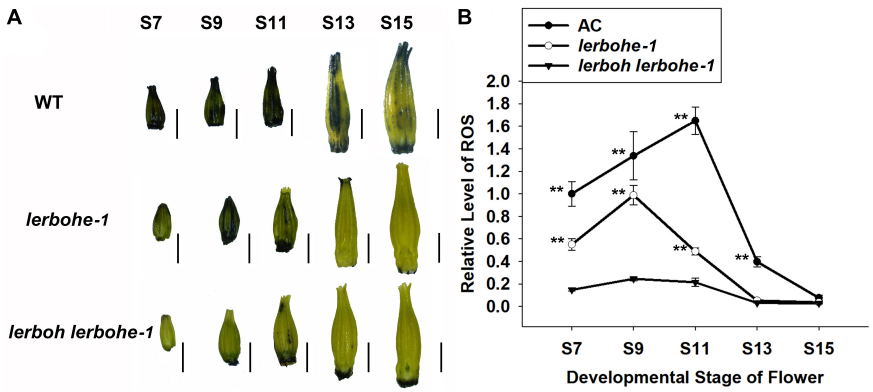


FIGURE 5 | *lerbohe* and *lerboh lerbohe* anthers displayed altered ROS accumulation. **(A)** NBT staining of anthers at different developmental stages. Scale bar: 1 mm. **(B)** Quantification of relative ROS levels calculated from mean pixel densities via Image J. The data was standard by the ROS content in stage7 (S7) of AC. The statistical difference between mutant and AC lines at the same stages was analyzed by Student's *t*-test and ** meant $P < 0.01$.

maturation of microspores (Pacini, 2010). A recent study revealed that *SlMS10* (a *bHLH* transcription factor) regulates meiosis and cell death of the tapetum during microsporogenesis in tomatoes, thereby influencing pollen abortion (Yu et al., 2020). Our male sterile mutants exhibited delayed PCD in the tapetum (**Figure 6**), which would create a starvation stress for the microspores. As a result, we observed dying pollen cells (with abnormal nuclei numbers) and subsequently inactive pollens with weird morphology (**Figures 4**, **6**), which demonstrated the key role of tapetum PCD in tomato pollen maturation. ROS is also regarded as a key regulator of the tapetum (Hu et al., 2011; Luo et al., 2013), and RBOH has been implicated in ROS production (Xie et al., 2014). In our work, *lerbohe* and *lerboh lerbohe* mutants displayed lighter NBT stain and delayed degradation of tapetum than WT (**Figures 5**, **6**), revealing a positive relationship between ROS accumulation and PCD during tapetum development. Taken together, we inferred a *LeRBOHE*/*LeRBOH*-ROS-PCD pathway in regulating tomato pollen maturation.

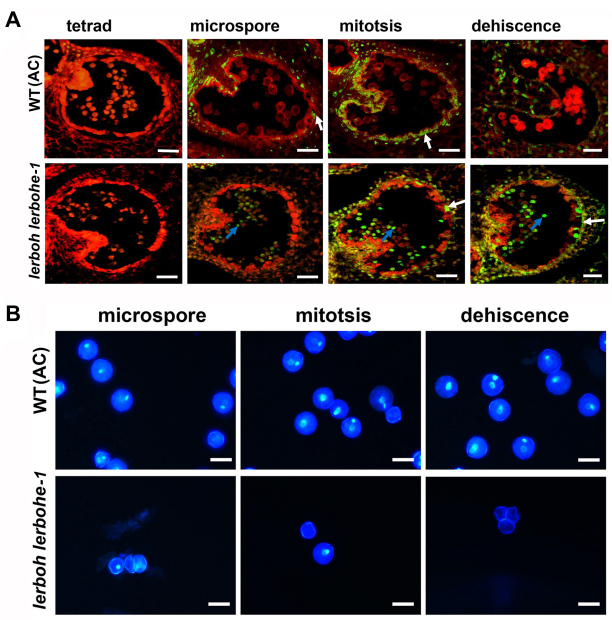


FIGURE 6 | *lerboh lerbohe* anthers exhibited delayed tapetum PCD and abnormal pollen nuclei. **(A)** TUNEL analysis of anthers. Green fluorescence, TUNEL; Red fluorescence, propidium iodide staining; white arrow, degraded tapetum layers; blue arrow, degraded pollens. Scale bar: 75 μm. **(B)** DAPI staining of pollens. The bright, white dots are the pollen nuclei. Scale bar: 25 μm.

LeRBOHE and *LeRBOH* Regulated Male Sterility Unequally

In *Arabidopsis*, RBOH constitutes a protein family consisting of many conserved members which have different expression patterns and influence ROS synthesis in different tissues (Sun et al., 2015). Among the 8 tomato *RBOH* genes, *LeRBOHE* and *LeRBOH* exhibit expression peaks in the anthers, with *LeRBOH* being specifically expressed only in the anthers (Yu et al., 2017). Unexpectedly, we did not observe any abnormal phenotypes in neither the anthers nor pollens of the *lerboh* line, which suggests *LeRBOH* is not essential for male reproduction (**Figure 2**). Interestingly, our results revealed the key role of *LeRBOHE* in tapetum development in the anthers, as knock out of *LeRBOHE* induced male sterility. This partially agrees with the trends seen with *Arabidopsis RBOHE* (Xie et al., 2014). However, *atrbohe* displays only partial pollen sterility in contrast to the complete male sterility seen with *lerbohe*, illustrating that *RBOHE* is critical during anther development in tomato. However, the decrease in ROS content was less severe in the anthers of *lerbohe-1* than in those of the *lerboh lerbohe* double mutant (**Supplementary Figure 5**). Moreover, we found that there were a few morphologically normal pollens in *lerbohe-1* (**Figure 3**), which were hardly observed in *lerboh lerbohe* anthers. In addition, the morphologically normal pollens in *lerbohe-1* were still enucleated just as the *lerboh lerbohe* ones

(**Supplementary Figure 6**). Together, these results indicate that there is a slight contribution of *LeRBOH* in regulating ROS synthesis and pollen development, although it has no essential effect on male sterility. Thus, we concluded that knocking out *LeRBOHE* was sufficient to generate a male sterile germplasm, but knockout of both *LeRBOH* and *LeRBOHE* rendered all pollens abnormal and non-functional.

According to the researches in many species such as Arabidopsis, rice, and tomato, some *RBOH* homologous express widely during the vegetative growth stage and are closely related to stress tolerance and disease resistance (Yoshiaki et al., 2005; Sun et al., 2015; Orman-Ligeza et al., 2016; Xu et al., 2021). Aside from the high-expression in anthers, some transcriptome data exhibits that *LeRBOH* and *LeRBOHE* also express more or less in vegetative tissues (Tomato Functional Genomics Database), which perhaps indicates their role beyond reproductive regulation. However, we observed no significant difference of vegetative growth phenotype among *lerboh*, *lerbohe*, *lerboh lerbohe*, and wild type tomatoes under normal cultivation conditions, probably due to the redundancy of other *RBOH* homologous expressed in tomato vegetative organs (Yu et al., 2017). In addition, whether *LeRBOH* and *LeRBOHE* play roles in regulating tomato stress response is still worth investigating in future.

CONCLUSION

We found the function of *LeRBOHE* and *LeRBOH* in anther development, revealing the LeRBOHE/LeRBOH-ROS-PCD pathway in regulating pollen maturation of tomatoes. Additionally, the success of disruption tomato male reproduction via editing of *RBOH* genes perhaps could be used in generating male sterile germplasms for tomato hybrid breeding in the near future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XD, HH, CM, and HX conceived the project, analyzed the data, and wrote the manuscript. XD and HH designed the sgRNAs and analyzed the phenotypes of the mutants. Other authors contributed to phenotype analysis and provided important suggestions for this work. HH, CM, and HX supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.817101/full#supplementary-material

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Conflict of Interest: XD, CL, XN, and HX were employed by the company BellaGen Biotechnology Co., Ltd.

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Highly Efficient Generation of Canker-Resistant Sweet Orange Enabled by an Improved CRISPR/Cas9 System

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Sweet orange (*Citrus sinensis*) is the most economically important species for the citrus industry. However, it is susceptible to many diseases including citrus bacterial canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) that triggers devastating effects on citrus production. Conventional breeding has not met the challenge to improve disease resistance of sweet orange due to the long juvenility and other limitations. CRISPR-mediated genome editing has shown promising potentials for genetic improvements of plants. Generation of biallelic/homozygous mutants remains difficult for sweet orange due to low transformation rate, existence of heterozygous alleles for target genes, and low biallelic editing efficacy using the CRISPR technology. Here, we report improvements in the CRISPR/Cas9 system for citrus gene editing. Based on the improvements we made previously [dicot codon optimized Cas9, tRNA for multiplexing, a modified sgRNA scaffold with high efficiency, citrus U6 (CsU6) to drive sgRNA expression], we further improved our CRISPR/Cas9 system by choosing superior promoters [*Cestrum yellow leaf curling virus* (CmYLCV) or *Citrus sinensis* ubiquitin (CsUbi) promoter] to drive Cas9 and optimizing culture temperature. This system was able to generate a biallelic mutation rate of up to 89% for Carrizo citrange and 79% for Hamlin sweet orange. Consequently, this system was used to generate canker-resistant Hamlin sweet orange by mutating the effector binding element (EBE) of canker susceptibility gene *CsLOB1*, which is required for causing canker symptoms by *Xcc*. Six biallelic Hamlin sweet orange mutant lines in the EBE were generated. The biallelic mutants are resistant to *Xcc*. Biallelic mutation of the EBE region abolishes the induction of *CsLOB1* by *Xcc*. This study represents a significant improvement in sweet orange gene editing efficacy and generating disease-resistant varieties *via* CRISPR-mediated genome editing. This improvement in citrus genome editing makes genetic studies and manipulations of sweet orange more feasible.

Keywords: CRISPR/Cas9, citrus, canker, HLB, *CsLOB1*, susceptibility gene, EBE, transcription activator-like effector

INTRODUCTION

Citrus is one of the most important fruit crops worldwide because of its delightful flavor and scent, as well as its health properties. Sweet orange (*Citrus sinensis*) is the most economically important species for the citrus industry. However, citrus production is facing many biotic (e.g., diseases and insects) and abiotic challenges (e.g., drought, flooding, acidity, salinity, heat, cold, drought, and nutrient deficits) (Li, 2009). Among them, citrus diseases such as citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) (Ference et al., 2018) and Huanglongbing (HLB, also known as citrus greening) caused by *Candidatus* Liberibacter asiaticus (Bové, 2006; Wang, 2019; Yuan et al., 2020) are causing devastating effects on the citrus industry. Most elite citrus varieties, including sweet orange and grapefruit varieties, are susceptible to canker and HLB disease. Genetic improvements of citrus *via* conventional approaches are challenging. Most citrus varieties result from human selection of natural mutations or natural hybridization rather than artificial hybridization. Hybridization-based breeding for citrus is hindered by long juvenile stage (3–10 years), a highly heterozygous nature, heterozygous nature of nucellar seedlings (clones of the female parent), and male or female sterility (Omura and Shimada, 2016). It has been suggested that CRISPR-mediated genome editing will revolutionize the genetic improvements of citrus and other tree crops (Dutt et al., 2020; Wheatley and Yang, 2020).

Citrus genome editing mediated by the CRISPR technology was first reported in 2014 (Jia and Wang, 2014a,b). The first biallelic mutant of citrus (Carrizo citrange, a rootstock variety) was reported in 2017 (Zhang et al., 2017a). The first biallelic/homozygous mutations of citrus scion varieties [Pummelo (*Citrus maxima*)] were reported in 2020 (Jia and Wang, 2020). It is noteworthy that Pummelo is highly homozygous (Wu et al., 2018) and relatively easy to generate biallelic/homozygous mutants compared to other citrus species. However, most elite citrus varieties including sweet orange are highly heterozygous and biallelic/homozygous mutants have not been reported to date or showed very low editing efficiency (Jia et al., 2017a, 2019a; Peng et al., 2017). We aimed to further improve the CRISPR/Cas system to make genome editing of elite citrus varieties more achievable and improve the disease resistance of sweet orange against canker. Utilization of resistant varieties is the most efficient and eco-friendly approach to control diseases. However, most commercial citrus varieties including sweet orange are susceptible to citrus canker (Favaro et al., 2020; Ference et al., 2020).

Improvement of genome editing efficiency of plants has been conducted *via* multiple approaches including improvements of the expression of Cas proteins and sgRNA using different promoters. Several promoters for driving Cas9 expression have been tested, such as 35S promoter, ubiquitin promoter (Castel et al., 2019), cell division-specific Yao promoter (Yan et al., 2015), egg cell-specific EC1.2/DD45 (Wang et al., 2015), and RPS5a promoter expressing at early embryonic stage (Tsutsui and Higashiyama, 2017; Ordon et al., 2020). Among them, the ubiquitin promoter and RPS5a promoter have significantly improved genome editing efficacy compared to the commonly used 35S promoter in Arabidopsis, tomato, rice, or maize (Stavolone et al., 2003; Cermak et al., 2017; Tsutsui and Higashiyama, 2017; Castel et al., 2019). Endogenous polymerase III promoters (U3 or U6) improve the expression of sgRNAs (Sun et al., 2015; Qi et al., 2018). In addition, other approaches including codon optimization of the Cas proteins (Li et al., 2014) and temperature optimization (Xiang et al., 2017) have shown promises to improve the efficacy of genome editing.

Xanthomonas citri subsp. *citri* causes the characteristic hypertrophy and hyperplasia symptoms on citrus tissues *via* secretion of PthA4, a transcriptional activator-like (TAL) effector, through the type III secretion system (Swarup et al., 1991; Yan and Wang, 2012; An et al., 2020). PthA4 enters the nucleus and activates the expression of the canker susceptibility gene *LATERAL ORGAN BOUNDARIES 1* (*LOB1*) *via* binding to the effector binding element (EBE) in the promoter region using its central nearly identical tandem repeats of 33–34 amino acids (Hu et al., 2014). The specific recognition between nucleotides and the repeat is determined by the 12th and 13th amino acids of each repeat, known as the "repeat-variable diresidue" (RVD) (Moscou and Bogdanove, 2009; Römer et al., 2009). Mutation of the EBE regions has been used to generate disease resistance crops against TAL effector-dependent pathogens such as bacterial blight of rice (Li et al., 2012; Oliva et al., 2019). In our previous study, genome modified Duncan grapefruit of the coding region of *LOB1* demonstrates canker resistance (Jia et al., 2017b). Mutation of both alleles of the EBE region of *LOB1* is required to abolish its induction by PthA4 and generate canker-resistant citrus plants (Jia et al., 2016, 2021; Jia and Wang, 2020). However, biallelic/homozygous mutation of the EBE region for elite varieties such as sweet orange is yet to be achieved. Most importantly, highly efficient generation of biallelic/homozygous mutation in elite varieties such as sweet orange and grapefruit has not been reported yet.

In this study, we improved the CRISPR/Cas9 system, which was used to generate six biallelic *CsLOB1* EBE mutant lines of Hamlin sweet orange. All biallelic *CsLOB1* EBE mutant plants (100%) are resistant against *Xcc*, representing a milestone in canker resistance development for elite citrus varieties. In addition, this improved CRISPR/Cas9 system efficiently (100%) edits the tobacco genome. Hence, this improved CRISPR/Cas9 system may be applied to other dicots besides citrus and tobacco.

RESULTS

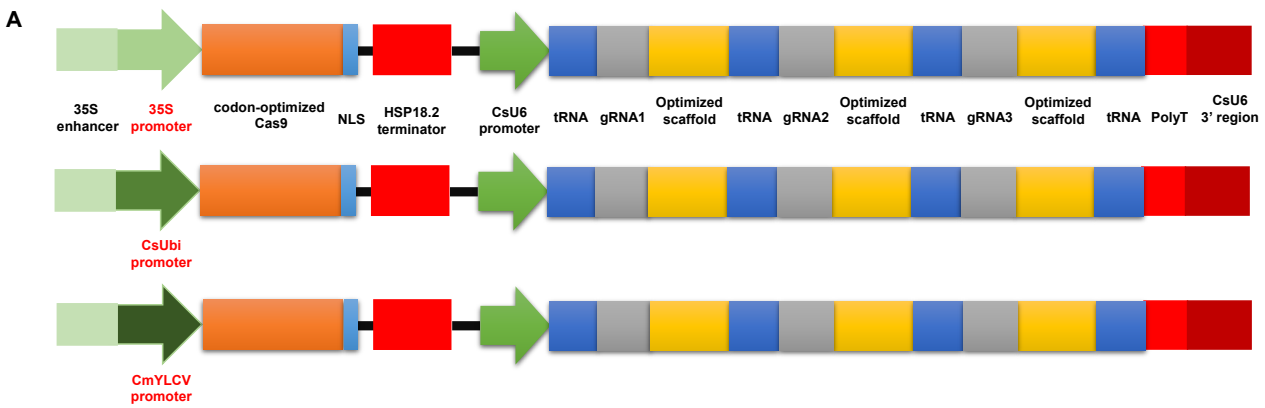
Improvement of CRISPR/Cas9 System for Citrus Genome Editing

First, we sought to improve the CRISPR/Cas9-mediated genome editing efficiency in citrus using the phytoene desaturase (*PDS*) gene as a target. The *PDS* gene was chosen for editing because of the obvious albino phenotype caused by homozygous or biallelic knockout mutation of the *PDS* gene (Zhang et al., 2017a; Huang et al., 2020). The albino phenotype serves as the functional editing (null mutation) readout. We first edited Carrizo citrange, a hybrid between *C. sinensis* "Washington" sweet orange and *Poncirus trifoliata*, which can be relatively easily transformed

(compared to sweet orange) and edited based on our previous experience (Jia et al., 2017a; Huang et al., 2020). A green fluorescence (GFP) visual maker was included in the constructs to further confirm successful plant transformation in addition to antibiotic marker selection. Inspired by previous reports showing that increased culture temperature can increase editing efficacy (LeBlanc et al., 2018; Malzahn et al., 2019; Milner et al., 2020), we sought to test if we can increase editing efficacy by increasing culture temperature. The editing efficiency was higher at 30°C for both gene editing in protoplasts (**Supplementary Figure 1A** and **Supplementary Table 4**) and in *Agrobacterium*-mediated transformed epicotyl tissue than that at room temperature (**Supplementary Figure 1**).

Previously, we improved the CRISPR/Cas9 system by codon optimizing the Cas9 gene, identifying citrus U6 (CsU6) promoter, using an improved gRNA scaffold, and inclusion of tRNA for multiplexing (Xie et al., 2015; Huang et al., 2020). Here we aimed to select a superior promoter for driving Cas9 expression. The 320 bp core region of the 35S promoter in the CRISPR/Cas9 construct was replaced with either the *Citrus sinensis* ubiquitin (CsUbi) or *Cestrum yellow leaf curling virus* (CmYLCV) promoter (**Supplementary Data 1**). The CsUbi promoter (Cs4g11190) was selected through mining of citrus RNAseq data (Ribeiro et al., 2021). Cs4g11190 is highly expressed in various stages of leaf development (**Supplementary Table 1**). The promoter region of CsUbi (Cs4g11190) was amplified from the citrus genome. The CmYLCV promoter (Stavolone et al., 2003) is a strong promoter and functions in both monocots and dicots. The upstream 538 bp regulatory region containing the enhancer elements of 35S was kept and fused with candidate promoters (**Figure 1A**) to enhance Cas9 expression and/or promote early expression of Cas9 in first dividing cells (Ow et al., 1987; Benfey et al., 1990). The editing efficacy of 35S, CsUbi, and CmYLCV driving Cas9 expression at 30°C was compared (**Figure 1A**). The albino phenotype was used as a readout for null mutations (**Figure 1B**). The 35S promoter produced 50% albino plants; the CsUbi promoter and CmYLCV promoter generated 80 and 89% albino plants, respectively (**Figure 1C** and **Supplementary Figure 2**), suggesting that CsUbi and CmYLCV promoters are superior to 35S for gene editing in citrus.

In all transformation events, we observed chimeric albino phenotype. It is common to observe chimera phenotypes during plant transformation of explants, such as epicotyledons, leaves, and stems. The chimera rate for the 35S, CsUbi, and CmYLCV was 25, 12, and 5.6%, respectively (**Figure 1C**). These results suggest that CsUbi and CmYLCV promoters drive an early expression of the Cas9 in the first transformed cells compared to 35S promoter. All constructs contained GFP. Not surprisingly, GFP florescence was observed to colocalize with albino phenotype. Therefore, for all the transformation experiments, only transformants with evenly distributed GFP florescence all over the plantlets were kept for further analyses.



Promoter	35S	CsUbi	CmYLCV
# of positive transformants	16	25	18
# of albino phenotype	8	20	16
# of chimera phenotype	4	3	1
# of wild type phenotype	4	2	1
Null mutation rate	50%	80%	89%

FIGURE 1 | Optimal promoters for driving Cas9 expression to improve gene editing efficacy in citrus. **(A)** 35S promoter, citrus ubiquitin promoter (CsUbi), and *Cestrum yellow leaf curling virus* (CmYLCV) promoter for driving Cas9 expression in the CRISPR constructs. **(B)** A representative picture showing albino phenotype, caused by null mutation of the *PDS* gene with CmYLCV promoter construct. The construct contains GFP as visual marker. **(C)** Summary of editing efficacy from three constructs shown in **(A)**.

The Improved CRISPR/Cas9 System Efficiently Edits Tobacco (*Nicotiana tabacum*)

The CmYLCV promoter is a strong constitutive promoter for heterologous gene expression in *Arabidopsis thaliana* and *Nicotiana tabacum* and in a wide variety of crops including *Lycopersicon esculentum*, *Zea mays*, and *Oryza sativa* (Stavolone et al., 2003). We hypothesized that the improved CRISPR/Cas9 system driven by CmYLCV works in other plant species, especially dicots. To test this hypothesis, we tested the construct in tobacco (*N. tabacum*), an allotetraploid species, considering its amenability to stable transformation. There are two *PDS* homologs, *PDS-A* and *PDS-B* in tobacco genome. Two gRNAs were designed in the conserved regions between the two homologs (**Figure 2A**). We obtained a total of 11 transformants with GFP. The transformants with green fluorescence all showed albino phenotype (**Figure 2B** and **Supplementary Figure 3**), indicating that we achieved tetra-allelic mutations for *PDS-A* and *PDS-B* in all positive transformants (**Figure 2C**). This result demonstrates that the improved CRISPR/Cas9 system achieves a 100% null mutation efficacy for *PDS* genes in tobacco. This result suggests that our improved CRISPR/Cas9 system may be able to efficiently edit genomes of other dicots, besides citrus and tobacco.

Highly Efficient Generation of Biallelic Mutation in Sweet Orange

Sweet orange is a hybrid between pummelo (*C. maxima*) and mandarin (*Citrus reticulata*), and most genes have two different alleles. Here, we used the improved CRISPR/Cas9 system that contains a codon optimized Cas9, tRNA for multiplexing (Xie et al., 2015; Huang et al., 2020), CsU6 promoter (Huang et al., 2020), improved sgRNA scaffold (Dang et al., 2015), and the CmYLCV promoter to edit the EBE in the promoter region of the canker susceptibility gene *CsLOB1* (Hu et al., 2014). The EBE region of sweet orange contains two different types/alleles. We aimed to edit both alleles to create null mutations.

Two different gRNAs for both type I and type II EBEs were designed. A third gRNA targeting a region upstream of the translation start codon ATG was also included (**Figure 3A**). The final construct contains these three gRNAs. Such a design aimed to create not only small indels, but also longer deletion mutations. We first transformed citrus protoplasts to confirm the functionality of the construct. As expected, the construct not only produced indel mutations, but also generated relatively long deletion mutations (**Figures 3B–D**).

Next, we performed a stable transformation of Hamlin sweet orange epicotyls. A total of 16 positive transformants were obtained. Two lines died during regeneration. Finally, we obtained 14 transgenic plants. We genotyped these 14 seedlings. Among them, 11 plants contained biallelic mutations in the EBE region (**Figure 4** and **Supplementary Figure 4**), representing a 78.6% biallelic mutation rate (**Supplementary Table 2**). The three heterozygous mutants contained both wild-type and edited EBEs. Subsequently, we micro-grafted these seedlings onto the rootstock variety Carrizo citrange. A total of six biallelic mutants and two heterozygous mutants were successfully grafted on the rootstock, whereas the rest did not survive.

Biallelic Mutants Are Resistant to *Xanthomonas citri* subsp. *citri*

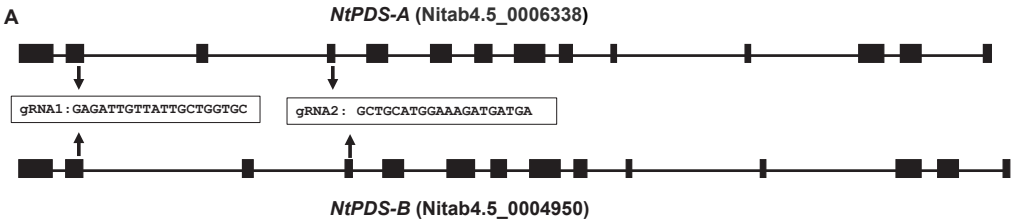
We hypothesized that the biallelic mutation of the EBE region of *CsLOB1* disrupts its binding to *Xcc* TAL effector PthA4, thus *Xcc* is unable to induce canker susceptibility gene *CsLOB1* for symptom development (Swarup et al., 1992; Hu et al., 2014). To test this hypothesis, wild-type Hamlin sweet orange plants, one heterozygous mutant line (line #11) and six biallelic mutant lines (line #1, 2, 3, 4, 5, 15) of Hamlin sweet orange were tested for resistance against *Xcc* strains (**Figure 5**). For each genotype, one half of the leaf was inoculated with wild-type *Xcc*, and another half was inoculated with *Xcc pthA4*:Tn5 mutant carrying the designer TALE dLOB2, which activates the expression of *LOB2* (Teper et al., 2020). *LOB2* is a *LOB1* homolog that causes canker symptoms when artificially induced, such as in the presence of dLOB2 (Zhang et al., 2017b; Teper et al., 2020). *Xcc pthA4*:Tn5 dLOB2 was included as a control. The onset of canker symptoms by *Xcc pthA4*:Tn5 dLOB2 on the same leave can exclude the possibility that lack of canker symptoms for biallelic mutants is owing to leaf age. At 8 dpi, all wild-type Hamlin leaves developed canker lesions for both wild-type *Xcc* and dLOB2 carrying *Xcc pthA4*:Tn5. No canker symptoms were observed for the biallelic mutant lines (line #1, 2, 3, 4, 5, and 15) when inoculated with wild-type *Xcc*. On the contrary, canker symptoms were observed on the other half of the leaves inoculated with *Xcc pthA4*:Tn5 dLOB2. The heterozygous mutant line #11 showed canker disease symptoms for both wild-type *Xcc* and dLOB2 carrying *Xcc pthA4*:Tn5 (**Figure 5**). Taken together, the present results demonstrate that the biallelic Hamlin sweet orange mutant lines are resistant to *Xcc*.

Xanthomonas citri subsp. *citri* Does Not Induce the Expression of *CsLOB1* in Biallelic Mutants

Next, we tested whether biallelic mutations of the EBE region of *CsLOB1* abolish its induction by *Xcc* using reverse transcription-quantitative PCR (RT-qPCR). The expression level of *CsLOB1* in wild-type Hamlin sweet orange was dramatically induced when inoculated with *Xcc*, which is consistent with previous findings (Hu et al., 2014; Teper et al., 2020). The induction of *CsLOB1* was obliterated in the biallelic mutant line Ham1 (**Figure 6A**). *Xcc pthA4*:Tn5 dLOB2 induced the expression of *LOB2* (**Figures 6B,C**), which is consistent with canker symptoms induced by *Xcc pthA4*:Tn5 dLOB2 on both wild-type and biallelic mutant lines (**Figure 5**) and with our previous result (Teper et al., 2020).

Analysis of Off-Targets

To investigate whether this improved CRISPR/Cas9 can introduce off-target mutations, we amplified and sequenced 20 potential off-target sites with 4 or fewer mismatches within the protospacers in all 6 canker-resistant Hamlin mutant lines. No potential off-targets containing less than 4 mismatches were



B



C

# of positive transformants	11
# of albino phenotype	11
# of wild type phenotype	0
# of chimera phenotype	0
Null mutation rate (tetra-allelic mutation)	100%

FIGURE 2 | The improved CRISPR/Cas9 system works efficiently for genome editing of tobacco (*Nicotiana tabacum*). **(A)** Gene structures of two *NtPDS* homologs from allotetraploid *Nicotiana tabacum*. Two gRNAs conserved in both *NtPDS-A* and *NtPDS-B* genes as indicated were designed for genome editing. **(B)** A representative picture shows the albino phenotype in genome modified *Nicotiana tabacum*. The construct contains GFP as visual marker. **(C)** Summary of editing efficacy.



FIGURE 3 | *CsLOB1* editing in Hamlin sweet orange protoplasts. **(A)** *CsLOB1* promoter contains two different types. The EBE region was highlighted with yellow; regions chosen for gRNAs were underlined; PAM was shown in red. The final CRISPR constructs contains three gRNAs as indicated. **(B)** PCR products generated by two primers spanning the EBE and last gRNA. The PCR product with an arrow was amplified from the deletion mutants. **(C)** Sanger sequencing results of the PCR product with the arrow in **(B)**. Twenty clones were sequenced. m1, 11 clones; m2, 5 clones; m3, 4 clones. **(D)** Sanger sequencing results of the PCR products marked by * in **(B)** that were cloned for colony sequencing. Ninety-four clones were sequenced. m4, 2 clones; m5, 2 clones; m6, 1 clone; WT, 89 clones.

found. Hence, it is very likely that there are no off-targets. The sequencing results showed that no mutations at potential off-target sites were detected (**Supplementary Table 5**).

DISCUSSION

In this study, we improved CRISPR/Cas9 system to achieve high efficacy for biallelic editing in citrus. The improved system contains a dicot codon-optimized Cas9, tRNA for multiplexing, an improved sgRNA scaffold with high efficiency (Dang et al., 2015), CsU6 to drive sgRNA expression, and superior promoters (CmYLCV or CsUbi promoter). The dicot codon-optimized Cas9 can work efficiently in tomato (Cermak et al., 2017), citrus, and *N. benthamiana*. The improved sgRNA scaffold contains a shorter T-stretch to avoid early termination and 5 nt extensions of the tetraloop for stability (Dang et al., 2015). Importantly, this improved CRISPR/Cas9 system has significantly increased the biallelic/homozygous mutation rates. For Carrizo citrange, the CsUbi and CmYLCV driven constructs achieved null mutation rates of 80 and 89%, respectively, *via* epicotyl transformation. In addition, the CmYLCV construct

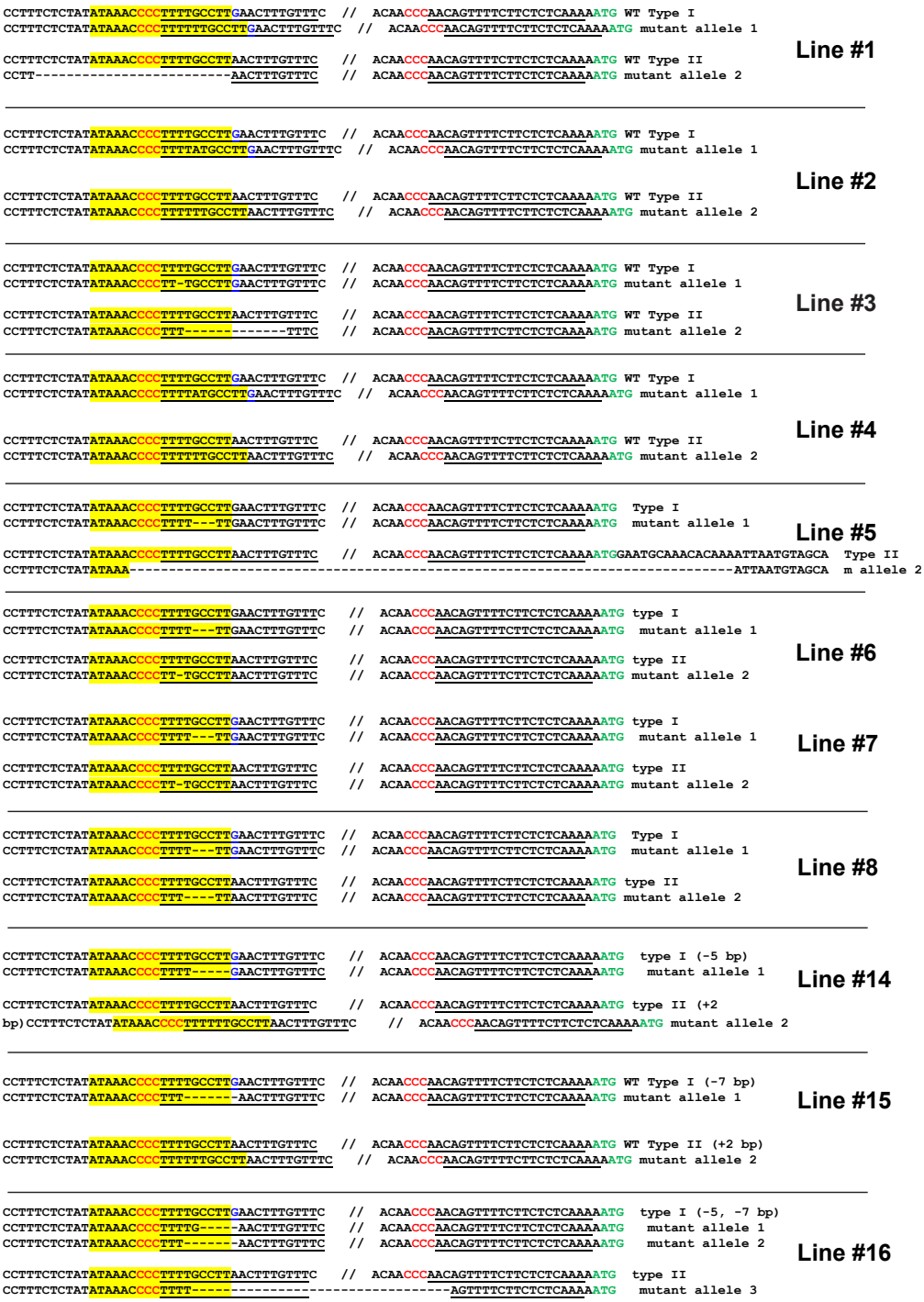
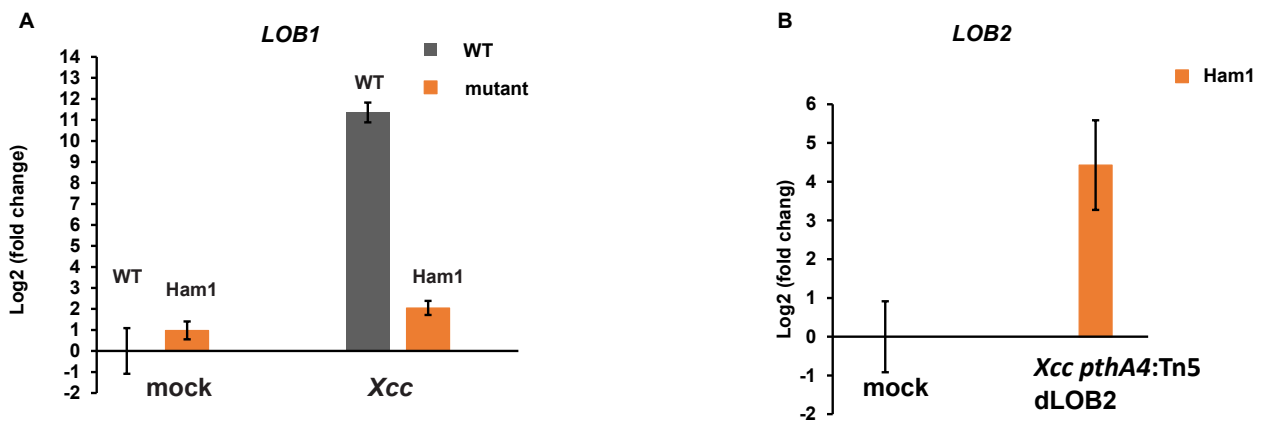


FIGURE 4 | Highly efficient generation of biallelic mutant citrus plants for Hamlin sweet orange. Genotyping of biallelic mutants. Mutations of the EBE region is the focus of the genotyping. The EBE region was highlighted with yellow; regions chosen for gRNAs were underlined; PAM was indicated in red; nucleotide G in blue is a SNP only in one allele, but not in another allele.

attained a 79% biallelic mutation rate in Hamlin sweet orange even under restrained gRNA options (EBE region). The difference in editing efficacy may also result from the differential transformation efficacy in different citrus genotypes. The transformation efficacy was reported to be 20.6% for Carrizo citrange (Pena et al., 1995), but ranged from 0.88 to 23.8 for sweet orange varieties (Poles et al., 2020) and was 2.5% for Hamlin sweet orange (Fávero et al., 2012). Previously, the biallelic mutation rate was reported to be 5.3% for grapefruit (Jia et al., 2021), 44.4% for Carrizo citrange (11.1% homozygous



FIGURE 5 | Biallelic mutants of the EBE region of *CsLOB1* of Hamlin sweet orange are resistant to *Xcc*. *Xcc* inoculation assay was conducted using fully expanded young leaves. Line #11 is a heterozygous mutant; other lines are biallelic mutants. For each line, left half of the tested leaf was syringe inoculated with wild-type *Xcc* (10^8 CFU/ml); the right half of the tested leaf was inoculated with *Xcc pthA4*:Tn5 carrying designer TAL effector dLOB2 (10^8 CFU/ml). Pictures were taken at 8 days post inoculation.



C

TAL effector	Promoter	Score	Target Sequence
PthA4	LOB1 promoter	8.42	TATAAACCCCTTTTGCCTT
dLOB2	LOB2 promoter	3.79	TAGTATAAATACCAACT

FIGURE 6 | Induction of *CsLOB1* by *Xcc* is abolished in a biallelic mutant line of Hamlin sweet orange. **(A)** RT-qPCR analyses of *CsLOB1* relative expression in leaf samples collected at 48 h post inoculation with indicated treatments. Mock, mock inoculation with buffer; *Xcc*, inoculation with wild-type *Xcc* (10^8 CFU/ml); dLOB2, inoculation with *Xcc pthA4*:Tn5 carrying designer TAL effector dLOB2 (10^8 CFU/ml). Each treatment has three biological repeats. Ham1, a representative biallelic mutant line #1 (aka Ham1). All expression levels were normalized to the WT mock. The GAPDH gene was used as an endogenous control. **(B)** RT-qPCR analysis of *LOB2* relative expression of leaf samples collected at 48 h post inoculation with indicated treatment. All expression levels were normalized to the Ham1 mock. The GAPDH gene was used as an endogenous control. **(C)** Finding promoter binding site for TAL effectors by using Target Finder (https://tale-nt.cac.cornell.edu/node/add/talef-off). Designer TAL effector dLOB2 shows affinity to the *LOB2* promoter, but not the LOB1 promoter.

mutation rate) (Huang et al., 2020), and 22.7–55% for Carrizo citrange (Zhang et al., 2017a). Thus, this improved CRISPR/Cas9 system we reported here represents a significant improvement compared to previous studies. In addition, this new system uses a citrus U6 promoter and a CmYLCV promoter to drive sgRNA or Cas9 protein, respectively. It has been recommended to use endogenous promoters to drive Cas proteins or sgRNA to improve efficacy or to address gene regulation concerns. It was reported that U6 promoters and two ubiquitin promoters identified in grapevine significantly increase the editing efficiency in grape by improving the expression of sgRNA and Cas9, respectively (Ren et al., 2021). Similarly, endogenous promoters for U6 and ubiquitin were successfully used for genome editing of maize and yielded mutation efficiencies ranging from 48.5 to 97.1% (Qi et al., 2018). The improved CRISPR/Cas9 system developed in this study can be used not only for editing the genome of citrus, but also for other plant species. We successfully edited tobacco *PDS* genes (100% null mutation) with the CmYLCV-driven construct. We anticipate that the modified CRISPR/Cas9 system we developed is efficient in genome editing of other plant species, especially dicots.

The CmYLCV promoter and CsUbi promoter driving the expression of Cas9 have significantly improved the citrus editing efficacy compared to that of the 35S promoter. It has been reported that strong and constitutive promoters isolated from the plant housekeeping genes such as ubiquitin or actin can achieve higher mutation rates in various monocot and dicot species (Hassan et al., 2021). The CmYLCV promoter was reported to function in both dicots and monocots and shows higher or comparable activities than the 35S promoter in driving the heterozygous expression (Stavolone et al., 2003). The CmYLCV promoter may drive early expression of the Cas9 in the first transformed cells as suggested by the fewest chimera observed. Similar to the 35S promoter, the CmYLCV promoter is an RNA polymerase II promoter. The CmYLCV promoter demonstrates similar efficacy as the Ubiquitin 2 promoter of switchgrass in transformation, reaching about 80 and 100% of protoplasts after 24 and 48-h, respectively, post transformation (Weiss et al., 2020). In addition to driving the expression of Cas proteins, the CmYLCV promoter has also been successfully used to drive the sgRNA array *via* Csy4-mediated processing or gRNA–tRNA array (Cermak et al., 2017).

We successfully obtained multiple biallelic canker-resistant Hamlin sweet orange mutant lines against *Xcc*, which is a TAL effector-dependent pathogen, *via* editing the EBE region of canker susceptibility gene *LOB1* (Hu et al., 2014). TAL effectors are critical virulence factors for most *Xanthomonas* pathogens (Boch and Bonas, 2010; Perez-Quintero and Szurek, 2019; An et al., 2020). TAL effectors are known to be subject to repeated recombination that allows them to recognize new targets or overcome the resistance resulting from mismatches between the TAL effectors and the EBE regions. It was reported that *Xcc* is able to overcome 2–5 mismatches between the repeat region of PthA4 and the EBE of *CsLOB1*, but is unable to overcome the resistance when the mismatch is equal or more than 7 (Teper and Wang, 2021). However, the previous study was conducted *via* an accelerated evolutionary approach that includes injection with high titers of *Xcc* than normally encountered in natural settings. In addition, the TALEs were cloned into pBBR1MCS5, a medium copy number vector (approximately 30 copies), whereas naturally occurring PthA4 is three copies in each bacterial cell (Teper and Wang, 2021). It is anticipated that the chance for *Xcc* to overcome the mismatch between PthA4 and the EBE region in natural settings is much lower than that observed *via* the accelerated evolutionary study (Teper and Wang, 2021). Additionally, the genome modified lines will enable us to investigate whether *Xcc* can overcome the resistance resulting from EBE mutations in natural settings in future studies. The biallelic mutants also can serve as a diagnostic tool to monitor emerging *Xcc* strains that target other susceptibility genes such as *LOB2* and *LOB3* (Zhang et al., 2017b; Teper et al., 2020). A similar strategy has been developed for *Xoo*/rice pathosystem (Eom et al., 2019).

Of note, the canker-resistant sweet orange lines contain the CRISPR/Cas construct, which is considered as transgenic and might not be suitable to be used for citrus production. Nevertheless, successful generation of canker-resistant sweet orange represents a milestone in the ultimate utilization of canker-resistant citrus varieties based on mutations of the coding or the EBE region of the canker susceptibility gene *LOB1*. Generation of canker-resistant citrus varieties will overcome the significant issues in canker management using copper-based products (Zhang et al., 2003), antibiotics (Hu and Wang, 2016; Li et al., 2019, 2020), and biocontrol bacteria. Such an approach has been successfully used to generate disease-resistant rice varieties (Oliva et al., 2019). For rice, the CRISPR/Cas construct can be easily removed by progeny segregation or back-crossing, which is not practical for citrus. This is because citrus has long juvenile stage. In addition, back-crossing leads to the loss of the elite quality of parental varieties. In future studies, we will aim to generate non-transgenic genome-modified canker-resistant citrus varieties *via* transient expression of the CRISPR/Cas construct. Such a method has been successfully used to generate non-transgenic genome-edited crops (Zhang et al., 2016; Chen et al., 2018; Lin et al., 2018; Veillet et al., 2019). Development of methods using *in vitro* assembled CRISPR/Cas9/gRNA ribonucleoprotein (Cas9/gRNA RNP) complexes for transient expression of the CRISPR/Cas9 system in citrus may also be an attractive future option given that this approach has proven successful in producing other crop plants that contain precisely edited genes and that are non-transgenic (Woo et al., 2015; Svitashev et al., 2016; Zhang et al., 2021). In addition, newly emerging DNA delivery technologies such as nanoparticles (Demirer et al., 2019; Lv et al., 2020) might help deliver our highly efficient constructs to achieve non-transgenic genome editing.

MATERIALS AND METHODS

Plant Materials

The seeds of Carrizo citrange were purchased from Lyn Citrus Seeds (Arvin, CA, United States). The seeds of sweet orange Hamlin were harvested from mature fruits in the groves of the Citrus Research and Education Center, University of Florida.

Constructs

To test the performance of different promoters for driving Cas9, we replaced the 320 bp core region of the 35S promoter in construct pC-CsU6-tRNA-3PDS, which was reported previously (Huang et al., 2020), with promoters used in this study. Specifically, the 35S promoter sequence was digested with *Bsp*EI and *Sal*I from pC-CsU6-tRNA-3PDS. CsUbi or CmYLCV (**Supplementary Data 1**) was PCR amplified and inserted into the same site to replace the 35S promoter. PC-CsUbi-PDS and PC-CmYLCV-PDS constructs were obtained and confirmed by Sanger sequencing. To make the *CsLOB1* EBE CRISPR construct, primers LOBPro3-F1/R1 and LOBPro3-F2/R2 (**Supplementary Table 3**) were used to make multiplex constructs using the transient vector pXH1-CsU6-tRNA *via* Golden gate cloning as described previously (Huang et al., 2020). Three gRNAs were included: one targeting type I EBE region of *CsLOB1*, one targeting type II EBE region of *CsLOB1* (Jia et al., 2016, 2017b; Jia and Wang, 2020), and one targeting a region upstream of ATG. The construct PXH1-LOB1-Pro3 was confirmed by Sanger sequencing. The CmYLCV promoter sequence (**Supplementary Data 1**) was cut from PC-CmYLCV-PDS with *Sph*I + *Sal*I and inserted in the same site of PXH1-LOB1-Pro3 to replace the 35S promoter to make the transient expression construct PXH1-CmYLCV-LOB1-Pro3. This construct was used for protoplast transfection.

For stable transformation, the cassette was cut from PXH1-CmYLCV-LOB1-Pro3 with *Fsp*I and *Xba*I and inserted into the *Zra*I/*Xba*I site of the binary vector PCXH2, which carries the GFP expression cassette to make the final construct PCXH2-CmYLCV-LOB1-Pro3. This construct, after transforming into *Agrobacterium* strain EHA105, was used for *Agrobacterium*-mediated citrus epicotyl transformation.

For tobacco *NtPDS* gene editing, we also used the same tRNA-based multiplex vector as described above. The Cas9 is driven by CmYLCV promoter, and the gRNAs for *NtPDS* are driven by CsU6 promoter. Two gRNAs were designed (gRNA1: GAGATTGTTATTGCTGGTGC and gRNA2: GCTGCATGGAAAGATGATGA) based on the conserved regions between the two *PDS* homologs in the genome of allotetraploid species *N. tabacum*. The final CRISPR construct was transformed into *Agrobacterium* strain EHA105, which was used for stable transformation of tobacco leaf discs.

Protoplast Transformation

Transformation of CRISPR constructs into embryogenic citrus protoplasts was performed as described previously (Huang et al., 2020).

Citrus Transformation

Agrobacterium-mediated transformation of citrus epicotyl was performed by following the protocol described previously (Jia et al., 2019b; Huang et al., 2020). After co-cultivation of epicotyls with *Agrobacterium*, the transformed epicotyls were incubated in regeneration medium at 30°C or at room temperature in the dark for 2 weeks before culturing under light at room temperature. For Carrizo *PDS* gene editing, two independent experiments were performed.

Genotyping of Citrus Transformants

Since the transformation constructs carry the GFP expression cassette, positive transformants were selected under GFP filter in addition to antibiotic selection (Kanamycin). Citrus genomic DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Pandey and Wang, 2019). Positive transformants were further confirmed by Cas9 amplification (primers in **Supplementary Table 3**). Detection of editing in the EBE region of *CsLOB1* promoter was performed by amplifying the target region (primers in **Supplementary Table 3**) with high fidelity DNA polymerase Q5 (New England Biolabs, Ipswich, MA, United States), followed by cloning of PCR products and sequencing.

Xanthomonas citri subsp. *citri* Inoculation

Xanthomonas citri subsp. *citri* wild-type strain 306 and dLOB2 containing *Xcc pthA4*:Tn5 (Yan and Wang, 2012; Hu et al., 2016; Teper et al., 2020) were suspended in 20 mM MgCl2 at 10^8 CFU/ml. The bacteria suspensions were syringe-infiltrated into young fully expanded leaves of wild-type sweet orange Hamlin sweet orange plants or mutant plants (Teper and Wang, 2021). Inoculated plants were kept in a temperature-controlled (28°C) glasshouse with high humidity. Pictures were taken 8 days post inoculation for disease resistance evaluation.

Reverse Transcription-Quantitative PCR

Leaves of wild-type and a biallelic mutant line #1 (hereafter named Ham1) with and without *Xcc* inoculation were sampled at 48 h post inoculation. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's manual. Extracted RNA was further treated with RQ1 RNase-Free DNase (Promega, Madison, WI, United States) to remove genomic DNA contamination, if any. The treated RNA was further purified with RNeasy Mini Kit (QIAGEN, Germantown, MD, United States). One microgram of RNA from each sample was used for cDNA synthesis by using QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative PCR was performed using SYBR Green PCR Master Mix in QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The citrus house-keeping gene *GAPDH* was used as an endogenous control. Quantification of gene expression was calculated by following the comparative *Ct* method (Pfaffl, 2001). Primers for RT-qPCR are listed in **Supplementary Table 2**.

Analysis of Potential Off-Targets

To analyze potential off-targets, we obtained the putative off-targets using a web-based software.[1] The potential off-target sites with four or fewer mismatches were chosen for analysis. Genomic DNA from *LOB1* EBE-edited Hamlin transgenic lines was used as template, and the primers listed in **Supplementary Table 3** were used to amplify the fragments spanning the off-targets.

[1]http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR

Finally, the PCR products were subjected to Sanger sequencing to identify off-target.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XH and NW designed and organized the study. XH performed the experiments. YW contributed to off-target experiments and grafting. XH and NW prepared the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.769907/full#supplementary-material

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Efficient Genome Editing in *Setaria italica* Using CRISPR/Cas9 and Base Editors

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The genome editing toolbox based on CRISPR/Cas9 has brought revolutionary changes to agricultural and plant scientific research. With the development of stable genetic transformation protocols, a highly efficient genome editing system for foxtail millet (*Setaria italica*) is required. In the present study, we use the CRISPR/Cas9 single- and multi-gene knockout system to target the *SiFMBP*, *SiDof4*, *SiBADH2*, *SiGBSS1*, and *SiIPK1* genes in the foxtail millet protoplasts to screen out highly efficient targeted sgRNAs. Then, we recovered homozygous mutant plants with most of the targeted genes through an *Agrobacterium*-mediated genetic transformation of foxtail millet. The mutagenesis frequency in the T_0 generation was as high as 100%, and it was passed stably on to the next generation. After screening these targeted edited events, we did not detect off-target mutations at potential sites. Based on this system, we have achieved base editing successfully using two base editors (CBE and ABE) to target the *SiALS* and *SiACC* genes of foxtail millet. By utilizing CBE to target the *SiALS* gene, we created a homozygous herbicide-tolerant mutant plant. The current system could enhance the analysis of functional genomics and genetic improvement of foxtail millet.

Keywords: CRISPR/Cas9, genome editing, base editing, *Setaria italic*, herbicide resistance

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INTRODUCTION

Foxtail millet (*Setaria italica*) is a very important food crop in Asia and Africa. Compared with other common grains, foxtail millet is rich in protein, vitamins, minerals, and fiber, and the content of some macroelements and microelements is also relatively high, which makes it a nutritious crop (Han et al., 2015). In addition to its excellent nutritional value, foxtail millet also tolerates various abiotic stresses well, has good resistance to pathogens, and exhibits high utilization of nitrogen. It is an environmentally friendly crop that uses less water or fertilizers, and it is a strategic reserve crop to deal with environmental changes such as drought (Lata et al., 2013). Foxtail millet is a diploid crop with a small genome (Zhang et al., 2012). Genome sequencing has been completed, and it has gradually been developed into a xerophytic C4 cereal model crop (Doust et al., 2009). Recently, the Wang group developed a foxtail millet variety "*xiaomi*" with small size and short life cycle by screening the traditional EMS mutant library, which has sped up the use of foxtail millet as a model plant (Yang et al., 2020). However, current basic research on foxtail millet is extremely weak, and

there is a lack of effective research methods. The emergence and development of genome editing technology provides new opportunities and hope for relevant research on foxtail millet.

CRISPR/Cas9 technology is the most effective gene editing system. The system is mainly composed of two parts: single guide RNA (sgRNA) and Cas9 endonuclease (Cong et al., 2013). With the help of sgRNA, specific targets that contain the protospacer-adjacent motif (PAM) are identified and cut, which results in double-strand breaks (DSB) in the host genome, that trigger non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR) pathways to repair the DNA damage. The NHEJ and MMEJ pathways usually generate targeted insertions/deletions (indels), but the HDR pathway introduces precise gene replacements or insertions (Chen et al., 2019; Tan et al., 2020). Base editing was developed based on the CRISPR/Cas9 system, the Cytosine Base Editor (CBE) and Adenine Base Editor (ABE), which is composed of a nickase Cas9 (nCas9) and a cytosine or adenine deaminase. This editing can achieve precise replacement of bases at specific sites (C to T or A to G) without producing double-strand breaks and then cause the substitution of amino acids at specific sites that changes the function of genes (Komor et al., 2016; Gaudelli et al., 2017). In the past 10 years, CRISPR/Cas9 system and the derived base editing systems have been used to do functional genomic studies, trait improvement and breeding in common crops such as rice, maize, wheat, and tomato (Shimatani et al., 2017; Zong et al., 2018; Zhang et al., 2019). However, a highly efficient genome editing system in foxtail millet has not been established yet, although there are a few relevant reports (Lin et al., 2018; Cheng et al., 2021; Zhang et al., 2021). Genome editing in foxtail millet was firstly demonstrated to disrupt *SiPDS* gene in transient protoplast assay, but no stable mutant was obtained (Lin et al., 2018). Cheng et al. (2021) generated a haploid inducer line by using CRISPR/Cas9 technology to target the *SiMTL* gene, with a mutagenesis efficiency of 26%. However, there are still no reports on the application of base editing in foxtail millet.

Here, we report the development of a highly efficient genome editing system with single, multiple gene knockouts and single base substitution using the CRISPR/Cas9 system, cytosine and adenine base editing systems in foxtail millet based on an *Agrobacterium*-mediated transformation. Six genes related to agronomic traits were selected as targets to establish the CRISPR/Cas9 knockout system. Foxtail millet bran protein (FMBP) is a peroxidase that has anti-colon cancer effects (Shan S. et al., 2014). Maize DNA-binding with one finger 1 (Dof1) increases carbon and nitrogen assimilation under low-nitrogen conditions (Kurai et al., 2011). In subsequent experiments, the homologous gene *SiDOf4* was chosen (Zhang et al., 2017). The Betaine aldehyde dehydrogenase 2 (BADH2) inhibits the synthesis of 2-acetyl-1-pyrroline, which is a major component in fragrance (Chen et al., 2008). The Granule bound starch synthase 1 (GBSS1) is responsible for the synthesis of amylose, and interruption of the *GBSS1* gene resulted in waxy maize (Gao et al., 2020). Inositol-pentakisphosphate 2-kinase 1 (IPK1) catalyzes the final step in phytate biosynthesis, which is an ideal target for phytate reduction (Shukla et al., 2009). For base editing experiments, the acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACC) that associated with herbicide tolerant were chosen as primary target genes (Li et al., 2018; Zhang et al., 2020). Homozygous or bi-allelic mutants were obtained in the T_0 generation, and the targeted mutations were transmitted to the next generation.

MATERIALS AND METHODS

Single Guide RNA Design and Vector Construction

For single gene knockout, the potential sgRNAs were designed using CRISPR-P online software[1] and the top two sgRNAs were selected based on the mismatch numbers with other potential off-target sites. Pairs of oligonucleotides of sgRNAs were synthesized, annealed, and cloned into corresponding *Bsa*I-digested pHUE411 plasmid (Xing et al., 2014). To construct the pH-CBE vector, the codon-optimized NLS-APOBEC1-XTEN-nCas9-UGI-NLS fusion protein were synthesized commercially and cloned into the pHUE411 backbone via Gibson Assembly to replace the Cas9 gene. To construct the pH-ABE, the codon-optimized ecTadA WT/7.10 deaminase and three copies of the NLS were synthesized commercially. The ecTadA WT/7.10, nCas9 and 3*NLS were then sequentially cloned into pHUE411 backbone via Gibson Assembly. For base editing experiments, the sgRNAs involved in herbicide resistance were selected and cloned into *Bsa*I-digested pH-CBE and pH-ABE, respectively.

To construct the multiplex genome editing vector using multicomponent transcriptional unit (hereafter MCTU) system to express the sgRNAs that targeted *SiDOf4*, *SiBADH2*, *SiGBSS1*, and *SiIPK1*, three fragments were prepared to assemble the sgRNAs array. MT1-Si9-BsF, MT1-Si9-F0, and MT0-BsR2 were used to amplify fragment 1 from pCBC-MT1T2 plasmid. MT2-Si12-BsF2, MT2-Si12-F0, and MT0-BsR3 were used to amplify fragment 2 from pCBC-MT2T3 plasmid. MT3-Si14-BsF3, MT3-Si14-F0, MT4-Si15-R0, and MT4-Si15-BsR were used to amplify fragment 3 from the pCBC-MT3T4 plasmid. The three fragments were then inserted into the pHUE411 vector using the Golden Gate method (Xing et al., 2014).

To construct the multiplex genome editing vector using polycistronic tRNA-gRNA (hereafter PTG) to express the sgRNAs that targeted *SiDOf4*, *SiBADH2*, *SiGBSS1*, and *SiIPK1*, five fragments were prepared to assemble the sgRNAs array. L5AD5-F/gRSi9-R, gRSi9-F/gRSi12-R, gRSi12-F/gRSi14-R, gRSi14-F/gRSi15-R, and gRSi15-F/L3AD5-R primer pairs were used to amplify five fragments from pGTR plasmid. Then the five fragments were ligated using the Golden Gate method. The products were amplified using the S5AD5-F/S5AD5-R primer pair, digested by *Fok*I and inserted into *Bsa*I-digested pGREB32 plasmid (Xie et al., 2015).

To construct the multiplex genome editing vector using Csy4 separated sgRNA arrays (hereafter Csy4) that targeted *SiDOf4*, *SiBADH2*, *SiGBSS1*, and *SiIPK1*, five fragments were prepared to assemble the sgRNAs array.

[1]http://crispr.hzau.edu.cn/CRISPR2/

oPvUbi1/CSY_gRNASi9, REP_gRNASi9/CSY_gRNASi12, REP_gRNASi12/CSY_gRNASi14, REP_gRNASi14/CSY_gRNASi15, and REP_gRNASi15/CSY_term primer pairs were used to amplify five fragments from pDirect-25H plasmid. Then, the resulting fragments were inserted into the pDirect-25H vector using the Golden Gate method (Cermak et al., 2017). The resultant constructs were confirmed by Sanger sequencing and further used for protoplast transfection or *Agrobacterium*-mediated transformations.

Protoplast Transfection

Foxtail millet protoplast transfection was performed as previously described (Shan Q. et al., 2014) with slight modifications. Mature seeds of foxtail millet were sterilized and grown in 1/2 MS solid medium at 26°C with a photoperiod of 16 h light (full spectrum LED light with ∼150 μmol m^{-2} s^{-1}) and 8 h dark for 16–20 days. The stems separated from seedlings were cut in cross section and incubated in enzyme solution (1.5% Cellulase, 0.3% Macerozyme, 0.6 M mannitol, 10 mM MES at pH 5.7, 1 mM $CaCl_2$, and 0.1% BSA) for 5 h with gentle shaking at 80 rpm. The stems of young seedlings were short, and usually about 10 seedlings were needed for each transformation. After digestion, the enzyme solution was filtered through a 45 μM nylon filter and discarded. Then, the digested tissues were washed 2–3 times with 50 mL W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 4 mM MES) and fresh protoplasts were released from the cutting edge. Protoplasts were collected by centrifuge at 70 *g* for 3 min and resuspended in MMG solution (0.4 M mannitol, 15 mM MgC_{l2}, and 4 mM MES). Twenty μg plasmids were delivered into 200 μL protoplasts using PEG-mediated transfection and incubated for 20 min in the dark at room temperature. Transfected protoplasts were washed with W5 and collected by centrifuge at 70 *g* for 3 min. Protoplasts were incubated in W5 in the dark at RM temperature. After 48 h incubation, the protoplasts were collected and genomic DNA was extracted using the CTAB method. In order to guarantee the success and a relative high transfection efficiency, a GFP plasmid was used for transfection as a positive control (**Supplementary Figure 1**).

Agrobacterium-Mediated Stable Transformation

The resultant constructs were transformed into wild type *Agrobacterium tumefaciens* strain EHA105 using a freeze/thaw method and selected on LB medium that contained kanamycin (50 mg/L) and rifampicin (25 mg/L). The clones were screed by colony PCR. Then, the plasmids in the positive clones were extracted and confirmed by Sanger sequencing. Transformation was carried out following Sood et al. (2020) with minor modification. Briefly, mature seeds of two varieties were de-husked, sterilized, and plated on callus induction medium at 26°C in the dark (i.e., Yugu1 and *xiaomi*; Both seeds obtained from the stock of Prof. Xingchun Wang's lab, College of Life science, Shanxi Agricultural University, China. All plant materials were provided free of charge and used for research only). After 3 weeks, the buds were removed and the induced calli were transferred to fresh callus induction medium for three rounds of subculture (2–3 weeks for each round) to obtain embryonic calli for transformation. Embryogenic calli were incubated with *Agrobacterium* strain EHA105 for 15 min, dried on filter paper and incubated in the dark for 3 days. The transformed calli were then selected with hygromycin (40 mg/L) for 1 month. Thereafter, surviving calli were regenerated at regeneration medium with hygromycin (15 mg/L) at 26°C with a photoperiod of 16 h light and 8 h dark for 6–8 weeks. The regenerated shoots were then plated on the rooting medium with hygromycin (20 mg/L). After 2 weeks, the transgenic plantlets were available for genotyping.

Genotyping and Detection of Mutations

Mutations that occurred in protoplasts assay were detected by two methods: PCR/T7EI assay and deep amplicon sequencing. The PCR/T7EI assay was conducted as described previously (Shan Q. et al., 2014). For deep amplicon sequencing, two rounds of PCR were performed as described previously (Liang et al., 2018). In the first round PCR, PCR products that contained the target site were amplified using gene-specific primers. In the second round PCR, primer sets with forward and reverse barcodes were used to limit the product size to within 200 bp for library construction and Illumina sequencing. NGS reads were analyzed using the Cas-Analyzer.[2] Mutation that occurred in transgenic plants were detected by PCR and subsequent Sanger sequencing. The sequencing chromatograms were deciphered by the DSDecode web tool (Liu et al., 2015).

Herbicide Resistance Test Assay

To detect the effective level of nicosulfuron herbicide, seedlings in the greenhouse of wild type *xiaomi* at the four-leaf stage were sprayed with 30 g ai ha^{-1} concentrations of nicosulfuron, which was an effective concentration of nicosulfuron as determined by the obvious death of wild-type seedlings. Base-edited plant T_1-1-12 with homozygous P170A mutation in ALS alleles were then used in the herbicide-resistance assay. Seedlings in the greenhouse at the four-leaf stage were treated with nicosulfuron at a concentration of 30 g ai ha^{-1}. Photos of plants were taken about 4 weeks after the herbicide treatments. At least three biological replicates were used for each treatment.

RESULTS

Development of CRISPR-Mediated Efficient Gene Knockout of Foxtail Millet

Due to the laborious and time-consuming tissue culture procedure, the protoplast assay system was established to test the activities of the sgRNAs that we designed. We first designed two sgRNAs to target each of the five endogenous genes, *SiFMBP* (*Seita.5G463200*), *SiDof4* (*Seita.1G303300*), *SiBADH2* (*Seita.6G151100*), *SiGBSS1* (*Seita.4G022400*), and *SiIPK1* (*Seita.3G025600*) (**Figure 1A**). The CRISPR-encoding constructs were introduced into protoplasts of foxtail millet using PEG-mediated transfection system that we established

[2]http://www.rgenome.net/cas-analyzer/#!

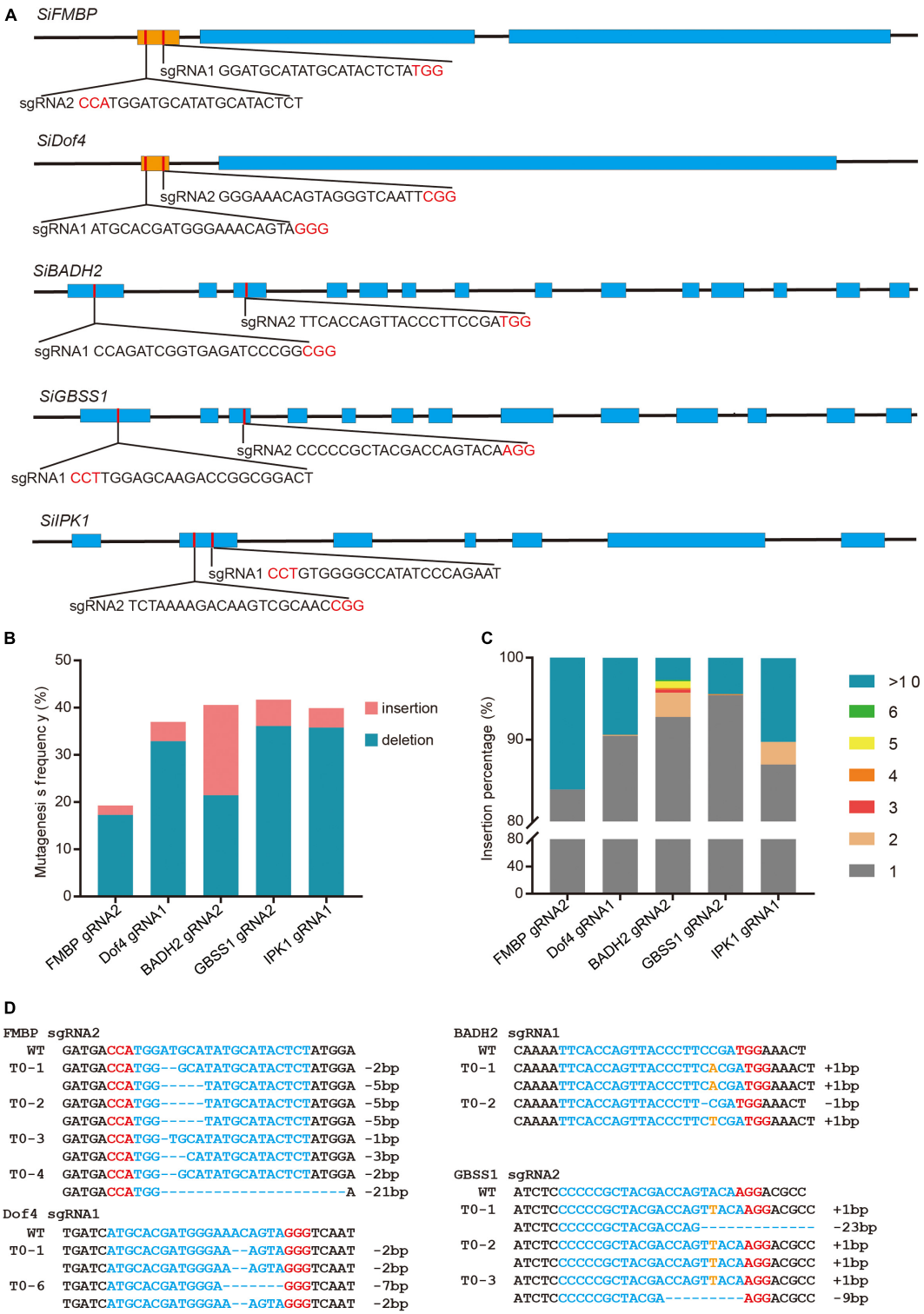


FIGURE 1 | Targeted mutagenesis in foxtail millet using the CRISPR/Cas9 system. **(A)** Schematic structures of the *SiFMBP*, *SiDof4*, *SiBADH2*, *SiGBSS1*, and *SiIPK* genes. The blue and brown boxes indicated exons and uORF, respectively. Two sgRNAs were designed to target each gene. The PAM sequences were highlighted in red. **(B)** Mutagenesis frequencies of *FMBP* sgNRA2, *Dof4* sgRNA1, *BADH2* sgRNA2, *GBSS1* sgRNA2, and *IPK1* sgRNA1 in transient protoplasts assay were analyzed by next generation sequencing. **(C)** Percentages of insertion mutation with different lengths tested in five sgRNAs using transient protoplast assay. **(D)** Sanger sequence analysis of representative T_0 mutants induced by *FMBP* sgRNA2, *Dof4* sgRNA1, *BADH2* sgRNA2, and *GBSS1* sgRNA2. The protospacer and protospacer-adjacent motif (PAM) sequences were highlighted in blue and red, respectively. Nucleotides inserted at the target sites were labeled orange.

following the protocol described previously (Shan Q. et al., 2014) (**Supplementary Figure 1**). Genomic DNA was extracted from the protoplasts incubated for 48 h after transfection and analyzed using a PCR/T7EI method (**Supplementary Table 1**). The mutagenesis efficiencies of the six target sites were determined by the band densities (**Supplementary Figure 2**). NGS was further performed to determine the specific mutation rate and pattern (**Figure 1B** and **Supplementary Figure 3**). In addition to the small indel mutations, there were a few mutation events with large fragments (>10 bp) inserted at the five sites (**Figure 1C**). Among them, the proportion of large insertion at the targets sites of *FMBP* sgNRA2 and *Dof4* sgRNA1 accounted for a relatively high rate (16.11 and 9.42%, respectively). Based on the sequence analysis, the insert fragments were all derived from the plasmids used for editing (**Supplementary Figure 4**).

Next, the sgRNAs (*FMBP* sgRNA2, *Dof4* sgRNA1, *BADH2* sgRNA2, and *GBSS1* sgRNA2) with a higher mutagenesis frequency that had been confirmed with protoplast transient assay for each target gene were selected to produce mutated plants using *Agrobacterium*-mediated transformation. We found that the mutation efficiencies of each target site in T_0 generation can be up to 100% (**Figure 1D** and **Table 1**). For *FMBP* sgRNA2, 2 out of 7 T_0 lines were confirmed as homozygous mutants and the remaining five lines had bi-allelic mutations. Most mutation types were small deletions within 5 bp, except for line #4 which had -21 bp deletion. This longer deletion may be due to MMEJ repair using the "TGG" micro-homology sequence. For *Dof4* sgRNA1, 6 out of 7 T_0 lines were homozygous mutants and the remaining one had bi-allelic mutations. All six homozygous mutants exhibited the same mutation types with -2 bp deletion, which was consist with deep sequencing results from the protoplast assay that showed the -2 bp deletion at the target site was the most frequent mutation type (**Supplementary Figure 3**). For *BADH2* sgRNA2, 5 out of 6 T_0 lines had desired mutations, in which two of them were homozygous mutants, and the others were bi-allelic mutants. For *GBSS1* sgRNA2, we obtained one homozygous and two bi-allelic mutants. Furthermore, the knockout construct with *Dof4* sgRNA1 was also delivered into another foxtail millet variety (cv *xiaomi*), two T_0 lines were regenerated and both of them contained the desired target homozygous or bi-allelic mutations (**Table 1**).

High heritability is of great significance in breeding. Three independent mutants that targeted *FMBP* and *Dof4* genes were selected randomly for self-pollination, and seeds were harvested. Ten T_1 progeny of each mutant line were detected using Sanger sequencing. Mutations that occurred in the T_0 generation were passed successfully to the next generation at a rate of 100% (**Supplementary Table 2**). Based on PCR amplification analysis of the T-DNA region of all T_1 generations, the proportion of progeny without transgenes was in the range of 20–30% (**Supplementary Table 2**). To analysis the off-target effects caused by CRISPR/Cas9, four top-ranked off-target sites of all the sgRNAs used in *S. italica* were predicted by using CRISPR-P (Liu et al., 2017). All the putative off-target sites contained three to four mismatches against the on-target sites (**Supplementary Table 3**). All the T_0 mutant plants that we obtained were analyzed using Sanger sequencing and no off-target effect was found.

Efficient Multiplex Genome Editing System for Foxtail Millet

For multiplex genome editing in plants, sgRNAs can be transcribed from several independent promoters or from a single polycistronic cassette by a single promoter (Cermak et al., 2017). Endonuclease Csy4 and transfer RNA are two RNA cleavage elements in the polycistronic cassette (Tsai et al., 2014; Xie et al., 2015). To achieve multiplexed gene editing in *S. italica*, we tested the MCTU, Csy4, and PTG systems using a protoplast transfection assay. For MCTU method, individual sgRNA cassettes were driven by different Pol III promoters: OsU3, TaU3, and AtU6; for Csy4 method, single transcript with sgRNAs separated by Csy4 hairpins was driven by PvUbi promoter; for PTG method, single polycistronic tRNA-sgRNA transcript was driven by the OsU3 promoter (**Figure 2A**). The multiplex knockout constructs that targeted the *Dof4*, *BADH2*, *GBSS1*, and *IPK1* genes simultaneously were delivered into protoplasts. The PCR/T7EI method test results showed that all four target sites had mutations (**Supplementary Figure 5**). Next, NGS was performed to analyze the mutation efficiency and profiles (**Supplementary Tables 1**, **4**). High indel mutagenesis efficiencies were observed at each target site, which ranged from 30.2 to 45.6%, this was comparable with that generated by single knockout constructs (**Figure 2B**). These results indicated that all the multiplex knockout systems worked effectively in *S. italica* protoplasts. Subsequently, MCTU system was chosen to generate multiple KO events. The MCTU vector was delivered into callus cells using an *Agrobacterium*-mediated transformation to create stable heritable mutants in the four target genes. In the T_0 generation, 4 out of 5 recovered plants contained quadruple mutations and the remaining one contained triple mutations (**Figure 2C**).

Achieved Effective Single-Base Editing in Foxtail Millet

Several base editors have recently been developed to directly introduce precise nucleotide substitutions in endogenous genes. We explored further whether the base editors work efficiently in foxtail millet. BE3 was the most widely used cytosine base editors, which consisted of the rat APOBEC1 deaminase fused with a Cas9 nickase and an uracil glycosylase inhibitor (UGI), introduced C:G to T:A transition into targeted sites (Komor et al., 2016). We codon-optimized the BE3 cassette and cloned it into the pHUE411 backbone to generate the CBE vector (**Figure 3A**). Previous studies found that the missense mutation that occurred in the *OsALS* gene in the P171 amino acid, such as P171S, P171F, and P171A, conferred different levels of herbicide resistance (Kuang et al., 2020; Zhang et al., 2020). By sequence alignment, we selected the P170 amino acid of the *SiALS* (*Seita.1G169700*) gene, which was homologous to OsALS-P171, as the candidate target site to test the cytosine base editors in foxtail millet. sgRNA was designed to target the SiALS-P170 site, and the spacer sequence was inserted into the CBE vector, generating ALS-P170-CBE (**Figure 3B**). The resulted construct was firstly tested in the transient protoplast assay. We performed deep amplicon sequencing to analysis the mutations. We found

TABLE 1 | Summary of T_0 plant characterization.

Target site	Varieties	Genome editing tool	No. of T_0 plants tested	Mutated T_0 lines: number, ratio (%)	Homozygous T_0 lines: number, ratio (%)	Bi-allelic T_0 lines: number, ratio (%)	Heterozygous T_0 lines: number, ratio (%)
FMBP sgRNA2	Yugu1	SpCas9	7	7, 100%	2, 28.6%	5, 71.4%	N.D
Dof4 sgRNA1	Yugu1	SpCas9	7	7, 100%	6, 85.7%	1, 14.3%	N.D
BADH2 sgRNA2	Yugu1	SpCas9	6	5 83.3%	2, 33.3%	3, 50%	N.D
GBSS1 sgRNA2	Yugu1	SpCas9	3	3, 100%	1, 33.3%	2, 66.7%	N.D
Dof4 sgRNA1	*xiaomi*	SpCas9	2	2, 100%	1, 50%	1, 50%	N.D
ALS sgRNA	*xiaomi*	CBE	2	1, 50%	N.D	N.D	N.D
ACC sgRNA	*xiaomi*	ABE	13	4, 30.8%	2, 15.4%	N.D	2, 15.4%

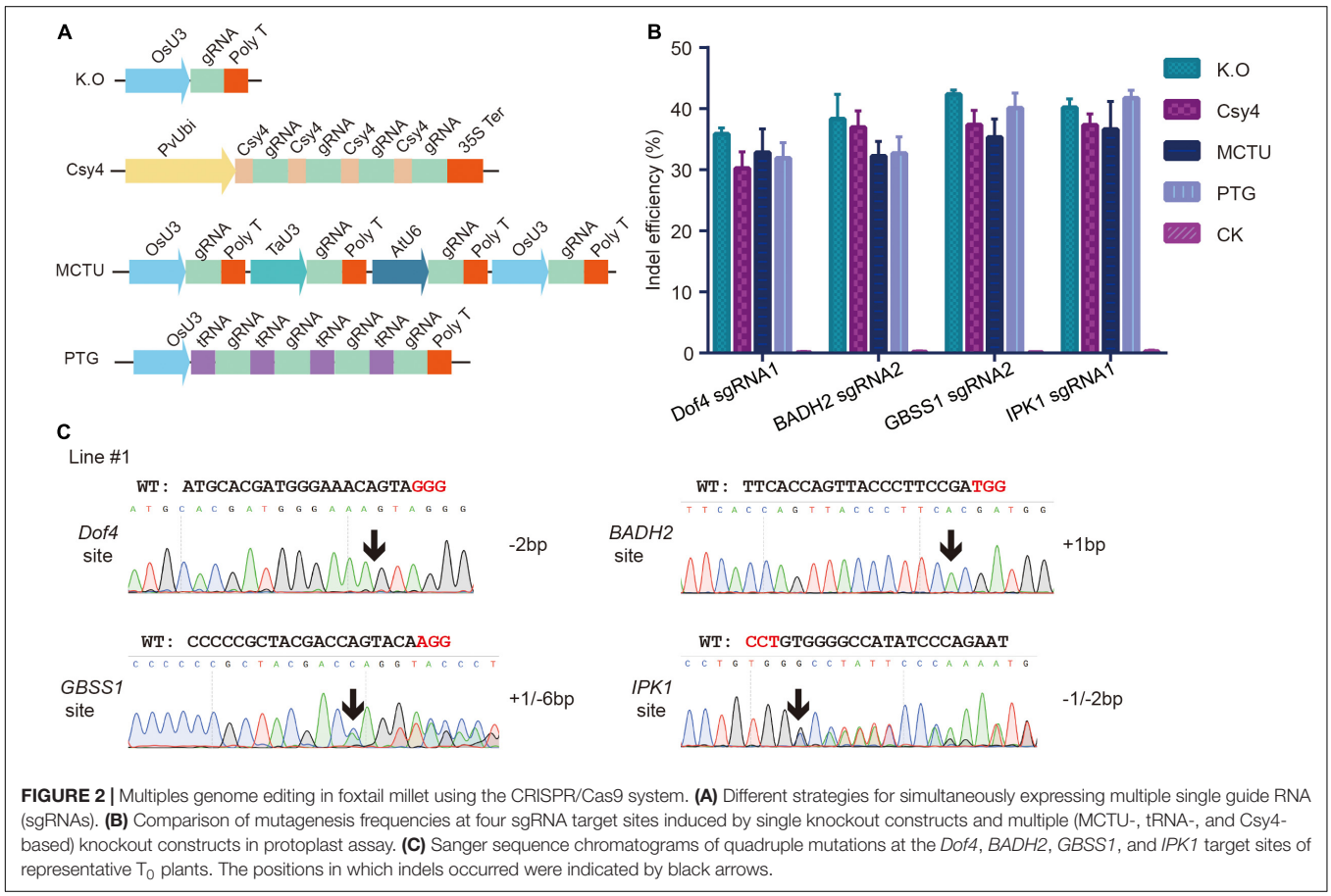


FIGURE 2 | Multiples genome editing in foxtail millet using the CRISPR/Cas9 system. **(A)** Different strategies for simultaneously expressing multiple single guide RNA (sgRNAs). **(B)** Comparison of mutagenesis frequencies at four sgRNA target sites induced by single knockout constructs and multiple (MCTU-, tRNA-, and Csy4-based) knockout constructs in protoplast assay. **(C)** Sanger sequence chromatograms of quadruple mutations at the *Dof4*, *BADH2*, *GBSS1*, and *IPK1* target sites of representative T_0 plants. The positions in which indels occurred were indicated by black arrows.

C to T conversions at positions 7–9 within the protospacer, with efficiencies of 0.01–0.14%. We also observed C7 to G7 byproduct at the target site (**Supplementary Figure 6A**). Then the ALS-P170-CBE vector was delivered into the callus cells of foxtail millet (cv *xiaomi*) through an *Agrobacterium*-mediated transformation. In T_0 generation, one line (T_0-1) with a non-canonical C to G mutation was obtained, which resulted in a P170A missense mutation (**Figure 3B** and **Table 1**). No sgRNA-dependent off-target mutations were detected (**Supplementary Table 3**). The mutant line was then selfed and seeds from this line were harvested. The T_1 homozygous, transgene-free P170A mutated plants were identified by PCR and Sanger sequencing. To test whether the P170A missense mutation conferred herbicide resistance, the homozygous T_1 mutants were sprayed with the ALS inhibitor nicosulfuron. Four weeks later, the P170A mutants exhibited normal phenotypes, but the wild-type plants almost died (**Figure 3C**). These results indicated that missense mutations at the SiALS-P170 site conferred herbicide tolerance in foxtail millet.

Adenine base editors, which consisted of dimerized wild-type and evolved bacterial tRNA adenine deaminase TadA (ABE7.10) fused with a Cas9 nickase, introduced A:T to G:C transition into targeted sites (Gaudelli et al., 2017). We codon-optimized the ABE7.10 cassette and cloned it into the pHUE411 backbone to generate the ABE vector (**Figure 3D**). Previous studies showed that the C2186R mutation in the *OsACC* gene

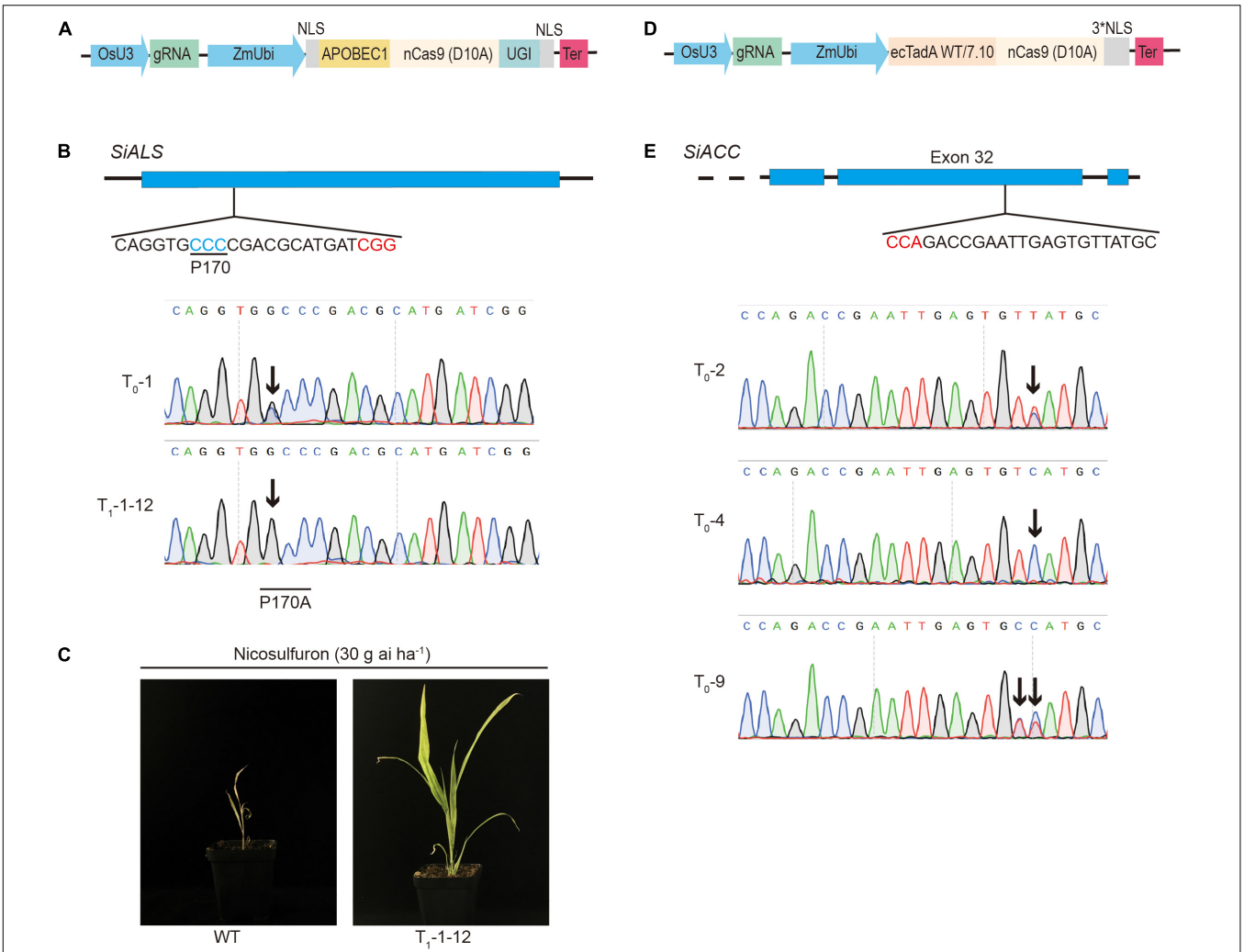


FIGURE 3 | Targeted nucleotide substitutions using cytosine and adenine base editors. **(A)** Schematic review of the cytosine base editor used in this study. **(B)** Targeted mutagenesis in the *SiALS* gene induced by CBE. The PAM sequence and P170 amino acid that conferred herbicide resistance were highlighted in red and blue, respectively. Sanger sequencing was used to analyze the T_0 transgenic plants. The mutated bases were indicated by black arrows. **(C)** Phenotypes of the homozygous P170A plants treated by nicosulfuron herbicide. **(D)** Schematic review of the adenine base editor used in this study. **(E)** Targeted mutagenesis in *SiACC* gene induced by ABE.

conferred tolerance to haloxyfop-R-methyl herbicide in rice (Li et al., 2018). To establish the adenine base editing system in foxtail millet, we chose the *SiACC* (*Seita.7G030200*) gene as the potential candidate target gene, and one sgRNA located at 32th exon was selected to target the C2088R amino acid, which corresponded to OsACC-C2186. The spacer sequence of the designed sgRNA was inserted into the ABE vector, generating ACC-C2186-ABE (**Figure 3E**). We observed A:T to G:C mutations at positions 3–8, with the efficiencies of 5.16–7.77%, in the transient protoplast assay (**Supplementary Figure 6B**). The ACC-C2186-ABE vector was then introduced into embryogenic callus cells by an *Agrobacterium*-mediated transformation as above, and 13 T_0 independent transgenic lines were generated. Sanger sequencing revealed that four mutants that carried A•T to G•C conversion were recovered with a mutation frequency of 30.8%. Three lines of them carried homozygous or heterozygous edits at position T5, and the other one carried heterozygous edits at position T5 and T6 (**Figure 3E** and **Table 1**). No desired C2088R missense mutation were obtained. Further efforts are still ongoing to produce the desired T8 to C8 mutations. We did not detect mutations in potential off-target regions (**Supplementary Table 3**). These results at least indicated that the adenine base editor was effective in inducing targeted nucleotide substitution in foxtail millet.

DISCUSSION

In recent years, genome editing toolboxes have developed rapidly and they have been applied in various plant species. Here, a highly efficient genome editing system to targeted indels or nucleotide substitution in foxtail millet was established

based on *Agrobacterium*-mediated editing reagent delivery. We successfully edited five endogenous genes using CRISPR/Cas9 and two endogenous genes using cytosine or adenine base editors in two varieties (Yugu1 and *xiaomi*). Mutations were heritable and could be transmitted faithfully to the next generation. In addition, we obtained one line with missense mutation P170A at the *ALS* gene that conferred herbicide resistance in foxtail millet. To our knowledge, this is the first study to demonstrate targeted mutagenesis in foxtail millet using base editing systems.

CRISPR/Cas9 induced DNA double-strand breaks at target loci is the most effective way to introduce specific genomic modifications. A transient protoplast assay enabled us to quickly test the mutation efficiency and pattern induced by genome editing reagents. We screened sgRNA, which had a higher targeted mutagenesis frequency for the same targeting gene in transient assay for the subsequent *Agrobacterium*-mediated transformation. In most cases, the mutation efficiencies in the T_0 generation were 100%, which was much higher than that reported previously in foxtail millet (Cheng et al., 2021). In addition, all the mutants we obtained were homozygous or bi-allelic (**Table 1**), which indicated that the current system enabled us to generate heritable knockout mutant even if a small number of transgenic plants were recovered.

In this study, we also performed Illumina sequencing to analyze the mutation patterns induced by CRISPR/Cas9 at five target sites. For *BADH2* sgRNA2 and *IPK1* sgRNA1, the predominant mutation types were single nucleotide deletion or insertion, which was consisted with previous studies in other species. However, for *FMBP* sgRNA2, *Dof4* sgRNA1, and *GBSS1* sgRNA2, two or three nucleotide deletions occurred most frequently (**Supplementary Figure 3**). These results indicated that the preference mutation pattern seemed to be related not only to CRISPR/Cas9, but also to the genomic context and the target sequence. The amplicon deep sequencing analysis of mutations that occurred in protoplasts showed that 2.72–16.11% of the insertion mutations were >10 bp and derived from the plasmid (**Figure 1C** and **Supplementary Figure 4**). Genome-edited crops without any foreign DNA sequence were the ideal products to avoid strict regulation (Li et al., 2019). As we described previously in bread wheat, CRISPR/Cas9 delivered as *in vitro* transcripts (IVTs) or ribonucleoprotein complex (RNPs) was another effective method to reduce off-target effects and avoid foreign DNA insertions in genome-edited crops (Liang et al., 2017, 2018). Further efforts will be forthcoming to establish a DNA-free genome editing system in foxtail millet.

The base editing systems are new types of gene editing technology, which can realize the irreversible replacement of a single base at a specific site of a gene to promote the improvement of agricultural crops. In this study, we established both CBE and ABE systems successfully in foxtail millet, and we found that the base editing efficiencies were relatively lower when compared with the CRISPR/Cas9 knockout system (**Table 1**). We used CBE to target the P170 locus of the *SiALS* gene of foxtail millet. In theory, CBE can cause the substitution of base C to T. Under different circumstances, it can also realize the substitution of C to G or C to A. Previous studies reported that CBE produced multiple mutation types of OsALS-P171, which included P171S (C7 to T7), P171F (C7C8 to T7T8 or C7C8C9 to T7T8T9), P171A (C7 to G7), and P171Y (C7C8 to T7A8). All the four missense mutations that occurred at OsALS-P171 conferred herbicide tolerance in rice (Kuang et al., 2020). In our experiments, we only obtained one mutant line that carried P170A mutation, mainly because few T_0 transgenic plants were obtained (**Table 1**). The genetic transformation system of foxtail millet still has much room for improvement. Plant regeneration is one of the limiting factors. It has been demonstrated that fine-tuning developmental genes, such as *BABY BOOM* (*BBM*), *WUSCHEL* (*WUS*), and GRF-GIF chimeras improved the regeneration efficiency of explant (Lowe et al., 2018; Debernardi et al., 2020). Hopefully these regeneration booster genes can also improve the transformation efficiency of foxtail millet when they are eventually implemented into the current genome editing system. Recently, several cytosine deaminases with various editing features have been utilized in CBE. BE3 with rAPOBEC1 prefer to edit TC not GC sequence motif with 3–9 editing window within the protospacer (Zong et al., 2017). While the PmCDA1 and hAID deaminases show no such sequence motif preference (Shimatani et al., 2017; Ren et al., 2018). Base editing based on human APOBEC3A exhibit high editing efficiency with broad editing window from 1 to 17 within the protospacer (Zong et al., 2018). Rationally designed human APOBEC3B showed remarkable precision and specificities in plants (Jin et al., 2020). In further experiments, protoplast assay could be used to evaluate the several deaminases at the same target and select the most effective one for stable transformation.

In the adenine base editing experiments, we chose to target the C2088R site of the *SiACC* gene of foxtail millet. Previous studies found that the C2168R (T to C) missense mutation in the *OsACC* gene conferred resistance to the herbicide haloxyfop-R-mithyl (Zhang et al., 2020). Although we successfully achieved the replacement of T5 and T6 positions, no base replacement occurred at the ideal T8 position, and no herbicide-resistant mutant was obtained (**Figures 3B,E**). This may be due to factor that base editors preferred to edit nucleotides at certain positions within the editing window. Recently, near-PAMless SpRY Cas9 variant was developed, which can be used to break through the limitations of PAM, adjust the appropriate editing window and preferences, and achieve precise editing of specific sites (Walton et al., 2020). In future experiments, more sgRNAs together with PAMless Cas9 variants can be designed and analyzed by the transient protoplast assay. The most effective one can be used for stable transformation to generate certain nucleotide substitution.

In summary, we have applied CRISPR/Cas9 gene editing technology and base editors successfully in foxtail millet. A protoplast-based system was developed to quickly evaluate the mutagenesis frequency and pattern of each target site in *S. italica* and a robust transformation system was implemented in local elite cultivars that produced stable mutated plants carrying targeted indels or nucleotide substitutions with high mutation frequency. We believe that valuable outcomes for foxtail millet genetic study and trait improvement for precise molecular breeding programs of foxtail millet can be achieved based on our system.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZL conceived and designed the project and wrote the manuscript with assistance from other authors. YW, ZL, and YR performed most of the experiments with assistance from LM and YG. YW, LM, YG, ZL, and YR analyzed experiments, read, and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.815946/full#supplementary-material

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METHODS
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An Efficient Hairy Root System for Validation of Plant Transformation Vector and CRISPR/Cas Construct Activities in Cucumber (*Cucumis sativus* L.)

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Hairy root induction system has been applied in various plant species as an effective method to study gene expression and function due to its fast-growing and high genetic stability. Recently, these systems have shown to be an effective tool to evaluate activities of CRISPR/Cas9 systems for genome editing. In this study, *Rhizobium rhizogenes* mediated hairy root induction was optimized to provide an effective tool for validation of plant transformation vector, CRISPR/Cas9 construct activities as well as selection of targeted gRNAs for gene editing in cucumber (*Cucumis sativus* L.). Under the optimized conditions including OD_{650} at 0.4 for infection and 5 days of co-cultivation, the highest hairy root induction frequency reached 100% for the cucumber variety Choka F1. This procedure was successfully utilized to overexpress a reporter gene (*gus*) and induce mutations in two *Lotus japonicus ROOTHAIRLESS1* homolog genes *CsbHLH66* and *CsbHLH82* using CRISPR/Cas9 system. For induced mutation, about 78% of transgenic hairy roots exhibited mutant phenotypes including sparse root hair and root hair-less. The targeted mutations were obtained in individual *CsbHLH66*, *CsbHLH82*, or both *CsbHLH66* and *CsbHLH82* genes by heteroduplex analysis and sequencing. The hairy root transformation system established in this study is sufficient and potential for further research in genome editing of cucumber as well as other *cucumis* plants.

Keywords: cucumber, hairy root, *Rhizobium rhizogenes*, CRISPR/Cas9, root hair-less, *CsbHLH66*, *CsbHLH82*, *ROOTHAIRLESS1*

INTRODUCTION

Originating in the Indian subcontinent, cucumber (*Cucumis sativus* L.) has become one of the top consumed vegetables widely grown in the world (Paris et al., 2012; Taha et al., 2020). However, cucumber cultivation currently has suffered from biotic and abiotic stresses (Liu et al., 2013; Chojak-Koźniewska et al., 2018; Fan et al., 2020; Taha et al., 2020). There

are various approaches used to improve cucumber production such as optimizing cultivation conditions (Taha et al., 2020), plant grafting (Elsheery et al., 2020), and breeding. Of which, modem breeding with the help of genetic engineering has been known as the most effective method because genetic variability of cucumber varieties is low 3–8% (Chee, 2001; Plader et al., 2007). The genome editing boosted by CRISPR/Cas systems has been widely applied on various crops as a powerful tool to improve plant traits and gene function studies (Zhang et al., 2021). In cucumber, there have been several reports of using CRISPR/Cas9 for targeted gene knockout (Chandrasekaran et al., 2016; Hu et al., 2017). However, applications of genome editing in cucumber have been challenged by less effective transformation procedures that are time consuming and labor extensive processes (Hu et al., 2017). Therefore, the improvement of procedures and the development of supporting tools are necessary to accelerate genetic engineering in cucumber.

Rhizobium rhizogenes-mediated hairy root cultures have been established and developed in different plant species for biomass, recombinant protein production, gene function studies, and secondary metabolite researches (Gutierrez-Valdes et al., 2020). In addition, hairy roots also could be used as materials for generating whole plants in certain reports (Trulson et al., 1986; Bueno dos Reis et al., 2007; Butler et al., 2020; Zhang et al., 2020). Recently, the hairy root transformation has been widely utilized to validate and optimize induced mutagenesis by the CRISPR/Cas9 system (Michno et al., 2015; Du et al., 2016; Jacobs and Martin, 2016; Li et al., 2019; Le et al., 2020). Both *in vitro* and *in vivo* cucumber hairy root cultures have been established for different research purposes (Shi and Lindemann, 2006; Anuar et al., 2011; Fan et al., 2020). However, the application of cucumber hairy root transformation for genome editing using CRISPR/Cas systems has not been reported yet. Moreover, it has been reported that hairy root induction frequency is affected by cucumber varieties, bacterial strains, and transformation methods (JingLong et al., 2017; Fan et al., 2020).

In this study, an efficient procedure for *in vitro* cucumber hairy root induction and transformation was established for a local cucumber cultivar. This system was also applied for the expression of a reporter gene (*gus*) as well as validation of CRISPR/Cas construct activity. The targeted mutations including heterozygous, homozygous, and biallelic indels in two targeted genes *CsbHLH66* and *CsbHLH82* was obtained at primary and propagated transgenic hairy roots. These results provide an effective tool for gene expression, gene function, and genome editing studies in cucumber.

MATERIALS AND METHODS

Plant Material

Seeds of a local cucumber (*Cucumis sativus* L.) cultivar Choka F1 were surface sterilized in 15% commercial bleach (bleaching solution containing 5% sodium hypochlorite, Duc Giang, VN) for 15 min, then washed 4–5 times in sterile water (Hoang et al., 2021). The sterilized seeds were soaked in autoclaved water for 3 h at 26°C. After removing seed coats, unwounded seeds were placed on MS30 medium (**Table 1**) for 2 days in the dark.

CRISPR/Cas9 Vector Construction for Hairy Root Assay

The *CsbHLH66* and *CsbHLH82* and other related protein sequences were discovered by the BlastP (NCBI) using the *Lotus japonicus* ROOTHAIRLESS1 (LjRHL1) protein sequence (Karas et al., 2009) as a reference. To induce targeted mutations in *CsbHLH66* and *CsbHLH82* genes, gRNAs were generated by the CCTop program (Stemmer et al., 2015). The CRISPR/Cas9 plant transformation vectors were constructed based on the pKSE401 vector (addgene plasmid # 62202), a gift from Qi-Jun Chen laboratory (Xing et al., 2014), in which, the *neomycin phosphotransferase II* (*ntpII*) gene was replaced by the *basta resistance* (*bar*) gene. The coding sequence of *bar* gene was amplified from the binary vector pZY102 (Zeng et al., 2004) using primer pairs: *Nco*I-*bar*-F and *Rsr*II-*bar*-R (**Supplementary Table S1**). The PCR amplifications were conducted using the Phusion High-Fidelity DNA Polymerase kit (Cat. No. F553L – Thermo Scientific) following the manufacturer's instructions. After purification, the PCR products were inserted into pKSE401 at *Nco*I and *Rsr*II sites. For gRNA insertion, *Bsa*I-*CsbHLH66* gRNA-gRNA scaffold-*U6* terminator-*Bsa*I and *Bsa*I-*U6* promoter-*CsbHLH82*-gRNA-*Bsa*I fragments were generated from the pKSE401 template by PCR using the listed primers (**Supplementary Table S1**). These two fragments were assembled into the destination vector pKSE401 using *Bsa*I enzyme sites. The final constructed vector was confirmed by sequencing and named pKSE401-CsbHLH82-66. The pKSE401-CsbHLH82-66 and pZY102 binary vectors were mobilized into *R. rhizogenes* strain K599 and used for cucumber hairy root transformation.

Rhizobium rhizogenes-Mediated Transformation and Hairy Root Induction

Rhizobium rhizogenes strains including ATCC 15834 (pRi15834) and K599 (pRi2659) wild-type or carrying binary vectors were used for bacterial mediated transformation. Briefly, single colonies

TABLE 1 | The list of media used in cucumber hairy root transformation.

Medium	Composition or references
MS	Murashige and Skoog, 1962
MS30	MS, 30 g/L sucrose and 7.5 g/L agar; pH 5.8
Re-suspension	0.5X MS, 30 g/L sucrose and 200 μM/L AS; pH 5.8
Co-cultivation	MS30, 200 μM AS, pH 5.8
Hairy root culture	MS30, 300 mg/L cefotaxime; pH 5.8
Hairy root selection	Hairy root culture medium added selective agents

AS, antibiotics and selective agents were added to medium after autoclaving.

were cultured in 5 ml liquid yeast extract peptone (YEP) medium supplemented with appropriate antibiotics (25 mg/L rifampicin for the *R. rhizogenes* strain K599; no antibiotic for the *R. rhizogenes* strain ATCC 15834; 25 mg/L rifampicin plus 50 mg/L kanamycin the *R. rhizogenes* strain K599 harboring pKSE401-CsbHLH82-66 vector; 25 mg/L rifampicin plus 100 mg/L spectinomycin for the *R. rhizogenes* strain K599 haboring pZY102 vector). The culture was shaken at 28°C and 200 rpm for 4–6 h, transferred to 100 ml of fresh medium and cultured overnight until OD_{650} reached 0.8–1.0. On the next day, the cells were harvested by centrifugation at 3,000 rpm for 15 min and re-suspended in the resuspension medium (**Table 1**) to get the OD_{650} of 0.4.

The cotyledons from 2-day-old seedlings were collected and used as explants for infection. The distal parts and proximal petioles were excised and removed. Then, the proximal parts of the cotyledons were cut in half along the midrib to get small pieces (**Figures 1A,B**). Cotyledon segments were kept on wet filter papers during the cutting time to avoid water loss. About 60–70 explants were soaked in 30 ml bacterial suspension for 30 min at room temperature (**Figure 1C**). Then, explants were removed from the extra liquid and placed on the co-cultivation medium (**Table 1**) covered by a filter paper (Whatman # 1001-090) on top. The culture plates were kept at 25 ± 2°C in the dark for 5 days. Next, explants were washed by autoclaved water, blotted by sterile filter papers, and placed on the hairy root culture medium (**Table 1**) for 15–20 days. Root tip fragments (2 cm) were cut and transferred to fresh medium for elongation and development. Transgenic hairy roots were obtained on the hairy root selection medium (**Table 1**) for 10–15 days (**Figure 1E**). Cucumber hairy root induction and selection were conducted at 25 ± 2°C under a light density of 2,000 lux with a photoperiod of 16/8 h.

GUS Histochemical Analysis

The pZY102 transformed hairy roots survived on the selection medium were collected for GUS histochemical analysis using 5-bromo-4-chloro-3-indolyl glucuronide buffer at 37°C for 16 h (Jefferson et al., 1987). GUS expression in cucumber hairy roots was observed and captured by a regular camera.

Identification and Characterization of Induced Mutant Hairy Roots

Root hair phenotypes were analyzed using the method described by Karas et al. (2005, 2009), in which root hairs were observed under ZEISS Stemi 305 stereo microscope and captured with AxioCam ERc 5 s digital camera. Genomic DNA was extracted from hairy roots using the CTAB method (Doyle and Doyle, 1987). Hairy roots with induced mutations in *CsbHLH* genes were detected by heteroduplex analysis on polyacrylamide gel electrophoresis (PAGE; Chen et al., 2012) with some modifications. Briefly, the spanning target regions on the *CsbHLH* genes were amplified using flanking specific primers (**Supplementary Table S1**). The PCR amplifications were conducted using DreamTaq Green PCR Master Mix (Cat. No. K1081 – Thermo Scientific) with the following conditions: initial denaturation at 94°C for 4 min; 30 cycles at 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, and a final extension

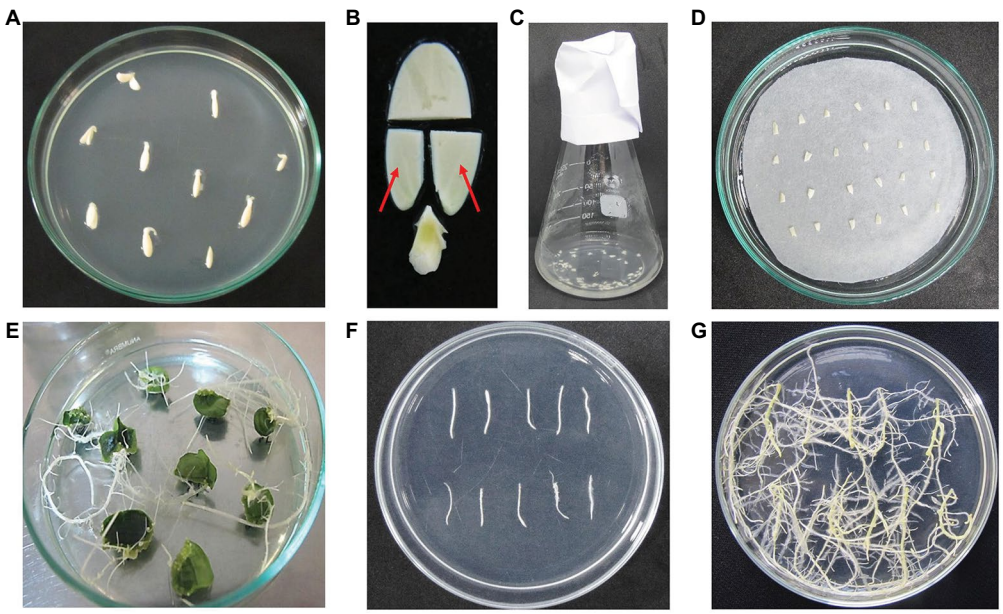


FIGURE 1 | Main steps of cucumber hairy root induction. **(A)** The 2-day-old seedlings; **(B)** cotyledon explants preparation. The red arrows indicate the cotyledon fragments used for bacterial infection; **(C)** cotyledon fragments were soaked in bacterial suspension; **(D)** the infected explants on co-cultivation medium; **(E)** hairy root formation at 20 days after co-cultivation; **(F)** hairy root fragments were transferred to a new medium; **(G)** hairy roots developed on hairy root culture medium.

at 72°C for 5 min. The mixture containing equal amplicons from wild type (WT) and transgenic hairy roots was denatured at 95°C for 10 min and slowly cooled down to room temperature to form heteroduplexes. The heteroduplexes were detected by PAGE using 12% polyacrylamide gel. The PCR amplicons were further purified and ligated to the pJET1.2 cloning vector (Thermo FisherScientific, United States) for sequencing. The sequencing was performed on the ABI3500XL system (Applied Biosystems). *CsbHLH* mutant sequences were compared to wild-type sequences using BioEdit 7.2.

Statistical Analysis

All statistical analyses were performed using SPSS software version 24.1. The significant analysis (value of $p < 0.05$) between experiments was performed by Student's *t*-test or one-way ANOVA followed by post-hoc Duncan's new multiple range tests.

RESULTS

Optimization of Cucumber Hairy Root Induction Procedure

In this study, cotyledon pieces from 2-day-old seedlings of ChokaF1 cultivar were first inoculated with *R. rhizogenes* K599 strain to evaluate the effects of co-cultivation duration on induced hairy root efficacy. After 3, 5, and 7 days of co-culture, explants were transferred onto hairy root induction medium. There were 21.1 ± 1.6% explants of the 5-day co-cultivation treatment that showed hairy root formation at 5 days on induction medium, and it dramatically reached 100% at 20 days after co-cultivation (**Figure 2A**). While the highest rate of explants in 3- and 7-day co-cultivation producing hairy roots after 20 days were 84.4 ± 1.6% and 65.6 ± 3.14%, respectively, and no more explants produced hairy roots if having the culture period prolonged. Bacterial overgrowth was also observed in the 7-day co-cultivation treatment (data not shown). Therefore, 5-day co-cultivation was sufficient for inducing hairy roots from the local cucumber ChokaF1 and further used for all cucumber hairy transformation experiments.

The effects of *R. rhizogenes* strain (ATCC 15834), a widely used *R. rhizogenes* strain to induce hairy roots in various plant species including cucumber (Shi et al., 2006; Shi and Lindemann, 2006), were also evaluated on the local cucumber cultivar in comparison to the K599 strain (**Figure 2B**). In this experiment, explants inoculated with ATCC 15834 strain produced hairy roots earlier than those infected with the K599 strain. About 33.3 ± 11.9% explants induced hairy roots (2–3 cm) after 1 week on hairy root induction medium, whereas no hairy root was observed from explants infected with K599. After 10 days, the rate of explants producing hairy roots was 34.4 ± 4.2% for K599, while it was double (61.1 ± 18.1%) for the ATCC 15834 strain. The number of ATCC 15834 inoculated explants generating hairy root was continuously higher than K599 treated ones after 15 days. However, after 20 days the highest hairy root induction frequency from ATCC 15834 infected explants was lower (83.3 ± 16.6%) than the ones from K599 (100%). In addition, the formation of calli were observed from wounding sites as well as whole explants which were inoculated with the ATCC 15834 strain. Whereas very few calli formed from K599 inoculation (**Supplementary Figure S1**).

From all the above results, an optimized procedure for cucumber *in vitro* hairy root induction was established, in which cotyledons of 2-day seedlings were infected with *R. rhizogenes* K599 and co-cultivated for 5 days. The total time to conduct the procedure is less than 1 month and the hairy root induction rate could reach up to 100%. Details of the procedure are presented in **Figures 1**, **3**.

Utilizing Cucumber Hairy Root System for Validating Plant Transformation Vector Activities and Transgene Expression

Utilizing the developed hairy root system, the expression of transgenes was conveniently assessed. K599 strain harboring binary vector pZY102 carrying a reporter gene (*gus*) and

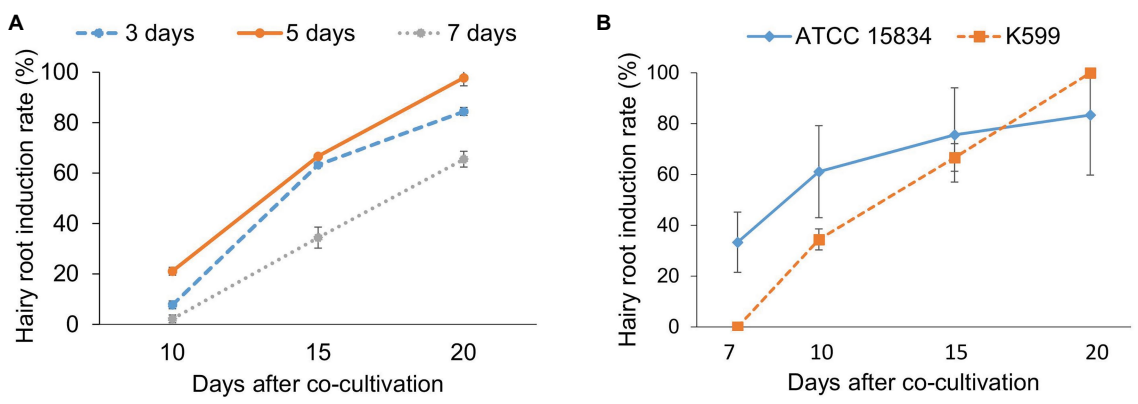


FIGURE 2 | The effects of co-cultivation duration and bacterial strains on cucumber hairy root induction. **(A)** Co-cultivation time and hairy root induction. **(B)** *Rhizobium rhizogenes* strains affect hairy root induction. Hairy root induction was observed and counted at different time points. Data are presented as mean ± SD of three replicates. About 30 explants were used for each treatment.

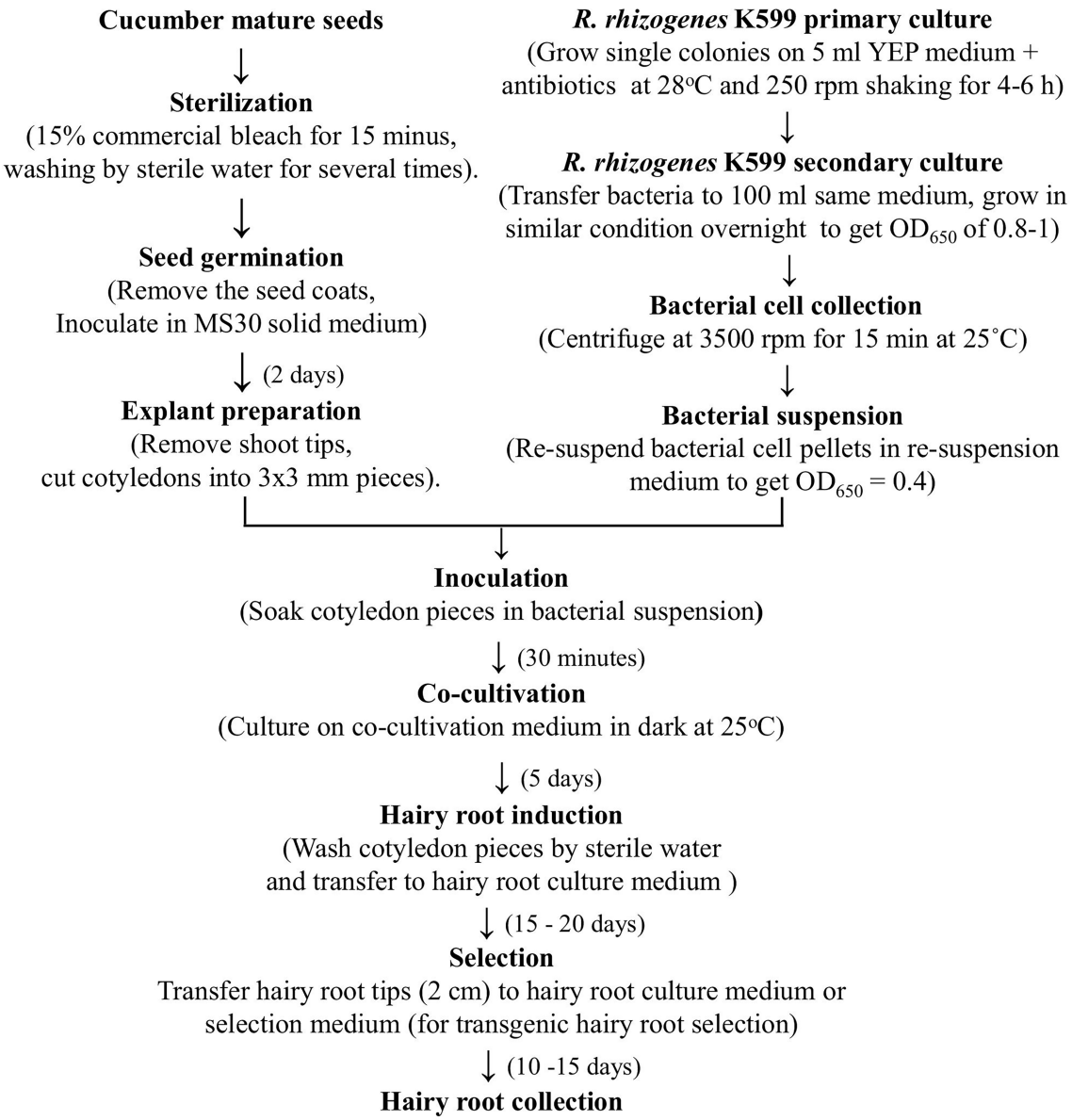


FIGURE 3 | The procedure for cucumber hairy root induction using cotyledons.

selective gene (*bar*) was transferred into cucumber cotyledon pieces. The induced hairy roots (2 cm) were removed from explants and cultured on the selection medium containing herbicide phosphinothricin (PPT) concentrations ranging from 0.5 to 5 mg/L. The herbicide-resistant hairy roots showed normal morphology and development, while non-resistant hairy roots were completely inhibited and turned yellow without elongation (**Figure 4A**). The analysis of *gus* gene expression in putative transgenic hairy roots (herbicide resistant ones) after 10 days on selection medium indicated that not all of them were positive for GUS staining (**Figures 4B,C**; **Supplementary Figure S2**). The number of selection-escape hairy roots was varied and depended on PPT concentration. On the medium with 3 or 5 mg/L PPT, 6.7 ± 1.6% of survived hairy roots escaped. This number raised up to 46 ± 7.2% and 48.7 ± 7.6% in the medium containing 1.0 and 0.5 mg/L PPT, respectively. On the 15th day in selection medium of 3 mg/L PPT, the number of GUS positive/negative hairy roots was not significantly different from hairy roots on the 10th day (**Figure 4C**). This result indicates that transgenic hairy roots could be effectively obtained in 3 mg/L PPT medium in 10 days with the lowest rate of escaping from the selection. Therefore, the activity of the binary vector and the expression of transgenes could be effectively analyzed using the cucumber *in vitro* hairy root system for 10–15 days.

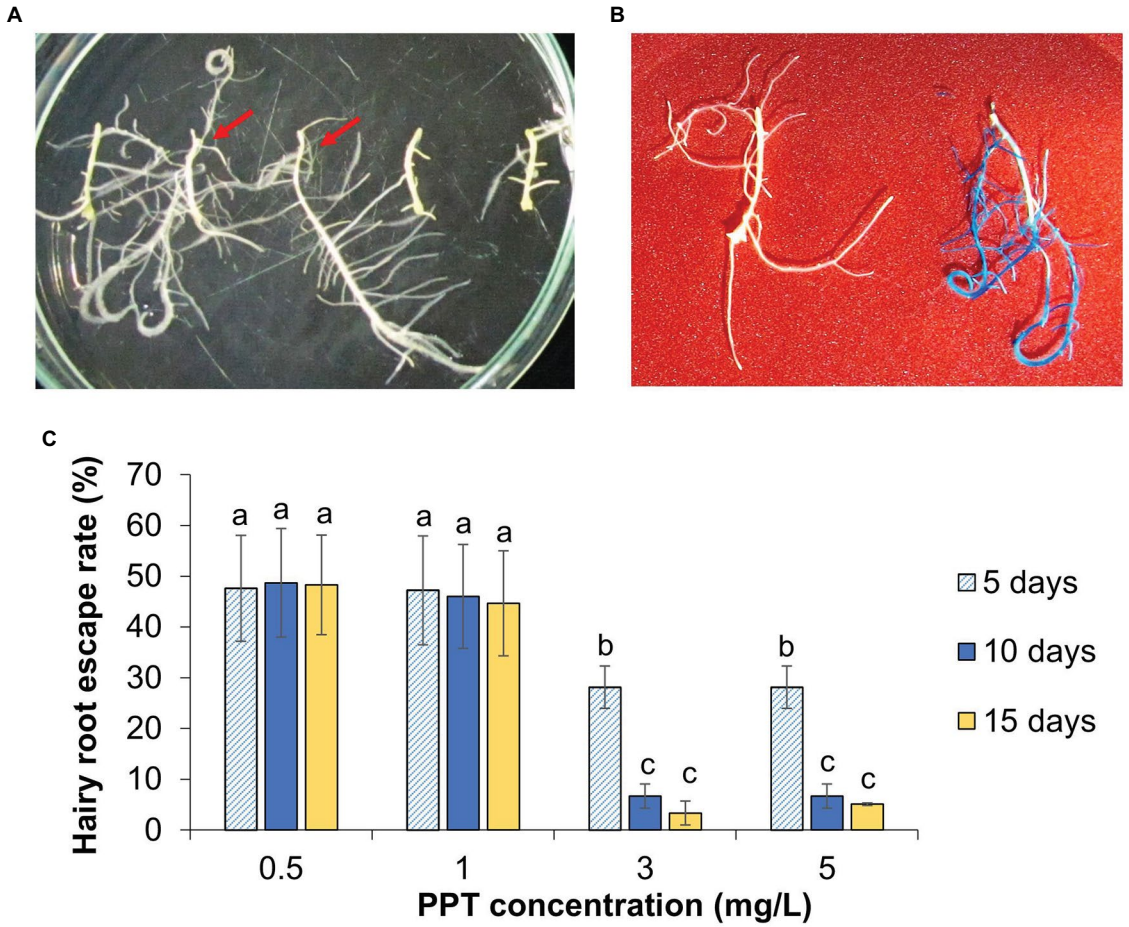


FIGURE 4 | The transgenic hairy root selection and histochemical analysis. **(A)** Hairy roots at 10 days on selection medium supplemented with 3 mg/L PPT. The red arrows indicate the PPT resistant hairy roots. **(B)** GUS histochemical analysis of non-transgenic (left) and transgenic hairy roots (right). **(C)** Effect of PPT concentrations on the selection efficiency. The escaped hairy roots are referred to the PPT resistant hairy roots without GUS staining. Data are presented as mean ± SD of three replicates. Different letters indicate significant differences based on one-way ANOVA analysis with Duncan's test ($p < 0.05$, $n = 30$).

Identification of Targeted Genes for CRISPR/Cas9 Induced Mutagenesis of Cucumber Hairy Root

In addition to testing the transgene expression, the developed hairy root induction system in this study was applied for genome editing studies utilizing the CRISPR/Cas9 system. Four candidate sequences, namely, CsbHLH82, CsBHLH66, CsUNE12, CsBHLH84, were identified when the LjRHL1 protein sequence (from *Lotus japonicus*, Karas et al., 2009) was used as a reference for searching on cucumber database (NCBI: ASM407v2) by protein BLAST tool. The phylogenetic tree was constructed using the four candidate sequences, the LjRHL1 sequence, and 3 ortholog sequences from *Arabidopsis thaliana* by the maximum likelihood method and JTT matrix-based model in MEGA X (Kumar et al., 2018). The result indicated that CsbHLH82 and CsbHLH66 were highly close to LjRHL1 (**Figure 5A**). Moreover, the bHLH domain sequences of the CsbHLH82 and CsbHLH66 were identical to the one of the LjRHL1 (**Figure 5B**). Therefore, both *CsbHLH82* and *CsbHLH66* were selected as the targets for mutagenesis in the cucumber hairy roots.

Inducing Targeted Mutations in Cucumber Hairy Roots by CRISRP/Cas9 System

The DNA sequences of both *CsbHLH82* and *CsbHLH66* genes were reconfirmed in the local cucumber variety for developing a CRISPR/Cas9 vector. Two gRNAs targeting the first exon of *CsbHLH66* and *CsbHLH82* were identified using the CCTop tool (**Figure 6A**) and inserted in the CRISPR/Cas9 expression binary vector (**Figure 6B**) with the purpose to induce individual and simultaneous targeted mutations of the two tested genes.

Targeted mutations of *CsbHLH82* and *CsbHLH66* were induced in hairy roots using the K599 strain that harbored the CRISPR/Cas9 vector. Each induced hairy root considered as an independent line was transferred to the selection medium. Phenotyping based on more than 80 hairy root lines indicated that there were three morphological groups (**Figure 6C**; **Supplementary Figure S3**) including (1) abundant root hairs like wild-type hairy roots (22.04%), (2) sparse and short root hairs (52.54%), and (3) root hair-less (25.42%).

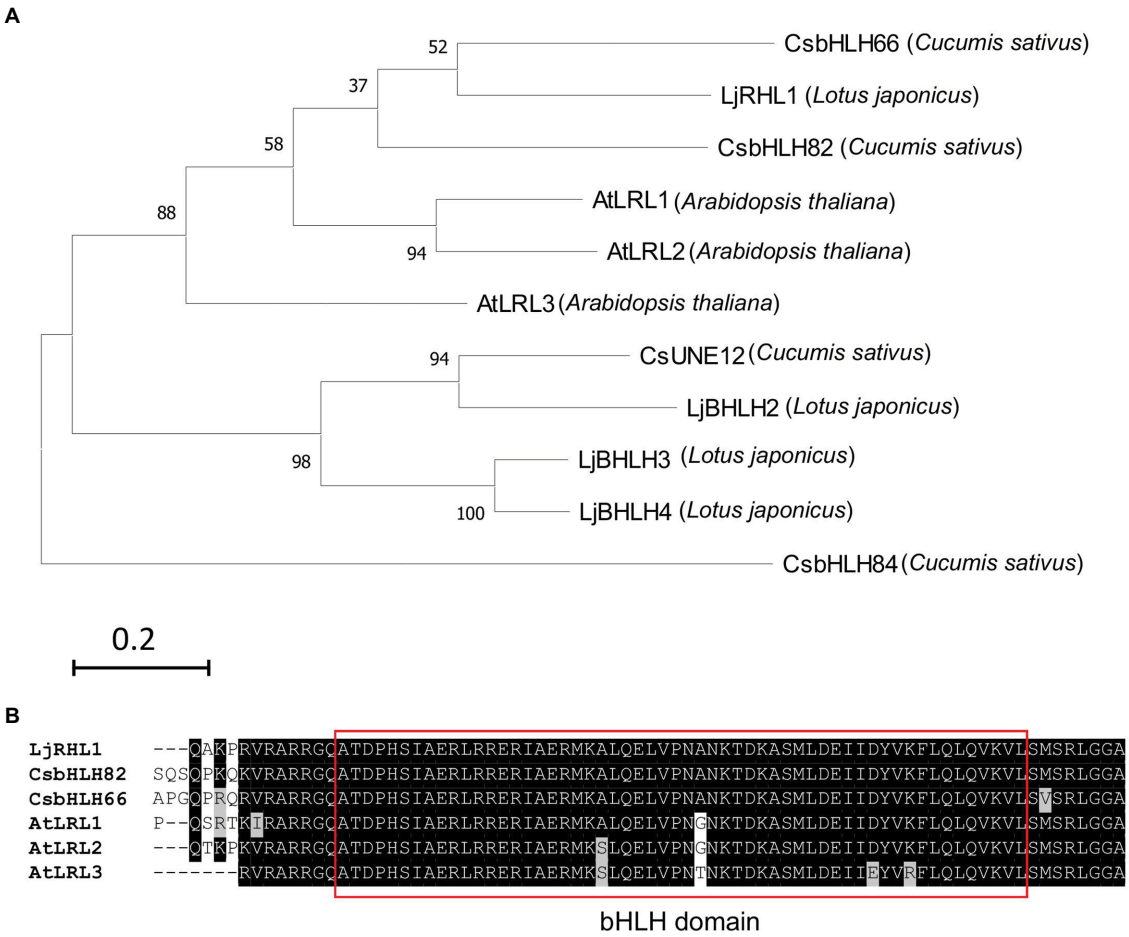


FIGURE 5 | The phylogenetic tree and sequence analysis of the LjRHL1 homologs. **(A)** Molecular phylogenetic tree of LjRHL1 protein sequence and its homologs were conducted by MEGA X using the maximum likelihood method and JTT matrix-based model. Numbers at nodes indicate the percentage bootstrap scores from 1,000 replicates. The scale bar represents 0.2 estimated number of amino acid substitution events per site. **(B)** Sequence alignment bHLH domain (in red box) of LjRHL1 in *Lotus japonicus* and its homologs in cucumber and Arabidopsis.

Heteroduplex analysis of 13 randomly selected hairy root lines from group 2 and 3 (**Figures 7A,B**) showed that all tested lines contained mutations on *CsbHLH82* upon the shifted DNA band observation on the PAGE gel as compared to the wild type hairy roots (**Figure 7A**). In the case of *CsbHLH66*, only 4 hairy root lines (3, 6, 10, 30) exhibited induced mutations (**Figure 7B**). Importantly, these 4 lines all belonged to group 3 having the root hair-less phenotype.

The induced mutations in *CsbHLH* genes of 3 lines (4, 15, 29) from the spare root hair group and 4 lines (2, 6, 9, 10) from the root hair-less group were further characterized *via* sequencing targeted amplicons (**Figures 7C,D**). For the *CsbHLH82* gene, induced mutations occurred in all tested hairy root lines (**Figure 7C**). Various types of indels were observed at the target locations, in which the deletion sizes ranged from −1 bp up to −33 bp and the insertions were from +1 to +10 bp. Different from *CsbHLH82*, targeted mutations in *CsbHLH66* were confirmed in only 5 of 7 tested root hair lines, including 2 mutant alleles in lines 2 and 15, which were undetectable in heteroduplex analysis because of only 1 bp indels (1 bp insertion or deletion). The 1 bp insertion and big deletions (−16 to −49 bp) in the *CsbHLH66* targeted gene were found in 3 tested hair-less root lines, while 1 bp deletion was found in one sparse root hair line 15 only (**Figure 7D**). Moreover, two mutant alleles (biallelic mutation) of targeted genes were also observed in many hairy root lines such as lines 6 and 10 for *CsbHLH66* gene and lines 2, 6, 15, and 29 of *CsbHLH82* gene (**Figures 7C,D**). The homozygous mutant allele was found in line 9 (for *CsbHLH82* gene). Three different alleles (chimeric alleles) of the *CsbHLH82* gene were also observed in hairy root line 4. Importantly, lines 6 and 10 contained null mutations of both the *CsbHLH82* and *CsbHLH66* genes. As a consequence, it is obvious that our developed hairy root system has a great potential for genome editing studies in cucumber.

The stability of induced mutations of targeted genes was further confirmed in *in vitro* propagated hairy roots. The root fragments from line 6 and 10 were cut and propagated in a new medium for the next generations. Heteroduplex, Sanger, and amplicon sequencing analysis were conducted using DNA

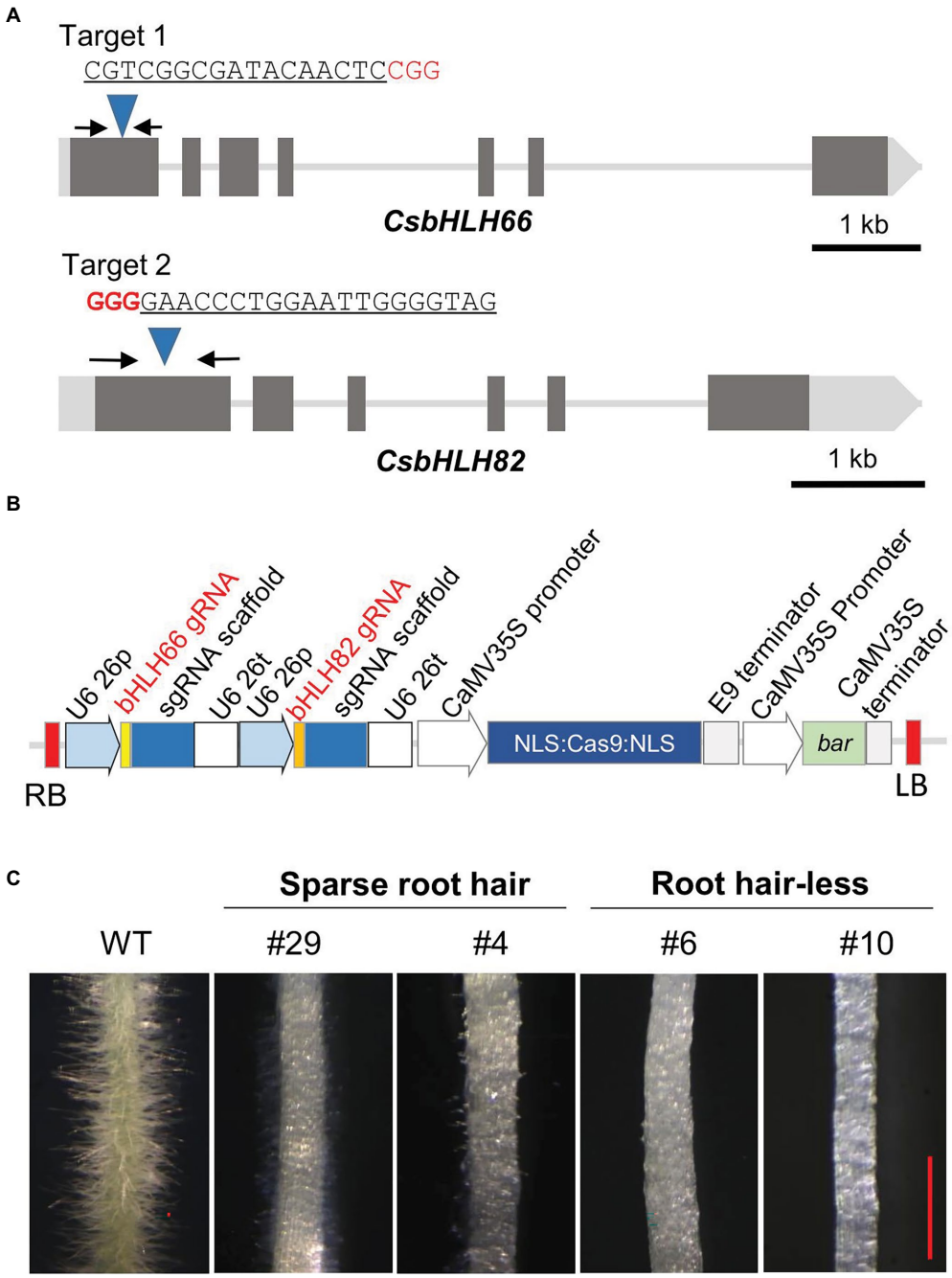


FIGURE 6 | CRISPR/Cas9 construction and phenotyping of mutant hairy roots. **(A)** Maps of *CsbHLH66*, *CsbHLH82* genes and target locations. The black arrows indicate primer binding sites for genotyping. **(B)** T-DNA regions of the CRISPR/Cas9 plant transformation vector. **(C)** Root hair phenotypes of WT and mutant hairy roots. The red scale bar indicates 1 mm.

extracted from the third propagation root lines. DNA band patterns in PAGE and sequencing data indicated that the mutations observed in the propagated lines were the same as in the primary roots (first propagation) for both *CsbHLH82* and *CsbHLH66* (**Supplementary Figure S4**). This result demonstrated that stably induced mutations were conducted in cucumber hairy roots.

DISCUSSION

Establishment of an Efficient Procedure for Cucumber *in vitro* Hairy Root Induction

In this study, an efficient procedure for cucumber *in vitro* hairy root induction was established. The hairy root induction frequency could reach 100% after 20 days of co-cultivation

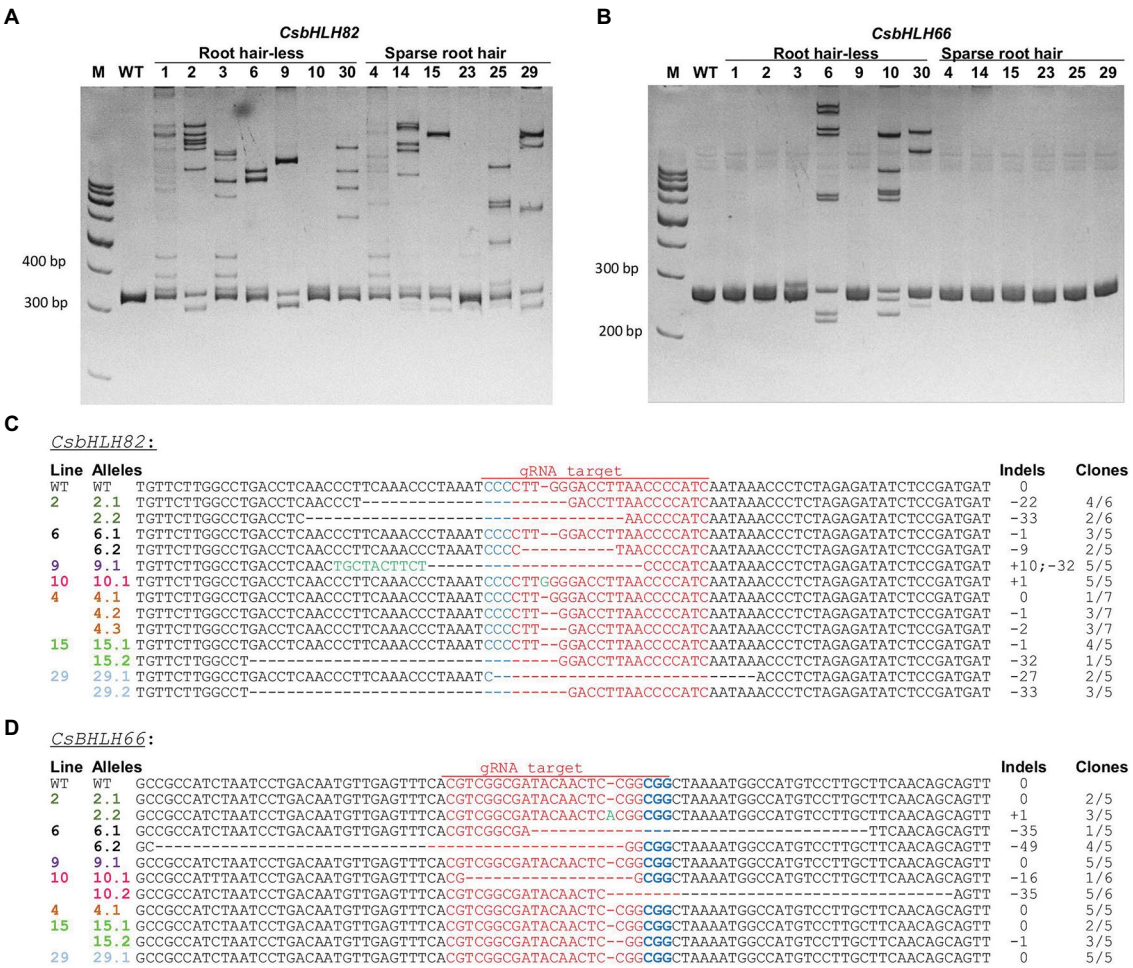


FIGURE 7 | Identification and characterization of induced mutations in cucumber hairy roots. **(A,B)** Heteroduplex analysis of *CsbHLH82* and *CsbHLH66*, respectively. M: 100 bp DNA Ladder; WT: wild type. **(C,D)** Expanding targeted sequences of *CsbHLH82* and *CsbHLH66* genes. Red letters indicate target sequences. Blue letters refer to PAM sequences. Green letters indicate inserted nucleotides. The indels indicate targeted sequence changes: 0 for no change, – for deletion, + for insertion. The number of clones used for sequencing was indicated.

using the *R. rhizogenes* K599 strain. Different factors including explants, co-inoculation, and co-cultivation conditions have been reported to influence plant transformation efficacy as well as hairy root induction. In our results, the 5 days co-cultivation was selected because of the highest hairy root induction efficiency. Co-cultivation duration extended to 7 days negatively influenced cucumber hairy root formation due to the overgrowth of bacteria after inoculation. This result is in agreement with Shi et al. (2006), in which 5 days of co-cultivation was considered to be optimal for cucumber *in vitro* hairy root induction. One other optimization in this study was the use of cotyledon pieces from 2-day-old seedlings as initial explants instead of 10-day-old seedlings as in previous studies (Shi et al., 2006; Shi and Lindemann, 2006) for hairy root generation. Such kind of explants was reported to be optimal for cucumber transformation and regeneration only (Nanasato et al., 2013). Apart from increasing the number of hair roots produced, the use of younger cotyledons also helps to shorten the time needed for hairy root induction procedure (<1 month).

Rhizobium rhizogenes K599 Strain Is Effective for Cucumber Hairy Root Induction

The hairy roots were effectively induced from the local cultivar Choka F1 using *R. rhizogenes* K599 strain. Bacterial strains have been known as an important factor for hairy root induction and transformation. In previous studies, different *R. rhizogenes* strains were used for *in vitro* hairy root induction in cucumber (Trulson et al., 1986; Shi et al., 2006; Shi and Lindemann, 2006; Anuar et al., 2011; JingLong et al., 2017) and the hairy root induction rates were variable depending on cucumber cultivars and transformation procedures. The hairy root induction rate of over 90% was reported by Shi et al. (2006) and Shi and Lindemann (2006) using the ATCC 15834 strain. Recently,

R. rhizogenes K599 strain had been utilized for inducing hairy roots in cucumber with the frequency of 95% under *in vivo* conditions (Fan et al., 2020). In our study, the K599 strain could facilitate 100% infected cucumber explants to produce strong hairy roots, much higher than that of ATCC 15834 strain (83.3 ± 16.6%). The formation of callus instated of hairy roots by ATCC 15834 strain (**Supplementary Figure S1**) might be a reason to lower the number of hairy roots produced. The fewer callus formed from K599 inoculation were coupled with the better hairy root morphology. Therefore, the K599 strain is most suitable for hairy root induction and transformation of the local cultivar.

A Convenient Tool to Assess the Activities of CRISPR/Cas9 Constructs in Cucumber

Hairy root systems have been used for genome editing of various plant species. For cucumber, the hairy root system has been applied for studying transgenic plant generation (Trulson et al., 1986), gene expression (Fan et al., 2020), promoter evaluation (Anuar et al., 2011), recombinant protein production (Shi and Lindemann, 2006), but not for genome editing yet. In this study, an efficient procedure for *in vitro* hairy root induction of a local cucumber cultivar (ChokaF1), which was successfully utilized for stable transformation (Hoang et al., 2021). This procedure was well performed for the expression of a reporter gene (*gus*) in a short time. The developed hairy root system was also effective for gene editing of cucumber using CRISPR/Cas9 construct. Targeted mutations of two endogenous genes (*CsbHLH66* and *CsbHLH82*) were induced accurately. Different indels of targeted genes were observed in tested hair roots including insertions, and larger and small deletions (**Figure 7**). All homozygous, heterozygous, and biallelic, as well as chimeric alleles could be obtained in the primary hairy roots (**Figure 7**). In addition, the targeting capability of different sgRNAs (single guide RNA) could be evaluated within 1 month, instated of several months or longer if using the transgenic plant approach. The targeted mutations induced in transgenic hairy roots were also observed in stable transgenic cucumbers (Chandrasekaran et al., 2016; Hu et al., 2017).

CsbHLH66 and *CsbHLH82* Genes Are Involved in Root Hair Performance of Cucumber

The root hair-less phenotype was observed in *Lotus japonicus* and characterized as a consequence of natural mutations in the *SjRHL1* gene (Karas et al., 2009). In *A. thaliana*, this phenotype was also found in the triple mutant of *AtLRL1*, *AtLRL2*, and *AtLRL3*, while sparse root hair phenotypes were caused by single or double mutations of these three genes (Karas et al., 2009). In our study, induced mutations in *CsbHLH82* resulted in the root hair spare phenotype (lines 15, 29), while simultaneous mutations of both *CsbHLH82* and *CsbHLH66* genes caused the root hair-less phenotype (lines 6, 10; **Figures 7C,D**). This result suggests that two bHLH transcription factors encoded by *CsbHLH82* and *CsbHLH66* are redundantly involved in regulating the root hair formation in cucumber. However, further research needs to be performed to characterize and confirm the contributions of *CsbHLH66*, *CsbHLH82*, and other *CsbHLH* genes in cucumber root hair formation at whole plant levels.

CONCLUSION

In this study, an effective protocol for cucumber hairy root induction and transformation was developed for a local cucumber cultivar. This hairy root system has been successfully used to evaluate the expression of transgenes and the activities of CRISPR/Cas9 systems within 1 month. The obtained result obviously provides a potential tool for transgene expression and genome editing studies in cucumber as well as other *Cucumis* plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

PD and HC conceived and supervised the study. PD, DN, and TH designed the experiments, analyzed the data and prepared the manuscript. CN conducted the phylogenetic tree and designed the gRNAs. NL and HT performed CRISPR/Cas9 vector constructions. TH conducted cucumber hairy root transformation. DN performed genotyping and phenotyping of transgenic hairy roots. HC, Y-HM, and CN revised and proofread the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.770062/full#supplementary-material

Supplementary Table S1 | Primers used in this study.

Supplementary Figure S1 | Cucumber hairy root induction by *Rhizobium rhizogenes* strain K599 and ATCC 15834. The photos were captured at 10 days after co-cultivation.

Supplementary Figure S2 | GUS histochemical analysis of the herbicide resistant hairy roots. WT: non-transgenic hairy roots grown on the PPT free medium; 1–14: Hairy root lines survived and developed on the selection medium with PPT.

Supplementary Figure S3 | Distribution of root hair phenotypes of tested cucumber hairy roots.

Supplementary Figure S4 | The stability of induced mutations in propagated cucumber hairy roots. **(A)** Heteroduplex analysis of *CsbHLH66* gene. **(B)** Heteroduplex analysis of *CsbHLH82* gene. M: 100 bp DNA Ladder; WT: wild type; P6 and P10: The original hairy roots of lines 6 and 10; 6-1, 6-1-1, 10-1, 10-1-1: Propagated roots from lines 6 and 10 at the second and third generations. **(C,D)** Expanding targeted sequences of *CsbHLH82* and *CsbHLH66* genes from propagated roots of the hairy root lines 6 and 10. Amplicon sequencings were conducted by the Sanger method. The expected sequences of mutant alleles were denoted upper chromatograms. **(E)** Clone sequencing of *CsbHLH82* and *CsbHLH66* targeted regions of propagated cucumber hairy roots at the third generation. Red and blue letters indicate target and PAM sequences, respectively. Green letters indicate inserted nucleotides.

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REVIEW
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Modified Gene Editing Systems: Diverse Bioengineering Tools and Crop Improvement

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Gene-editing systems have emerged as bioengineering tools in recent years. Classical gene-editing systems include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9), and these tools allow specific sequences to be targeted and edited. Various modified gene-editing systems have been established based on classical gene-editing systems. Base editors (BEs) can accurately carry out base substitution on target sequences, while prime editors (PEs) can replace or insert sequences. CRISPR systems targeting mitochondrial genomes and RNA have also been explored and established. Multiple gene-editing techniques based on CRISPR/Cas9 have been established and applied to genome engineering. Modified gene-editing systems also make transgene-free plants more readily available. In this review, we discuss the modifications made to gene-editing systems in recent years and summarize the capabilities, deficiencies, and applications of these modified gene-editing systems. Finally, we discuss the future developmental direction and challenges of modified gene-editing systems.

Keywords: modified gene editing system, CRISPR, BEs, PE, genome engineering, transgene-free

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INTRODUCTION

The world's population is increasing rapidly, so food security has been greatly challenged. With the problem of climate change, the world's arable land is also under threat. Therefore, increasing food production per unit of cultivated land area is a crucial strategy to maintain global food security.

Improving crops is an important way of improving agricultural productivity. The traditional breeding method obtains crops with excellent agronomic traits through natural mutation and cross-breeding. This method has been widely used in human history, and many excellent crop resources have been obtained (Liu et al., 2020). However, cross-breeding can only utilize traits already acquired by natural mutations in the crop, limiting it at the genetic level (Witkin, 1969; He et al., 2012). Chemical or radiation mutagenesis has been widely used to expand the gene pool potential. Nevertheless, this method still requires considerable labor and time to identify and obtain favorable traits through mutation breeding. In contrast, transgenesis allows transgenic elements to be inserted into a plant genome to express or silence a target gene, generating better crop traits or more efficient herbicide resistance and is labor-saving. However, genetic modification can also cross species boundaries, favorable traits may disappear generation by generation in actual agricultural production, and the insertion of exogenous fragments makes genetically modified crops subject to

government supervision and public suspicion (Raman, 2017). Therefore, new methods of molecular breeding are of great importance.

Unlike transgenesis, gene editing allows targeted gene manipulation at a specific locus, so traits do not depend on transgenic elements. This technique generates transgene-free crops with favorable traits and stable inheritance in actual agricultural production (Chiu et al., 2013). Currently, the main gene-editing technologies include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat sequences (CRISPR) with CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) (Hou et al., 2013; Joung and Sander, 2013; Gupta and Musunuru, 2014). As the newly established gene-editing system, CRISPR/Cas9 has been verified and applied in crops including, rice, maize, wheat, tomato, and potato (Jiang et al., 2013; Brooks et al., 2014; Shan et al., 2014; Zhu et al., 2016). Classical gene-editing systems typically target the crop genome, cause double-strand breaks, and then edit at the target site during DNA repair of the crop itself. Mutations obtained in this way are generally random and negatively impact crop research and breeding. To better meet the needs of crop research and breeding, gene-editing systems have been modified in various ways. These modified gene-editing systems include Cas13a, mitoTALENs, base editors (BEs), and prime editors (PEs) and enable more accurate and reliable gene editing while expanding target locations to include RNA, mitochondrial, and chloroplast genomes (Komor et al., 2016; Pereira et al., 2018; Anzalone et al., 2019). Multi-target editing makes genome engineering possible, and several different modification or transformation strategies make transgene-free crops more efficient to obtain, where transgene-free crops have significant commercial value. In this **article**, the modification and characteristics of gene-editing systems are reviewed, and their application in crops is introduced.

CLASSICAL GENE-EDITING SYSTEMS

Presently, various gene-editing tools are employed, among which ZFNs and TALENs were developed earlier.

Zinc-finger nucleases are derived from a region containing multiple zinc finger structures that can bind to DNA and from a non-specific nuclease region. A single zinc finger recognizes three specific bases, so by combining zinc finger structures, the DNA binding domain can recognize specific sequences in multiples of three bases. TALENs are similar to ZFNs in that their DNA binding regions are composed of specific conservative repeats, each of which contains 34 amino acids, and the 12th and 13th amino acids form repeat variable di-residues (RVDs), where one RVD can only recognize specific bases (Tomas et al., 2011). After the ZFNs or TALENs specifically recognize the base sequence, the target is cleaved by the nuclease domain, and the genes are edited under the action of the plant's own DNA repair mechanism (Radecke et al., 2010; Gupta and Musunuru, 2014). Typically, ZFNs or TALENs are often used in pairs to cause double-strand breaks (DSB).

Clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 is essentially the immune mechanism of bacteria or archaea and is composed of three parts. The first is CRISPR RNA (crRNA) complementary to the targeted sequence. The second is trans-activating crRNA (tracrRNA), which binds both crRNA and Cas9. To make CRISPR-Cas9 easier to use in practical applications, CRISPR-Cas9 was modified at the molecular level. CrRNA and tracrRNA were linked by stem-loop, combining the two into a single guide RNA (sgRNA), which significantly reduced the structural complexity of the CRISPR-Cas9 gene-editing system (Jinek et al., 2012). The third part is Cas9, which contains two domains, namely the HNH domain and the RuvC-like domain. The HNH domain cuts the complementary strand of crRNA, while the RuvC-like domain cuts the opposite strand of double-stranded DNA. When CRISPR-Cas9 works, sgRNA carries the Cas9 protein to recognize the target containing a protospacer adjacent motif (PAM) NGG at the 3′ end and cleaves, resulting in DSB (Jacobs et al., 2015; Yu et al., 2016). Later, during DSB repair, the sequence is inserted by action of homologous recombination (HR), or the insertion, deletion, or alteration of bases is caused by action of non-homologous end joining (NHEJ) (**Figure 1A**; Bengtsson et al., 2017).

Cas12a is also used in CRISPR. The CRISPR-Cas12a (Cpf1) system uses crRNA to target sequences and has unique advantages. Firstly, Cas12a is smaller than standard Cas9, making it easier to enter the nucleus for editing. Secondly, CRISPR-Cas12a editing produces sticky ends, which is conducive to the deletion of larger fragments and the joining of foreign fragments. Thirdly, CRISPR-Cas12a can process pre-crRNA to form mature crRNA, making its multi-target editing system easier to construct and more efficient (Hu et al., 2017). Moreover, by modifying crRNA, the editing efficiency of CRISPR-Cas12a is improved, allowing it to edit genomic loci that have hitherto been difficult to edit (Hu et al., 2020). However, the PAM sequence identified by CRISPR-Cas12a is TTTV, resulting in only a 10th of the number of target sequences in plant genomes compared to CRISPR-Cas9. In addition, studies have shown that Cas12a is more sensitive to temperature than Cas9, so it needs to be modified in the many crops that grow at lower temperatures (Malzahn et al., 2019).

To date, classical gene-editing techniques have been applied in many crops, including rice, corn, wheat, potato, tomato, grape, and citrus, providing technical support for crop quality improvement, resistance to biological stress, resistance to abiotic stress, and re-domestication (Wang T. et al., 2019; Xu et al., 2019).

MODIFIED GENE-EDITING SYSTEMS

Many modifications to the classic gene-editing systems have been made to overcome their limitations. An example is CRISPR interference systems and CRISPR activation systems, which regulate gene expression rather than edit genes. Four strategies have a high potential for modifications with gene editing capability. The first strategy is to enable gene-editing systems to be applied to RNA, mitochondrial, or chloroplast genomes. The

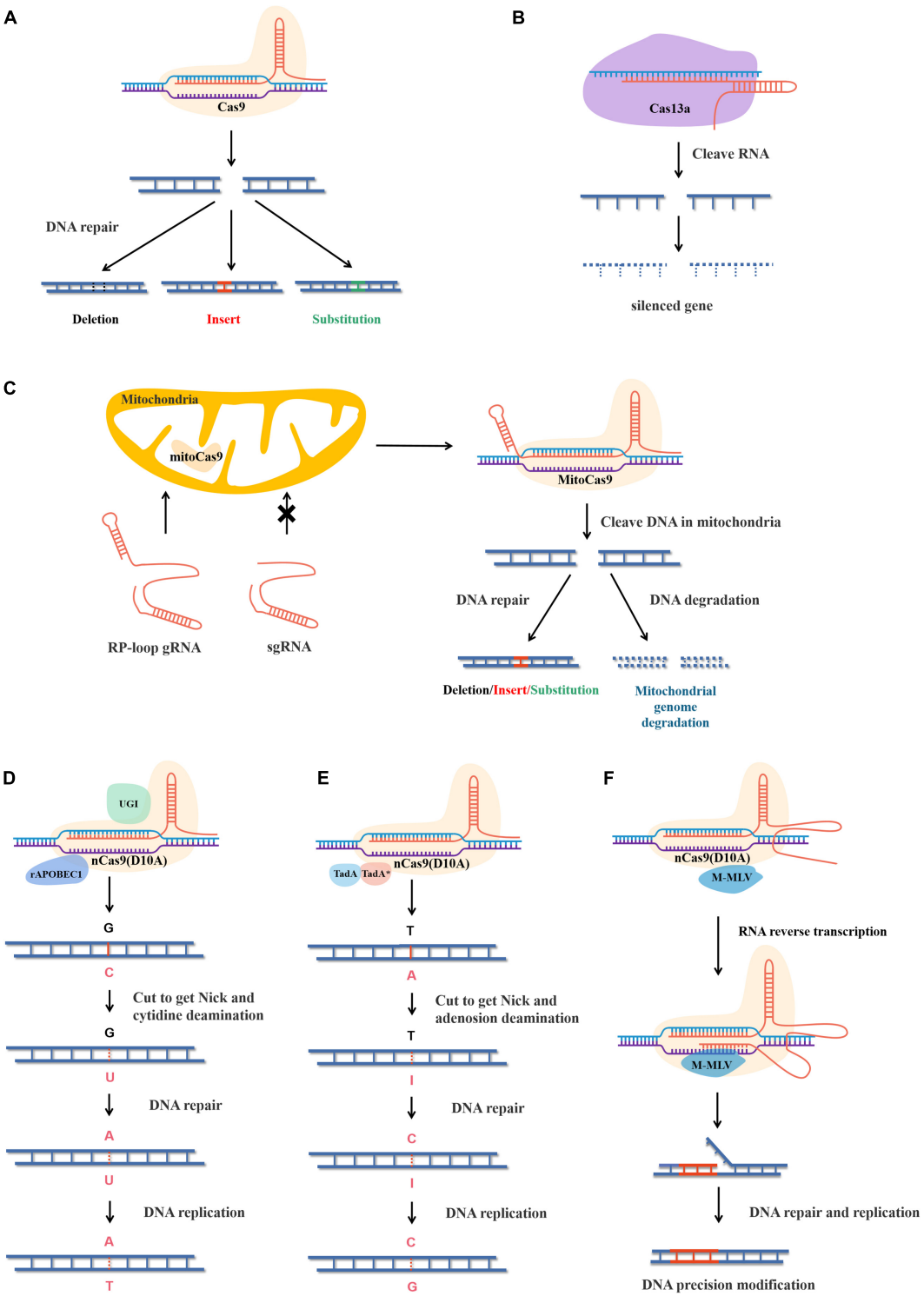


FIGURE 1 | Structure and principle of different gene editing systems. **(A)** CRISPR-Cas9 cleave double-stranded DNA and causes DSB and the genes are edited under the action of the plant's own DNA repair mechanism. **(B)** Rp-loop structure promotes sgRNA entering the mitochondrial and CRISPR-MitoCas9 result mitochondrial gene editing or genome degradation. **(C)** CRISPR-Cas13a cleaves ssRNA and causes ssRNA degradation. **(D)** The CBEs replaces bases with cytosine deaminase and T-A replace C-G under the action of plant DNA repair. rAPOBEC1, rat cytidine deaminase; GUI, uracil glycosylase inhibitor. **(E)** The adenine base editor replaces bases by adenine deaminase and G-C replace A-T under the action of plant DNA repair. TadA, wild-type *Escherichia coli* tRNA adenosine deaminase. TadA*, mutant TadA. **(F)** nCas9(D10A) cleaves single strands of DNA to form Nick, and pegRNA acts as a template to replace the sequence under the action of reverse transcriptase. Precise modification of target sequences by plant DNA repair. M-MLV, Moloney mouse leukemia virus reverse transcriptase.

second strategy is to change the gene-editing ability so that the gene-editing system can accurately complete the replacement of bases or the replacement and insertion of long sequences. The third strategy is multi-target editing, which would make genome engineering possible. The fourth strategy is to integrate the system with other transgenic elements or change transformation strategies to make transgene-free crops more readily available.

Cas13: Tools for Editing RNA

In classical gene-editing systems, Cas9 and Cas12a are both type II single-component effector proteins that target DNA. In the progressive exploration of immunity in prokaryotes, the Type VI system has been elucidated. This system is characterized by the effector protein C2c2, which contains a higher eukaryotic and prokaryotic nucleotide-binding (HEPN) domain. HEPN domains are characterized by RNA enzyme activity, so C2c2 has the ability to target and edit RNA, and was named Cas13a in subsequent studies (Abudayyeh et al., 2016).

Cas13a targets sequences located 28 nt downstream of crRNA, resulting in single-stranded RNA (ssRNA) degradation and inhibiting target genes at the transcriptional level (**Figure 1B**). In addition, the Cas13b system has been reported to significantly improve its inhibitory efficiency compared with Cas13a in mammalian cells (Cox et al., 2017). Interestingly, cleavage of Cas13a also leads to degradation of non-target RNA *in vitro* or in prokaryotic cells, which often leads to apoptosis, but is highly specific in eukaryotic cells. In plants, Cas13a is suitable for the modification of rice, with a maximal knockdown of 78% (Abudayyeh et al., 2017).

Compared to gene silencing methods, such as RNAi or artificial microRNA, Cas13 is highly specific and, more importantly, does not depend on the plant's own immune function. RNAi or artificial microRNA pairs dsRNA with target RNA in plants and binds with the plant Argonaute (AGO) and Dicer-like (DCL) enzymes to form an RNA-induced silencing complex (RISC), thus completing the silencing of target genes. Therefore, RNAi or artificial microRNA cannot be used to study genes related to the silencing system. Cas13 can complete the silencing of target genes only by itself, without limitations from target genes. In addition, Cas13 can also be used for RNA virus inhibition. Studies have shown that Cas13 can significantly inhibit *Tobacco mosaic virus* in tobacco leaves, so Cas13 has a high breeding potential for disease resistance in crops (Mahas et al., 2019).

Cas13 can also be modified to perform more complex functions to meet production or research needs. dCas13 was obtained by inactivating Cas13 by mutating the HEPN conserved domain and fusing dCas13 with the human adenosine deaminase acting on RNA 2 (ADAR2) deaminase domain to obtain a BE for ssRNA in mammalian cells (Cox et al., 2017). Compared with DNA editing, RNA editing is more prone to recovery and post-transcriptional regulation, which makes it highly safe and ensures its safety in the treatment of genetic diseases. However, this also makes it difficult to use in agricultural traits. dCas13can also fuse with fluorescent proteins to locate target RNA in cells, providing a new tool for RNA localization studies (Abudayyeh et al., 2017). Overall, Cas13 is more accurate than traditional silencing methods such as RNAi, but its application in crops needs further exploration.

Gene Editing in Mitochondria and Chloroplasts

Mitochondria are responsible for plant respiration and are associated with many secondary metabolites, so they could greatly improve crop yield and quality if they can be modified (Balaban et al., 2005). Chloroplasts are directly responsible for photosynthesis and are directly related to crop yield (Chen et al., 2020). However, mitochondria and chloroplasts have relatively independent genomic and DNA repair systems and membrane systems, making traditional gene-editing methods unsuitable.

The first established method to modify mitochondria used TALENs, which were expressed in the nucleus and then transferred to the mitochondria to edit the mitochondrial genome. In rice and rape, mitoTALENs were used to precisely knock out *ORF79* and *ORF125*, which proved to be cytoplasmic male sterility genes (Kazama et al., 2019). In *Arabidopsis thaliana*, the mitoTALENs technique was used to destroy two mitochondrial genes, *AtP6-1* and *AtP6-2*, which proved that conventional mitoTALENs were more effective than single-molecule mito-compact TALENs (Arimura et al., 2020). mtZFNs were also proven useful for mitochondrial gene editing in animal cells (Gammage et al., 2018b). However, attempts to apply CRISPR/Cas9 to mitochondrial genomes encountered some difficulties, mainly because sgRNA was difficult to transport through the mitochondria membrane into the mitochondrial matrix (Gammage et al., 2018a). A recent study showed that sgRNA with short hairpin structures could be transported into the mitochondrial matrix at low levels and complete mitochondrial genome editing (Loutre et al., 2018). In a further improvement, by adding the 20-nucleotide stem-loop structure of *RNase P* to the 5′ end of sgRNA, the efficiency of sgRNA entering the mitochondrial matrix was effectively improved (**Figure 1C**; Hussain et al., 2021). In general, current methods exist to edit mitochondrial genomes, but they generally cause DSB, which results in long sequence changes or overall degradation during mitochondrial repair.

Although there is currently no CRISPR/Cas9-based mitochondrial BE, sgRNA with stem-loop makes it possible to establish this method. Due to difficulties encountered with sgRNA in earlier studies, new methods were developed for mitochondrial BEs. RNA-free DddA-derived cytosine base editors (DdCBEs) were produced by fusing half of the split-DddA transcription activator-like effector array protein with a uracil glycosylase inhibitor (UGI). In human mitochondria, this method can substitute C-G base pairs with A-T base pairs with high precision and efficiency (Mok et al., 2019).

Another strategy for regulating mitochondria modifies mitochondria-related RNA-binding proteins, which are often involved in mitochondrial RNA editing. In *A. thaliana*, RNA processing factor 2 (RPF2) was engineered to target *NAD6* for cleavage, virtually eliminating *NAD6* expression, leading to the accumulation and activity of complex I (Colas et al., 2018). In addition, RNA-binding proteins are involved in base

conversion rather than RNA cleavage in mitochondria, providing a possibility for mitochondrial RNA base conversion (Yang et al., 2020). If the sgRNA transport method of Cas13 could be established, high-precision mitochondrial RNA editing or a base conversion system could also be established.

Compared with mitochondria, gene-editing systems in chloroplasts have developed more slowly. Although there are methods to transform chloroplasts and express sgRNA directly in chloroplasts (Ralph Bock et al., 2019), gene editing in chloroplasts is rarely reported. The first chloroplast gene editing was completed in Chlamydomonas, and the donor DNA was successfully integrated into psaA by transforming a plasmid containing the Cas9 expression cassette and a plasmid containing the donor DNA. Comparing single-target editing of intact Cas9 expression cassette samples with that of Cas9 or sgRNA deletion, single nucleotide polymorphisms in intact Cas9 expression cassette samples were not significantly increased, and no deletion mutations were found (Yoo et al., 2020). This result suggests that chloroplasts lack the NHEJ pathway, preventing the most commonly used gene-editing strategies from being implemented in chloroplasts.

Another feasible chloroplast gene-editing strategy is base editing. DdCBEs have also been applied in chloroplasts. In *A. thaliana*, ptpTALECDs were obtained by modifying DdCBEs to make them suitable for *A. thaliana*, confirming that the base editing system could efficiently edit *16S*, *rpoC1*, and *psbA* (Nakazato et al., 2021). As with mitochondria, chloroplast BEs based on CRISPR-Cas9 have not yet been developed, but based on what is available, the transformation of BEs in chloroplasts should allow base editing.

In general, mitochondrial and chloroplast editing is still in its infancy, and one of the difficulties lies in the shortcomings of the mitochondrial and chloroplast repair mechanisms. A feasible solution is to avoid DSB through BEs. However, mitochondrial and chloroplast editing has great breeding potential, especially for chloroplasts, which are the sites of photosynthesis and encode many key proteins. Rubisco, for example, is a key enzyme in the Calvin cycle. However, due to the complex biogenesis of its large subunit, the exogenous expression has been unable to produce good effects. Therefore, direct modification of Rubisco by gene editing may radically improve crop yield (Bracher et al., 2017).

Base Editors: Precise Substitution of Bases

Clustered regularly interspaced short palindromic repeats based on NHEJ always produces random mutations with low accuracy, and HR requires donor DNA, making the system complex and inefficient (Hess et al., 2017). To overcome these limitations, researchers are working to develop editing techniques with higher efficiency, reliability, and accuracy. BEs are a newly developed precise genome-editing technique that can achieve irreversible base conversion at specific sites (Gao, 2018). After mutating Cas9 (D10A), the Cas9 nickase (nCas9) had single-strand cutting ability only. BEs are a complex consisting of the nCas9 protein, guide RNA (gRNA), and a base deaminase domain capable of converting specific base pairs (Komor et al., 2016).

Base editors are divided into two types, cytosine BEs (CBEs) and adenine BEs (ABEs). CBEs were developed first and can transform C into U. Thus, a U-G base pair can be converted into a T-A base pair during DNA repair and replication. CBEs edit bases by cytosine deaminase and contain UGIs. UGIs, derived from *Bacillus subtilis* bacteriophages, can block the activity of human uracil DNA glycosylase (UDG), preventing U from being repaired to C and reverting to a C-G base pair (**Figure 1D**; Molla and Yang, 2019). The earliest CBE does not contain a UGI and uses the dead Cas9 (dCas9) mutant with no cutting capability, making its C-T base-pair substitution inefficient at only 0.8–7.7% (Komor et al., 2016). In a later version, the efficiency of CBE was greatly improved by the addition of UGI and the replacement of dCas9 with nCas9 (Chen et al., 2017).

Adenine BEs replace A with I, converting I-T base pairs to C-G base pairs during DNA repair and replication. Unlike CBEs, DNA glycosylase inhibitors are not required in ABEs; however, natural adenine deaminases cannot accept DNA. With further understanding of base deaminases, the heterodimer formed by the fusion of RNA adenosine deaminase (TadA) and its mutant deoxyadenosine deaminase (TadA*, W23R, H36L, P48A, R51L, L84F, A106V, D108N, H123Y, S146C, D147Y, R152P, E155V, I156F, and K157N) was found to have a good catalytic capacity for adenine deaminase (Gaudelli et al., 2017). A highly efficient ABE was obtained by fusing this heterodimer with nCas9 (**Figure 1E**).

Compared with general gene editing, base editing requires higher accuracy and is often used to edit specific bases on genes. Therefore, diversification of the PAM sequence has been a hot research topic. One strategy is to look for Cas9 proteins in other species. Presently, the most widely used Cas9 is *Streptococcus pyogenes* Cas9 (spCas9), whose PAM sequence is NGG (Yu et al., 2016). However, Cas9 proteins with different PAM sequences have been found in other species through homology comparison. For example, the NmCas9 protein found in *Neisseria meningitidis* recognizes the PAM sequence NNNGMTT (Hou et al., 2013). StCas9 found in *Streptococcus thermophilus* recognizes the PAM sequence NNAGAAW (Deveau et al., 2008). However, having a longer PAM sequence does not increase the number of targets. Another strategy is to modify spCas9 through protein structure and protein-directed evolution. This strategy diversifies the PAM sequence, resulting in NG, GA, GGA, NGC, NGT, GAT, GAA, CAA, GAG, NGA, NNG, NGAG, and NGCG (Endo et al., 2018; Hu et al., 2018a,b; Ge et al., 2019; Li J. et al., 2019; Wang J. et al., 2019; Zhong et al., 2019; Qin et al., 2020; Xu et al., 2020; Zhang et al., 2020). It is worth noting that different modifications have different effects on specificity. xCas9 improves the ability of DNA specific recognition and reduces off-target efficiency, while SpRYCas9 improves off-target efficiency (Hu et al., 2018a; Walton et al., 2020). Studies in plants have shown that the editing efficiency of xCas9 on atypical PAM sequences (GAA, GAT, and GAG) was lower than for the typical PAM sequence (NGG), but Cas9-NG maintained a higher editing efficiency on the atypical PAM (NG), thus improving the BEs (Endo et al., 2018; Wang J. et al., 2019). In addition to xCas9 and Cas9-NG, the PAM sequence of the BEs was diversified to NAG, NGA, NNNRRT,

NRRH, NRCH, and NRTH by mutating Cas9 in subsequent studies (Hua et al., 2019; Li et al., 2021). In the latest research, the CRISPR-SpRY Toolbox even breaks the restriction of PAM sequences and can be edited PAM-free (Ren et al., 2021). Other studies improved the editing efficiency of xCas9 through enhanced sgRNA.

Notably, shortening the PAM sequence can cause Cas9 to target its own sgRNA sequence, thus reducing editing efficiency or leading to off-target recognition, requiring optimization of the sgRNA sequence (Zhang et al., 2020). Overall, the PAM sequence diversity of BEs has been greatly enriched, vastly improving its applicability in practical production.

Base Editors: Acquisition of Herbicide Resistance and Agronomic Traits Improvement

Presently, BEs have many applications in crops (**Table 1**); the most effective and promising application is the acquisition of herbicide resistance, mainly through accurate modification of acetolactate synthase (ALS), one of the key enzymes in amino acid biosynthesis. There are multiple bases in the *ALS* gene, whose substitution causes plants to acquire resistance to different herbicides (Durner et al., 1991). By CBE editing of Pro to Ser, plants acquire sulfonamide herbicide resistance, which has been widely used in crop cultivation. The strategy has been used to achieve sulfonamide herbicide resistance in wheat (*TaALS*, P174S), watermelon (*ClALS*, P190S), tomato (*SlALS1*, P186S), and potato (*StALS1*, P186S); transgene-free plants were obtained for watermelon, tomato, and potato (Shimatani et al., 2017; Tian et al., 2018; Veillet et al., 2019b). Imidazolinone herbicide resistance was achieved through the CBE mutation *AtALS* S653N much later, mainly owing to the base mutation site being outside the editing window, resulting in low editing efficiency. Finally, imidazolinone herbicide resistance plants were obtained in *A. thaliana* (Dong et al., 2020). In addition to editing *OsALS* to achieve herbicide resistance, haloxyfop-R-methyl herbicide resistance was achieved in rice by mutating C2186R in *OsACC* using the ABE7.10 system (Li et al., 2018).

Several agronomic key traits have also been improved by BEs. For example, the successful application of CBE to edit eIF4E in *A. thaliana* has greatly improved the plant's resistance to disease (Bastet et al., 2019). *OsSBEIIb* and *SSI* were edited in rice and potato, respectively, resulting in decreased starch synthesis (Li et al., 2017; Veillet et al., 2019a). While gene inactivation sometimes leads to plant death, precise replacement by base editing modifies genes rather than inactivates them, making base editing hugely advantageous for studying or improving key genes in crops. However, there are still some shortcomings. BEs, especially CBEs, have been widely used, and ABEs have good specificity, but there is evidence that early CBEs are off-target at the whole gene level, resulting in a large number of C substitutions in plant genomes (Jin et al., 2019). To reduce off-target effects, the deaminase domain in BE3 was replaced with a member of the APOBEC3 cytidine deaminase family. This modification proved to have good specificity in rice (Jin et al., 2020).

Prime Editors: Precise Modification of DNA Sequence

Non-homologous end joining always results in random DNA repair, and base editing struggles to cover all sequence modifications, so PE was invented to modify DNA sequences precisely. PE is a versatile and precise genome-editing method that uses nCas9 fused to an engineered reverse transcriptase, while template RNA is linked to sgRNA to form prime editing guidance RNA (pegRNA), which both specifies the target site and encodes the desired editing sequence. After nCas9 cleaves the single strand of DNA, template RNA paired with the sequence near the editing site, and the pegRNA template complementary strand is resynthesized under the action of Moloney murine leukemia virus (M-MLV) to complete accurate editing (**Figure 1F**; Anzalone et al., 2019). To date, PE has been applied in rice, wheat, corn, and tomato (Jiang et al., 2020; Lin et al., 2020; Lu et al., 2021a). However, prime editing efficiency in plants was significantly lower than reported in human cells. Therefore, various strategies have been undertaken to optimize prime editing efficiency. Improvements to the components of PE, including optimization of pegRNA length, alteration of the engineered reverse transcriptase, and enhancement of the pegRNA promoter were performed (Jiang et al., 2020; Lin et al., 2020, 2021). Also, the same edits were made using different pegRNA on the template and antisense strand (Lin et al., 2021). These methods improved prime editing efficiency in plants. In the latest study, pairs of pegRNA were able to precisely delete 710 bp or precisely replace a sequence of 108 bp (Anzalone et al., 2021). In general, the great potential of prime editing remains to be explored.

Multi-Target Editing: Genome Engineering

For CRISPR/Cas9 systems, multi-target editing is easily implemented with multiple sgRNAs. In plants, there are two main strategies to obtain multiple sgRNAs: one strategy is to use multiple promoters to express different sgRNAs, the other strategy is to use specific sequences (tRNA, Cys4, and HDV-HH) to separate different sgRNAs and cut them into independent sgRNAs *in vivo* (Xing et al., 2014; Xie et al., 2015; Gasparis et al., 2018). At present, multi-target editing is mainly used for simultaneous editing of multiple genes or saturation knockout of a single gene, which can sometimes lead to genomic changes, such as deletion, duplication, or inversion of fragments (Zhou et al., 2014).

Currently, gene editing mainly silences genes through early termination of the coding frameshift, but this method produces truncated proteins that, in some cases, are functional and can affect research and breeding (Qing et al., 2020). In addition, mutation of individual bases *via* gene editing sometimes fails to cause changes in the secondary structure of lncRNA, so there will be no functional changes. One way to avoid these disadvantages is to delete these long fragments so that the gene disappears completely. Although many large fragment deletion results have been reported, these cases are often random and difficult to apply in practice. A feasible approach is to design multiple targets

TABLE 1 | Application and improvement of BEs.

Plant species	Target gene	BE	PAM	Editing efficiency (%)	Contributions	References
Oryza sativa	*FTIP1e* *ALS*	Target-AID	NGG	4.3–85.7	Generation of imazamox herbicide resistance	Shimatani et al., 2017
Oryza sativa	*NRT1.1B* *SLR1*	APOBEC1	NGG	2.7–13.3	Regulate the conversion and utilization of nitrogen	Lu and Zhu, 2017
Oryza sativa	*OsCDC48* *OsNRT1.1B* *OsSPL14*	PBE	NGG	0.5–7.0	Improve editing efficiency by using nCas9	Zong et al., 2017
Oryza sativa	*OsPDS* *OsSBEIIb*	BE3	NGG	20	Reduced starch synthesis	Li et al., 2017
Oryza sativa	*OsCERK1* *OsCERK2* *OsCERK3* *ipa1* *pi-ta* *BRT1*	rBE3 rBE4	NGG NGA	10.5–38.9	Increase PAM sequence diversity	Ren et al., 2017
Oryza sativa	*OsSPL14* *OsSPL16* *OsSPL17* *OsSPL18* *SLR1*	ABE-P1	NGG	4.8–61.3	ABE was improved for rice	Hua et al., 2018
Oryza sativa	*OsRLCK185* *OsCERK1*	rBE5	NGG	2.1–27.8	CBE is optimized for GC AC sequences	Ren et al., 2018
Oryza sativa	*OsMPK6* *OsMPK13* *OsMPK2* *OsMPK6* *Tms9-1*	rBE14 rBE15 rBE17 rBE18	NGG	4.30–62.26	Developed a fluorescence-tracking adenine base editor	Yan et al., 2018
Oryza sativa	*OsACC* *OsALS* *OsCDC48* *OsDEP1,* *OsNRT1.1B-T1*	pABE	NGG	5.8–59.1	Herbicide resistant rice was obtained by ABE for the first time	Li et al., 2018
Oryza sativa	*OsCDC48* *OsNRT1.1B-T1*	PBE	NGG	44.1–82.9	Expand edit window	Zong et al., 2018
Oryza sativa	*OsGL1-1* *OsNAL1*	PBE	NGG	75	Intron shearing is interfered with by BE	Li Z. et al., 2019
Oryza sativa	*OsNGN1*	nSpCas9-NGv1-AID nSpCas9-NGv1-AID nSpCas9-NGv1-APOBEC-UGI	NG	6.3–91.1	Increase PAM sequence diversity	Endo et al., 2018
Oryza sativa	*OsSPL14* *OsSPL16* *OsSPL18* *GRF4* *OsSPL17* *sTOE1* *OsIDS1* *OMTN1* *SNB* *sSPL13*	ABE-P1-5 CBE-P3 CBE-p5 pKKH-Cas9 pVQR-Cas9	NAG NGA NNNRRT	2.6–74.3	Increase PAM sequence diversity	Hua et al., 2019
Oryza sativa	*OsDEP1*	Cas9-NG (D10A)-PmCDA1	NG	30.4–45.0	Increase PAM sequence diversity	Zhong et al., 2019
Oryza sativa	*IPA1* *Pikh* *WX*	NRRH-eBE3 NRCH-eBE3 NRTH-eBE3	NRRH NRCH NRTH	2.08–79.17	Increase PAM sequence diversity	Li et al., 2021
Oryza sativa	*OSPDS* *OsALS* *OsDSP1*	CRISPR–SpRY	PAM-free	5.3–79.0	Break the PAM sequence constraint	Ren et al., 2021
Triticum aestivum	*TaLOX2*	PBE	NGG	1.5–5.2	Improve editing efficiency by using nCas10	Zong et al., 2017
Triticum aestivum	*TaDEP1* *TaGW2*	pABE	NGG	0.4–1.1	ABE was applied in wheat for the first time	Li et al., 2018

(Continued)

TABLE 1 | (Continued)

Plant species	Target gene	BE	PAM	Editing efficiency (%)	Contributions	References
Triticum aestivum	*TaALS* *TaMTL*	A3A-PBE	NGG	16.7–22.5	Expand edit window	Zong et al., 2018
Triticum aestivum	*TaALS*	PBE	NGG	22–78	Improve screening efficiency through co-editing	Zhang et al., 2019
Solanum tuberosum	*StGBSS-T6*	A5A-PBE	NGG	6.5	Expand edit window	Zong et al., 2018
Solanum tuberosum	*SSI protein*	CBE	NGG	71	Reduce potato starch content	Veillet et al., 2019a
Solanum tuberosum	*StALS* *StALS2*	Target-AID	NGG	100	Generation of sulfonylurea herbicide resistance in transegene-free potato	Veillet et al., 2019b
Solanum lycopersicum	*DELLA* *ETR1*	Target-AID	NGG	26.2–53.8	Altered hormone regulation	Shimatani et al., 2017
Solanum lycopersicum	*SlALS1* *SlALS2*	Target-AID	NGG	71.4	Generation of sulfonylurea herbicide resistance in transegene-free tomato	Veillet et al., 2019b
Arabidopsis thaliana	*AtALS*	BE3	NGG	1.7–7.6	The edited plants were obtained by subculture	Chen et al., 2017
Arabidopsis thaliana	*AtALS* *AtPDS* *AtFT* *AtLFY*	BE6.3 BE7.8 BE7.9 BE7.10	NGG	0.3–10	ABE has been verified in plants	Kang et al., 2018
Arabidopsis thaliana	*eIF4E*	CBE	NGG	67.9	Enhanced arabidopsis resistance to disease	Bastet et al., 2019
Arabidopsis thaliana	*AtMTA*	PBE	NGG	49.1	Intron shearing is interfered with by BE	Li Z. et al., 2019
Arabidopsis thaliana	*AtALS*	CBE	NGG	14.3–66.7	Generation of imidazolinone herbicide resistant	Dong et al., 2020
Zea mays	*ZmCENH3*	PBE	NGG	0.3–3.7	Improve editing efficiency by using nCas11	Zong et al., 2017
Citrullus lanatus	*ClALS*	BE3	NGG	22.6	Transgene-free tribenuron herbicide resistant watermelon was obtained by CBE in watermelon	Tian et al., 2018
Brassica napus	*BnALS* *BnPDS*	BE6.3 BE7.8 BE7.9 BE7.10	NGG	0.6–8.8	ABE has been verified in plants	Kang et al., 2018

with the expectation of achieving fragment deletion between the targets. This strategy has been successfully established in rice and soybean (Wang et al., 2017; Cai et al., 2018). Furthermore, large fragment deletion efficiency can be significantly improved using microhomology-mediated end joining in rice by designing targets near microhomologous sequences (Tan et al., 2020). However, the deletion of mature and efficient large fragments is currently only reported in crops with high editing efficiency, mainly rice and soybean, and there are few reports in crops with low editing efficiency. The large fragment deletion method still requires improvement.

Multi-target editing can also cause the inversion of large segments of chromatin. In maize, by designing six targets, three on each side, 75.5 Mb were inverted on chromosome 2, simulating the mutation that occurs in nature in maize (Schwartz et al., 2020). In addition, in rice, through the design of exquisite targets, *CP12* and *PPO1* exchanged positions through a 911 kb inversion, and a new expression box was formed between the promoter of *Ubiquitin2* and *HPPD* through a 338 kb duplication. Due to the high expression levels of original *CP12* and *Ubiquitin2* in leaves, the change of promoter increased the expression levels of *PPO1* and *HPPD* by tens of folds, which enabled rice to have sufficient herbicide resistance in field tests without adverse effects on other important agronomic traits (Lu et al., 2021b). This method confirmed a new strategy for upregulating target gene expression independent of transgenic elements.

In summary, multi-target CRISPR/Cas9 has a high potential for chromatin engineering, both to simulate natural mutations and to make knockout results more reliable. However, chromatin engineering can only be applied to crops with high editing efficiency, such as rice. Application in other crops requires optimization of editing efficiency or interference with DSB repair.

Modified Gene-Editing Systems Help Obtain Transgene-Free Crops

Obtaining transgene-free plants can be of great value for the commercialization of crops; in addition, transgene-free plants can be more easily used for other gene function studies in scientific research. Currently, CRISPR transgenic plants are

obtained using Agrobacterium-mediated transformation. The Cas9-expression cassette and sgRNA-expression cassette with resistance screening genes are transferred into the plant genome, resulting in transgenic plants expressing Cas9 and sgRNA, allowing complete editing on the genome. Thus, the majority of T0 plants contain transgenic elements. Traditionally, the acquisition of transgene-free plants relies mainly on the genetic segregation of plants after self-fertilization. If only one copy of the CRISPR transgene is inserted into the genome in the T0 plants, 25% of transgene-free plants can be obtained in the T1 generation according to Mendelian genetics. However, transgenes often result in multiple copies of insertions that may be distributed on different chromosomes, making it extremely difficult to obtain transgene-free plants by self-fertilization (Lowe et al., 2009). Crossing T0 plants with wild-type plants would effectively eliminate the transgene, but it takes an extra generation to reach the goal of obtaining transgene-free and edited plants. In general, there have been many experiments to isolate transgenic free plants from the progenies of T0 plants using conventional genetic segregation, but it is laborious and time-consuming.

In the isolation of transgene-free plants, the most time-consuming and labor-intensive work is planting, sampling, DNA extraction, and identification. Therefore, a method to identify or screen whether the seeds are genetically modified would avoid much unnecessary labor. An effective way to reduce the workload is to marker transgenic plants by fluorescence. In *A. thaliana*, by adding the mCherry fluorescent marker gene into the transgenic elements, the red fluorescence of mCherry can be used to identify and isolate transgene-free seeds (**Figure 2A**; Gao et al., 2016). This method has also been applied in rice, effectively reducing the amount of work required to obtain non-transgenic plants (Chang et al., 2016). In addition, GFP fluorescent protein has been used to identify transgenes. Through the 2A peptide, a picornavirus, which has been shown to have self-splitting properties in various animal cells and tissues, linking GFP to Cas9 can produce transgenic elements expressing both Cas9 and GFP without the addition of a promoter. This method can also be used to analyze the expression of Cas9 because Cas9 and GFP are expressed simultaneously by protein translation, which enables a side-analysis of editing efficiency through fluorescence intensity (Wang and Chen, 2020).

Direct transgene-free seeds can also be obtained by self-elimination. The BARNASE gene encodes a toxic protein with nuclease activity, whose expression can kill plant cells. The promoter of *REG2* was found to be specifically expressed in early rice embryos, while the rice male gametophyte-specific lethal protein CMS2 can disrupt mitochondrial function during the development of male gametophytes, leading to male sterility. By adding a REG2: *BARNASE* and 35S: *CMS2* expression cassette into the transgenic element, the transgenic male gametes and embryos were directly killed, thus ensuring that the seeds obtained were non-transgenic (**Figure 2B**; He et al., 2018). Compared with fluorescence screening, this method can directly obtain non-transgenic seeds without manual screening. However, the death of many seeds will lead to a decrease in the number of plant progeny, reducing the sample size obtained.

Agrobacterium mediates transient transgene expression when transgenic elements enter plant cells (Amoah et al., 2001; Krenek et al., 2015). With the help of transient transgene expression, sgRNA and Cas9 can be edited directly in plant cells without integrating the transgenic elements into the plant genome, providing a method for directly obtaining transgene-free plants in the T0 generation (**Figure 2C**). Using this strategy, transgene-free plants have been successfully obtained in tobacco, tomato, and potato (Chen et al., 2018; Danilo et al., 2019; Veillet et al., 2019b). The key point of this strategy is to stop using antibiotic screening after plants are edited through transient transformation so that transgene-free cells can survive. In general, 4.9–10% of transgene-free edited plants could be obtained by this method. The biggest advantage of this method lies in the direct acquisition of transgene-free edited plants in the T0 generation, without the need to separate transgenic elements in the plant progeny. However, there are four types of T0 plants: edited plants with transgenic elements, edited plants without transgenic elements, unedited plants containing transgenic elements, and wild-type plants. It is laborious to identify and isolate these four types of plants, and the number of edited plants would decrease significantly due to non-resistance screening, so this strategy remains expensive. One possible modification is to combine transient transformation with fluorescent proteins to screen non-transgenic plants during tissue culture, thereby reducing unnecessary labor.

In animal cells, the most commonly used method is to combine purified recombinant Cas9 with *in vitro* transcribed sgRNA to form cas9-sgRNA RNP complex *in vitro* and deliver the complex *in vivo* with gene gun or microinjection (Burger et al., 2016; Suzuki et al., 2018). RNP can also be used to edit plants, typically in protoplasts. Compared with plasmids, RNP does not require transgenic elements, so all the obtained plants are transgene-free (**Figure 2D**). Edited protoplasts have been obtained from apple and soybean using this strategy (Malnoy et al., 2016; Kim et al., 2017). However, compared with the production of edited protoplasts, the regeneration of protoplasts into plants is complicated, and many plants cannot complete this process. Methods of editing cells by bombarding immature embryos or calli with gene guns and regenerating plants by tissue culture also exist, but these methods do not enable plants to acquire resistance genes and, therefore, cannot be screened with antibiotics or herbicides. Under the condition of no selection pressure, the proportion of edited plants obtained by this method is low, so requires much effort to screen. Therefore, this method has not been widely adopted in the laboratory.

The low efficiency of RNP may be because most cells are not transformed when bombarding calli with RNP owing to the structure of calli. It is possible to improve this efficiency if a new RNP conversion method is established. In animal cells, one potential strategy is to encapsulate RNP by adenovirus proteins and use viral mechanisms to transform RNP into cells. A large amount of RNP can be wrapped in the virus, significantly improving the transformation efficiency. The efficiency of both Cas9 knockout and base editing can be significantly improved by this method (Baisong et al., 2019; Pin et al., 2019). At present, this method has not been applied in plants, and

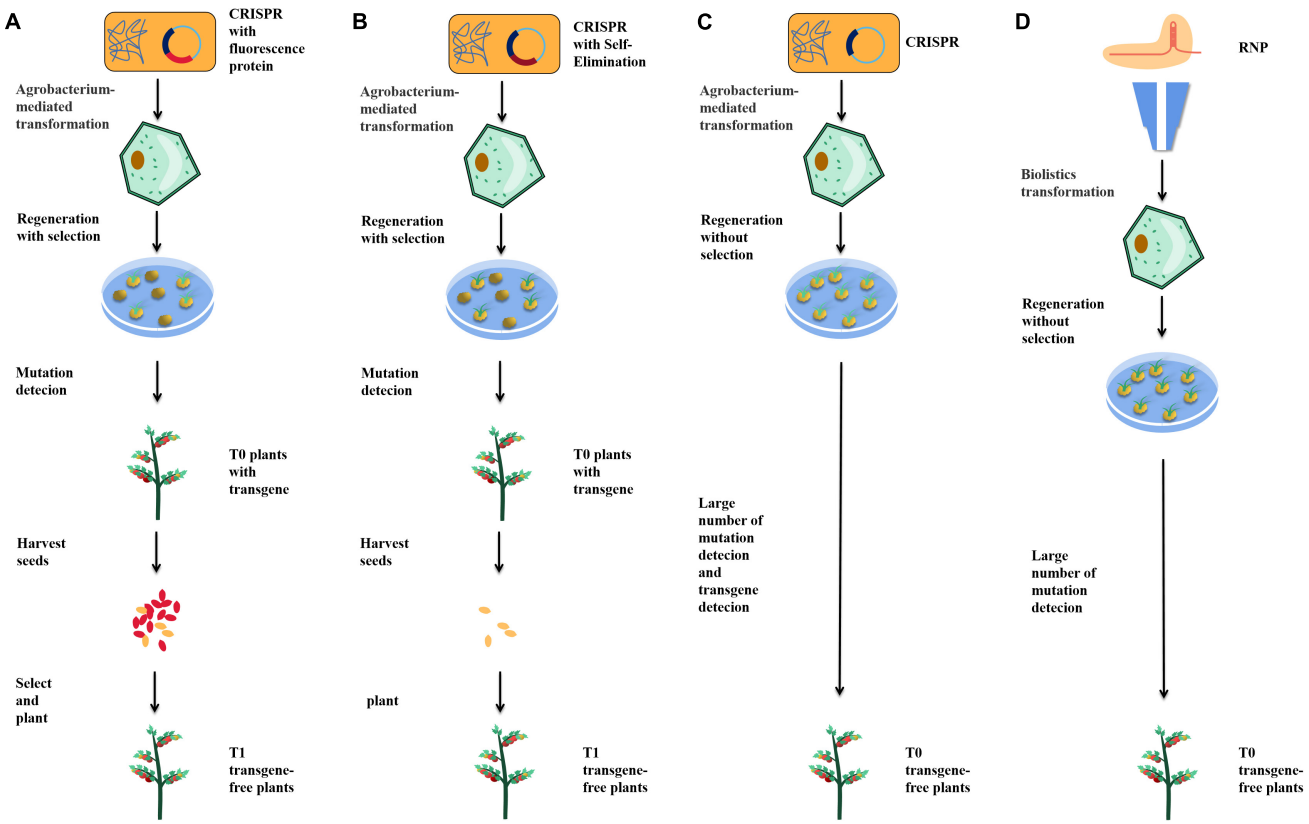


FIGURE 2 | Strategies to help obtain transgene-free plants. **(A)** Fluorescence labeling screening. **(B)** Self-elimination. **(C)** Agrobacterium mediate transient transgene expression. **(D)** Bombardment-mediated RNP delivery.

further research is needed to select and understand how to encapsulate the virus.

CONCLUSION AND FUTURE PERSPECTIVES

Traditional breeding methods are no longer able to meet the food security challenges posed by population growth owing to their highly laborious and time-consuming techniques. Compared with traditional breeding, molecular breeding, especially gene-editing systems, has the advantages of being highly efficient and safe. At present, classical gene-editing systems have been successfully applied to improve disease resistance, stress resistance, and quality traits of crops (Wang T. et al., 2019). Gene-editing systems have also achieved some success in plant synthetic biology and plant microbial engineering (Gao, 2021).

However, classical gene-editing systems have their limitations. They often rely on NHEJ to make editing random, which results in poor performance when studying lethal genes (Zhang et al., 2021). Also, changes in individual bases mean that the secondary structure of RNA often does not change, making studies on plant lncRNAs rely on the deletion of large fragments, which has a very low probability. In contrast, the modified gene-editing system gives researchers more options. BEs can precisely replace bases, while PEs can precisely replace or insert sequences at target sites, which allow lethal genes to be modified rather than inactivated, thus preventing crop death. Genomic engineering can make lncRNA knockout more efficient and reliable. Cas13 has a more precise and broad range of targets that can replace traditional gene silencing techniques. Mitochondrial and chloroplast editing regulates photosynthesis and respiration, including key genes for crop yield. Optimized transgene-free access makes it easier to commercialize edited crops. In general, with the efforts of many researchers, modified gene-editing systems provide new technical means for crop research and breeding.

Like classical gene-editing systems, many modified systems are limited by species. The vast majority of systems performed well in rice but poorly in other species. On the one hand, as the main grain, rice is of high research value, so is often selected as the research target. But on the other hand, the same gene-editing system tends to be more efficient in rice and significantly less efficient in other crops, showing species-specificity. Therefore, the optimization of gene-editing systems for different species has high research value and will be an important research direction in the future. In addition to promoter and codon preference, a possible cause of the inefficiency is crop culture temperature, which is lower for most crops than rice. There is evidence that both Cas9 and Cas12a are temperature sensitive. Therefore,

lifting the temperature limit may be helpful for the optimization of gene-editing systems.

The combination of different CRISPR modifications can also significantly increase the power of gene-editing systems. For example, paired pegRNAs PEs obtained by combining multi-target editing with PEs can modify longer sequences than classical PEs. BEs have excellent performance in mitochondrial and chloroplast editing owing to the defect in DSB repairability. PAM-free causes CRISPR to target itself and edit, which reduces editing efficiency and increases off-target recognition. If PAM-free is combined with Agrobacterium-mediated transient expression or bombardment-mediated RNP delivery, this deficiency may be overcome, and transgene-free plants could be obtained directly. However, compared with classical gene-editing, the editing efficiency of modified gene-editing systems will be reduced, and combinations of modified gene-editing systems may lead to lower editing efficiency. Improving transformation efficiency and screening efficiency could compensate for this issue.

Many gene-editing systems have high potential. Therefore, it is expected to see further applications of gene-editing systems in crop research and breeding in the future, which could contribute enormously to solving the problem of human food security.

AUTHOR CONTRIBUTIONS

GZ completed literature review and writing of this review. HZ reviewed the review and directed the revisions. Both authors contributed to the article and approved the submitted version.

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Functional Differentiation of *BnVTE4* Gene Homologous Copies in α-Tocopherol Biosynthesis Revealed by CRISPR/Cas9 Editing

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Tocopherols are essential nutrients for human health known as vitamin E. Vitamin E deficiency can have a profound effect on human health, including the central nervous system and cardiovascular and immune protection. Multiple enzymatic steps are involved in the conversion between different forms of tocopherols. Among them, γ-tocopherol methyltransferase encoded by gene *VTE4* catalyzes the conversion of γ- to α-tocopherol or δ- to β-tocopherol isoforms. However, the gene copies and their functional contribution of *VTE4* homologs in *Brassica napus* were not elucidated. To this end, different mutation combinations of four putative *BnVTE4* homologous copies were generated by using CRISPR/Cas9 genome editing technology. Editing of those *BnVTE4* homologs led to a significant change of the α-tocopherol content and the ratio between α- and γ-tocopherol compared with wide-type control. Analysis of the different combinations of *BnVTE4*-edited homologs revealed that the contribution of the *BnVTE4* individual gene displayed obvious functional differentiation in α-tocopherol biosynthesis. Their contribution could be in order of *VTE4.C02-2* (BnaC02G0331100ZS) > *VTE4.A02-1* (BnaA02G0247300ZS) > *VTE4.A02-2* (BnaA02G0154300ZS). Moreover, the *VTE4.A02-1* and *VTE4.A02-2* copies might have severe functional redundancies in α-tocopherol biosynthesis. Overall, this study systemically studied the different effects of *BnVTE4* homologs, which provided a theoretical basis for breeding high α-tocopherol content oilseed rape.

Keywords: α-tocopherol, *BnVTE4*, homologous copies, functional diversification, CRISPR/Cas9

INTRODUCTION

Tocopherols and tocotrienols are also called vitamin E. In this study, we focus on the analysis of tocopherols only. Tocopherols are lipid-soluble strong antioxidants that are involved in the protection of oxidative damage of membrane lipids by scavenging singlet oxygen and other reactive oxygen species (ROS) (Trebst et al., 2002; Schneider, 2005; Fritsche et al., 2017). Thus, tocopherols are presumed to be key scavengers of senescence or stress-induced lipid radicals and ROS in plants. Their strong antioxidant capacity can reduce lipid peroxy radicals to corresponding hydrogen

peroxide to avoid lipid peroxidation of polyunsaturated fatty acids. The high antioxidative property exerts a protective role in multiple plant stress responses such as cold and drought stress (Janeczko et al., 2018; Ma et al., 2020). Most importantly, this effect can also be extended to human health. Vitamin E is an essential nutrient in the human daily diet. Vitamin E deficiency primarily causes muscle atrophy and reproductive and neurologic dysfunctions (Martin et al., 2013; Kumar et al., 2018), whereas the adequate intake of vitamin E can prevent neurological diseases, cataracts, coronary heart disease, atherosclerosis, diabetes, Parkinson's disease, Alzheimer's disease, and vision diseases (Lloret et al., 2019; Rozanowska et al., 2019). Thus, biofortification of vitamin E in crop plants is not only beneficial for human health but also for plant stress response.

Based on the different numbers and positions of methyl substituents on the aromatic ring, tocopherols are defined as four isoforms, namely α-, β-, γ-, and δ-tocopherol (α-T, β-T, γ-T, and δ-T in short). Although those tocopherols have similar antioxidant activities *in vitro*, the vitamin E activity *in vivo* is significantly different. Among them, α-T possesses the highest vitamin E activity *in vivo* and is also a common type of tocopherol in the European diet (Galmés et al., 2018). It has been proven that α-T supplementation can improve cell-mediated immunity, and only α-T is selected to set the recommended dietary allowance (RDA) of vitamin E for Americans (Meydani et al., 1990; Meydani et al., 1997; Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds, 2000; Ranard and Erdman, 2018). Tocopherols are only synthesized on the inner chloroplast membrane of photosynthetic organisms, including plants, green algae, and some cyanobacteria. Undoubtedly, a daily supplement of α-T derived from plant-based food is a safe and natural way to ensure human health.

Oilseed rape (*Brassica napus* L., AACC, 2n = 38) is one of the most important resources of edible vegetable oil in the world, accounting for about 16% of the total global vegetable oil production (Cheng et al., 2019). High-quality rapeseed oil, containing multiple beneficial nutrients including vitamin E, is the dominant edible vegetable oil in China, Europe, and Canada. A daily supplement of vitamin E *via* rapeseed oil is the safest and most effective way to keep the nutritional requirement for the human body. Therefore, genetic improvement of vitamin E content has been considered a key breeding objective (Cheng et al., 2019; Yan et al., 2021). However, oilseed rape is a typical allotetraploid crop, and most of the genes have multiple homologous copies with redundant or diverse functions (Chalhoub et al., 2014; Braatz et al., 2017; Zaman et al., 2019, 2021; Li et al., 2021). Clarification of the detailed roles of each homologous copy is the basis of the further breeding application.

The biosynthesis pathway of tocopherols has been extensively studied in the model plant *Arabidopsis thaliana* (Valentin et al., 2006; Vom Dorp et al., 2015). Tocopherol biosynthesis begins with the formation of homogentisic acid (HGA), which is catalyzed by p-hydroxyphenylpyruvic acid dioxygenase (HPPD/PDS1) and is derived from the deamination of tyrosine (Norris et al., 1995, 1998; Tsegaye et al., 2002). Phytyldiphosphate (PDP) is formed by phytol kinase, and phytyl—P kinase catalyzes the formation of phytol (Valentin et al., 2006; Vom Dorp et al., 2015). HGA and PDP are condensed to 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) catalyzed by the enzyme homogentisatephytyltransferase (HPT), which is encoded by *VTE2* gene (Venkatesh et al., 2006). The 2-methyl-6-phyty-1,4-benzoquinol methyltransferase (MPBQ MT) methylates MPBQ to 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ), while MPBQ and DMPBQ are transformed into γ- and δ-tocopherol, respectively, by tocopherol cyclase (TC). TC is encoded by the *VTE1* gene, and MPBQ MT is encoded by *VTE3* gene (Porfirova et al., 2002; Semchuk et al., 2009). Then, the γ-Tmethyltransferase (γ-TMT) encoded by *VTE4* gene catalyzes the conversion of δ- to β-T and γ- to α-T (Porfirova et al., 2002; Bergmüller et al., 2003; DellaPenna and Pogson, 2006; Hunter and Cahoon, 2007; **Figure 1**). Thus, *VTE4* directly affects the content of α-T and γ-T. However, the putative functional differentiation of the *VTE4* gene in *B. napus* is still unclear.

In this study, four putative copies of the *BnVTE4* gene have been identified from the latest oilseed rape genome database. The functional contribution of *BnVTE4* homologs in the α-T biosynthesis is studied by generating different mutation types using CRISPR/Cas9 genome editing technology. This study will shed new light on the breeding application of high α-T content in oilseed rape.

MATERIALS AND METHODS

sgRNA Design and Vector Construction

Four homologous copies of the *BnVTE4* gene were retrieved in the *B. napus* genome database,[1] namely BnaC02G0197500ZS (VTE4.C02-1), BnaC02G0331100ZS (VTE4.C02-2), BnaA02G0247300ZS (VTE4.A02-1), and BnaA02G015430ZS (VTE4.A02-2). Two sgRNAs with minimal off-target effects were designed using CRISPR-P 2.0[2] at the conserved sequence positions of the third and fourth exons, namely S1 (GGTGAGCATATGCCTGACA) and S2 (CCATGGGAGCAGAACCTCT). The sgRNA assembly and vector construction were performed as a previous report (Xing et al., 2014; Ma et al., 2015).

Plant Material and Genetic Transformation of Oilseed Rape

The qualified genome editing vector was transferred into *Agrobacterium tumefaciens* strain (GV3101) by the heat shock method. The vector containing two sgRNAs was introduced into *B. napus* L. variety "Zhongshuang 6" by the *Agrobacterium*-mediated transformation (Li et al., 2018). The selection marker was Kanamycin. The T_0 generation mutants were planted in the artificial climate room and grown under a photoperiod of the 16 h light/8 h dark at a temperature of 22°C, and the T_1 generation was planted in the field of the Hanchuan transgenic base, Hubei, China.

[1] http://cbi.hzau.edu.cn/bnapus/

[2] http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR

Identification of Positive Mutants

Plant genomic DNA was extracted from leaves by the CTAB (hexadecyltriethyl ammonium bromide) method. We used *NPTII* gene-specific primers NPTII-F (5′-GATGGATTGCACGCAGGT-3′) and NPTII-R (5′-TCGTCAAGAAGGCGATAGA-3′) for PCR reaction to identify positive transgenic plants.

To identify whether the *BnVTE4* gene of the positive transgenic plants had been edited, gene-specific primers (**Supplementary Table 1**) were used to amplify the DNA sequence containing the target site by PCR, and then Sanger sequencing was used to identify the mutants. The heterozygous mutants were determined by the Hi-TOM platform (Liu et al., 2019). Hi-TOM sequencing consists of two rounds of PCR. In the first round of PCR, gene-specific primers (**Supplementary Table 1**) were used to amplify the genomic sequence of about 500–2,000 bp around the 4 copies of the target site. In the second round of PCR, gene-specific primers containing Hi-TOM adaptor primers (**Supplementary Table 1**) were used to amplify the 80–300 bp genomic region around the target site. The products of the second round were sequenced by the company.[3]

Tocopherol Extraction and Analysis

Tocopherol extraction was performed according to the reported method with slight modification (Yu et al., 2016; Xu et al., 2019). A total of 200 mg seeds were placed in a 2 ml centrifuge tube with 1 steel bead of a 5-mm diameter and grounded for 5 min at 60 Hz using a rapid grinder. An accurate 60 mg aliquot was weighed from the ground seeds. Three biological replicates were set up. Tocopherols were extracted by adding 1.5-ml hexane. The mixture was sealed and shaken for 4 h in the dark and then extracted at 4°C for 12 h. The mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was filtered through a.22-μm organic membrane.

Determination of tocopherols was carried out on high-performance liquid chromatography (HPLC, Waters). Agilent liquid chromatography column ZORBAX RX-SIL (4.6 mm × 250 mm) was used, and the temperature was set at 30°C. The mobile phase was n-Hexane:isopropanol (99:1, v/v) at a flow rate of 1 ml/min. The sample composition was determined qualitatively and quantitatively by UV light at 292 nm. Standards (95%, pure HPLC) for α- and γ-T were purchased from Merck, and all the standards and samples were in 5 μl injection volumes.

Statistical software SPSS v22.0 was used to analyze the data, and one-way ANOVA was employed to comparatively analyze the differences between α- and γ-T of different copy mutation combination materials and wild-type rape seeds ($p < 0.05$).

RESULTS

Sequence Analysis and Vector Construction for *BnVTE4* Gene

Genomic sequence analysis showed that the *BnVTE4* gene possesses four homologous copies in *B. napus*, each of which is composed of six exons and five introns. Two sgRNAs (named S1 and S2) were designed in their conserved sequence regions located in the third and fourth exons, respectively (**Figures 2A,B**).

Generation of Different *BnVTE4* Mutation Types in Oilseed Rape

In order to elucidate the possible functional differentiation of different homologous copies of the *BnVTE4* gene during α-T synthesis, we screened different mutation types of *BnVTE4* editing in the T_1 generation. Five editing types with different mutation combinations were obtained, named *bnvte4-1, bnvte4-2*, *bnvte4-3*, *bnvte4-4*, and *bnvte4-5*, respectively. Sequencing results indicated that *VTE4.C02-1* and *VTE4.C02-2* copies were homozygous mutations or wild type (WT) (**Figure 3A**). All five editing types had a homozygous mutation in the *VTE4.C02-1* copy with a single base insertion leading to a frame shift. The *VTE4.C02-2* copy also had homozygous mutations with single-base insertions leading to a frame shift in *bnvte4-1, bnvte4-3*, *bnvte4-4*, and *bnvte4-5*, except for *bnvte4-2* that was not mutated (**Figure 3**). *VTE4.A02-1* and *VTE4.A02-2* copies had heterozygous mutations in some editing types, and Hi-TOM high-throughput sequencing was employed to verify the editing frequency and amino acid changes at the targeted sites (**Figures 3B–F** and **Supplementary Figures 1, 2**). *VTE4.A02-1* in *bnvte4-1* and *bnvte4-4* was unmutated (WT). One nucleotide deletion in the *VTE4.A02-1* copy of *bnvte4-2* caused 64% of the frame shifts (**Figures 3A,C**). Both *bnvte4-3* and *bnvte4-5* had one or several nucleotide deletions and one nucleotide insertion in the *VTE4.A02-1* copy, 78 and 83%, respectively (**Figures 3A,D,F**), resulting in frame shifts or amino acid deletions. The *VTE4.A02-2* copies of *bnvte4-1* had a deletion of one nucleotide, resulting in frame shifts (**Figures 3A,B**). The *VTE4.A02-2* copies of *bnvte4-2* had a deletion of one nucleotide, resulting in frame shifts that accounted for only 6%, and the one nucleotide substitution without amino acid change accounted for only 1% (**Figures 3A,C**). Both *bnvte4-3* and *bnvte4-5* had one or more nucleotide deletion and one nucleotide insertion in the *VTE4.A02-2* copy, accounting for 88 and 90%, respectively (**Figures 3A,D,F**), resulting in frame shifts or amino acid deletions. The *VTE4.A02-2* copy of *bnvte4-4* had one or more nucleotide deletions, resulting in frame shifts of just 12% (**Figures 3A,E**).

Determination of Tocopherol Content in *BnVTE4* Mutant Types

The five *BnVTE4* T_1-mutated lines and WT control were grown and harvested under the same condition with good growth and no significant difference from the control (**Supplementary Figure 3**), and mature seeds were used to determine the content and composition of tocopherols. HPLC results showed that the contents of α-T and γ-T were successfully detected in both WT control and T_1 seeds (**Figure 4**). Consistent with a previous report (Zhang et al., 2007), the β- and δ-tocopherol contents were extremely low, which were neglected in the subsequence analysis.

[3]http://121.40.237.174/Hi-TOM/

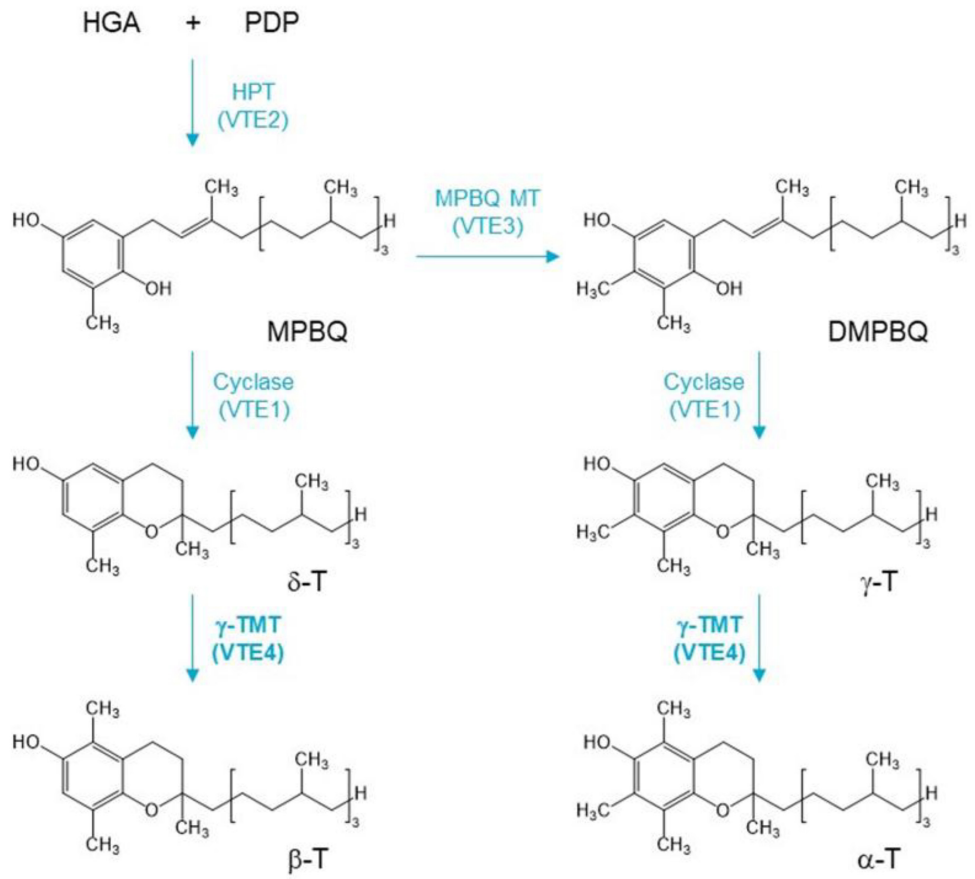


FIGURE 1 | A simplified pathway of vitamin E isoform conversion. HGA, homogentisic acid; PDP, phytyldiphosphate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinone; α-T, β-T, γ-T, and δ-T are α, β, γ, and δ isoforms of tocopherol. *VTE* genes are described in the text. γ-Tocopherol methyltransferase (γ-TMT) gene *VTE4* (bold) is the target gene to be edited in this study.

As shown in **Figure 4**, α-T content in *BnVTE4* mutant lines was substantially decreased and significantly lower than that in WT ($p < 0.05$, **Figure 5A**). The reduction of α-T content in the *bnvte4-2* mutant type was the lowest one among these five mutant types. The reduced α-T content was accompanied by a significant increase in γ-T content compared to the WT ($p < 0.05$, **Figure 5B**), except for the *bnvte4-2* mutant type, which had a similar level of γ-T compared to WT. Nevertheless, the ratios of α- to γ-T (α-/γ-T) in all five *BnVTE4* mutant types were only 0.1–0.5, which were significantly lower than 0.67 in WT oilseed rape ($p < 0.05$, **Figure 5C**). This result confirmed that the mutations in the *BnVTE4* gene can significantly affect the conversion of γ-T to α-T.

Comparing the mutations in individual copies of the *BnVTE4* gene (**Figure 3A**) with the change in tocopherol composition (**Figure 5**), we found that the homologous copies of the *BnVTE4* gene had different contributions in α-T biosynthesis. *bnvte4-3* and *bnvte4-5* lines had both homozygous mutations in *VTE4.C02-1* and *VTE4.C02-2* copies, and the editing efficiency in *VTE4.A02-1* and *VTE4.A02-2* copies were both more than 50%. There was no significant difference in α-T and γ-T content, as well as the α-/γ-T ratio between these two lines. The *bnvte4-1* and *bnvte4-5* differed in the mutation of the *VTE4.A02-1* copy. The *bnvte4-1* had no mutation in *VTE4.A02-1*, while *bnvte4-5* had 83% editing efficiency in this gene. The *bnvte4-1* showed significant differences in α- and γ-T contents and the α-/γ-T ratio compared with *bnvte4-5*, implying the contribution of the *VTE4.A02-1* copy to α-T synthesis.

The *bnvte4-1* and *bnvte4-4* were only different at the mutation in the *VTE4.A02-2* copy. The *VTE4.A02-2* copy in *bnvte4-1* was completely mutated, while the editing efficiency of *bnvte4-4* was only 12%, but the α-T and γ-T contents and the α-/γ-T ratio were not significantly different. This suggested that the contribution of the *VTE4.A02-2* copy might be low. Alternatively, the wild-type copy of *VTE4* in *bnvte4-1* and *bnvte4-4* might dominate the contribution, leading to the effect of the *VTE4.A02-2* mutant being insignificant.

VTE4.C02-1 and *VTE4.A02-2* copies in *bnvte4-2* and *bnvte4-4* had similar mutation patterns, while the other two *VTE4* genes had different mutation profiles. The *VTE4.C02-2* copy in *bnvte4-2* was WT, and its *VTE4.A02-1* copy had an editing efficiency of 64%, while *VTE4.C02-2* in *bnvte4-4* was completely mutated, and its *VTE4.A02-1* copy was not mutated. The α-T and γ-T contents and the α-/γ-T ratio between *bnvte4-2* and *bnvte4-4*

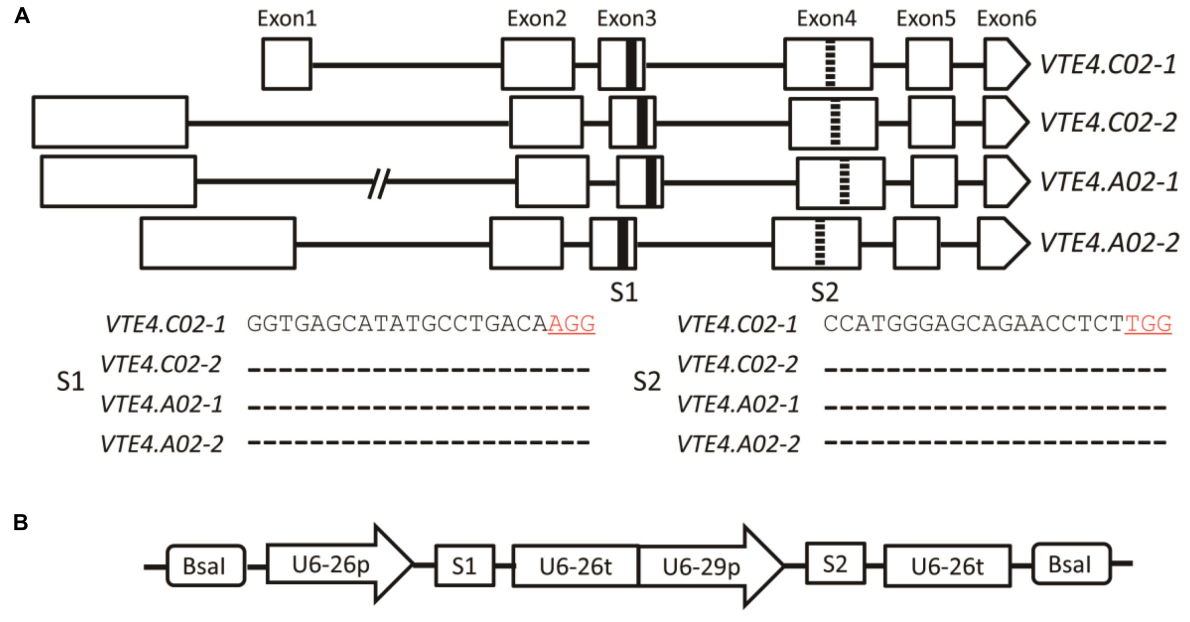


FIGURE 2 | A schematic diagram of *BnVTE4* gene editing vector construction. **(A)** Rectangular boxes indicate exons of *VTE4.C02-1*, *VTE4.C02-2*, *VTE4.A02-1*, and *VTE4.A02-2* copies; black horizontal lines represent introns; thickened black vertical lines show sgRNA1 (S1) targeting Exon3; thickened dashed lines indicate sgRNA2 (S2) targeting Exon4. **(B)** A schematic diagram of the *BnVTE4* gene editing vector. sgRNA1 is initiated and terminated by U6-26p and U6-26t, respectively, sgRNA2 is initiated and terminated by U6-29p and U6-26t, respectively.

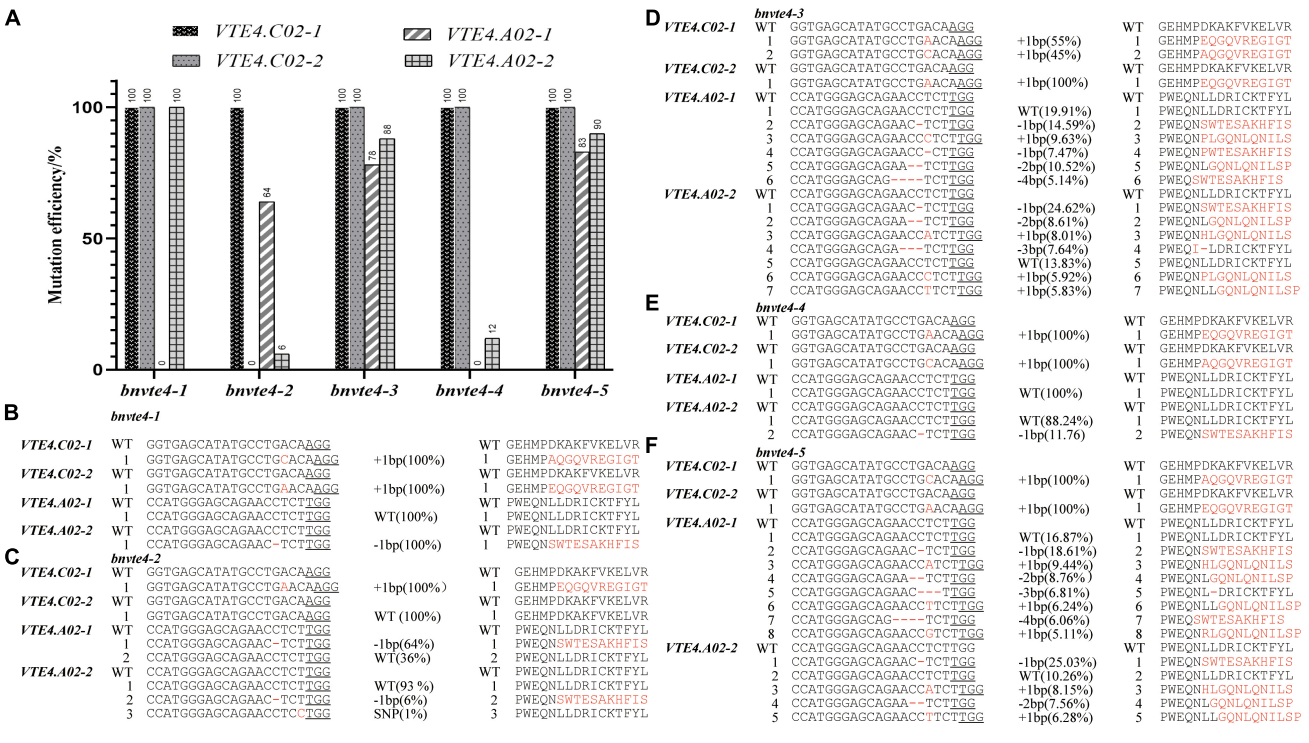


FIGURE 3 | Mutation type, mutation efficiency, and amino acid alterations in homologous copies of the *BnVTE4* gene in the T_1 generation. **(A)** Mutation efficiency of each copy of the *BnVTE4* mutant. **(B)** Mutation types and amino acid alterations in homologous copies in the *BnVTE4* gene of *bnvte4-1*. **(C)** Mutation types and amino acid alterations in homologous copies in the *BnVTE4* gene of *bnvte4-2*. **(D)** Mutation types and amino acid alterations in homologous copies in the *BnVTE4* gene of *bnvte4-3*. **(E)** Mutation types and amino acid alterations in homologous copies in the *BnVTE4* gene of *bnvte4-4*. **(F)** Mutation types and amino acid alterations in homologous copies in the *BnVTE4* gene of *bnvte4-5*.

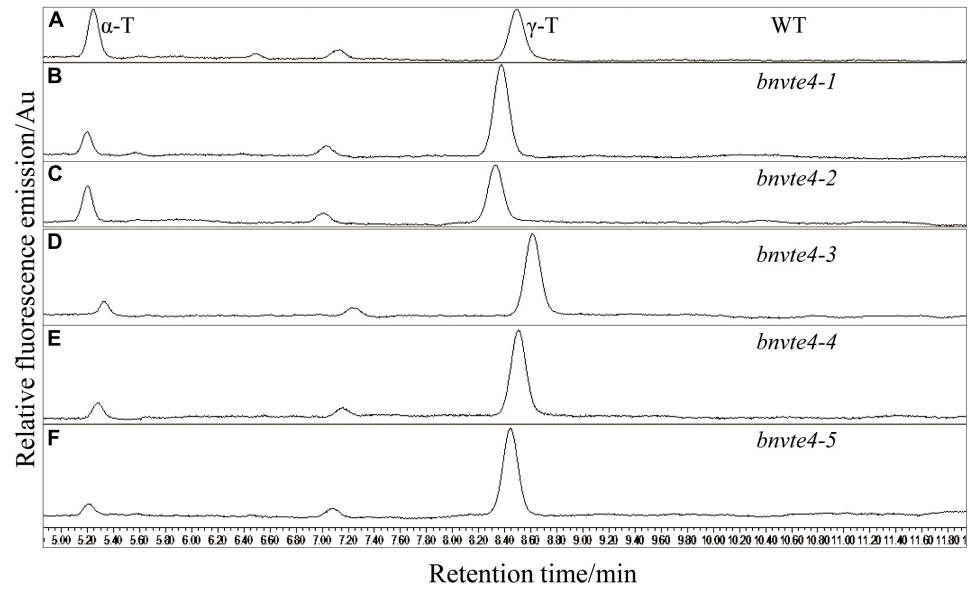


FIGURE 4 | Determination of the components and content of tocopherol by HPLC. The peak at 5.2 min indicated α-T, the peak at 8.5 min indicated γ -T and the peak area indicated the content of tocopherol. **(A)** Two types of tocopherols in the wild type control (WT) were determined by HPLC. **(B)** Two types of tocopherols in *bnvte4-1* were determined. **(C)** Two types of tocopherols in *bnvte4-2* were determined. **(D)** Two types of tocopherols in *bnvte4-3* were determined. **(E)** Two types of tocopherols in *bnvte4-4* were determined. **(F)** Two types of tocopherols in *bnvte4-5* were determined.

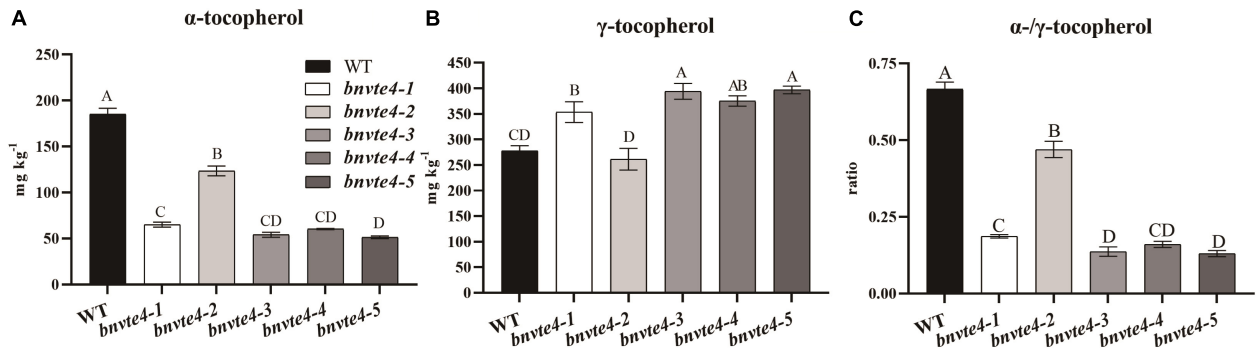


FIGURE 5 | The α-and γ-T content and the ratio in T_1 transgenic mutants. **(A)** The α-T content of the mutants. **(B)** The γ-T content of the mutants. **(C)** The α-/γ-T ratios of mutants.

were significantly different. The α-/γ-T ratio of *bnvte4-2* was higher than that of *bnvte4-4*, suggesting that the contribution of *VTE4.C02-2* might be greater than that of *VTE4.A02-1*.

The α-T and γ-T contents, and the α-/γ-T ratio of *bnvte4-3*, *bnvte4-4*, and *bnvte4-5* were not significantly different. In terms of mutation type, *bnvte4-3* and *bnvte4-5* were similar. *VTE4.C02-1* and *VTE4.C02-2* copies in *bnvte4-4* were fully mutated, same as in *bnvte4-3* and *bnvte4-5*. However, *VTE4.A02-1* and *VTE4.A02-2* copies did not exhibit mutation type, which again demonstrated that *VTE4.A02-1* and *VTE4.A02-2* copies might have a minor contribution.

It is particularly worth mentioning the obviously different tocopherol profile of *bnvte4-2* to other mutant types. *bnvte4-2* had similar mutations to other lines except for the *VTE4.C02-2* copy that was WT other than homozygous mutation in other lines. Considering the lowest reduction of α-T in *bnvte4-2* (from 185 mg/kg in WT to 123 mg/kg), while other lines had reduced α-T to ∼60 mg/kg, the key difference was the WT copy of *VTE4.C02-2* compared to a homozygous mutation in other lines. This again suggested the great contribution of *VTE4.C02-2* in α-T synthesis.

DISCUSSION

Tocopherol, especially α-tocopherol, is not only an important scavenger of stress-induced oxidative damage but an essential nutrient for human health. Genetic improvement of high-quality rapeseed oil with multiple vitamins such as α-tocopherol is an effective way to ensure the daily nutritional requirement of the human body. However, the complex genomic structure of oilseed rape that most of the genes have multiple homologous gene

copies with putative redundant or diverse gene functions is one of the open questions for breeding application.

CRISPR/Cas9 technology has several inherent advantages in the precisely studying gene functions and subsequent application in crop plants (Lee et al., 2018; Hirohata et al., 2019; Zhai et al., 2019), especially polyploidy crops such as oilseed rape. In our previous studies, the highly efficient genome editing platform has been established to dissect the functional diversity of different homologs in oilseed rape (Li et al., 2018; Zaman et al., 2019; Cheng et al., 2021). Many independent case studies suggested that the homologous gene copies, although possess similar genomic information, often exert different effects in a particular trait (Okuzaki et al., 2018; Zhai et al., 2019, 2020; Ahmar et al., 2021; Chen et al., 2021).

To elucidate the contribution of the *BnVTE4* gene in α-T biosynthesis, its four homologous gene copies were studied in detail by using CRISPR/Cas9 genome technology. To generate different mutation combinations of *BnVTE4* homologs, two sgRNAs were designed to target their conserved regions in exon 3 and exon 4 of the coding sequences. Sequencing data demonstrated that the majority of *BnVTE4*-mutated lines showed homozygous mutation types in *VTE4.C02-1* and *VTE4.C02-2* homologous copies (**Figure 3A**), which suggested that our designed sgRNA1 had high mutation capacity on the genomic region of *BnVTE4* homologs. However, it was very difficult to obtain homozygous mutation types in *VTE4.A02-1* and *VTE4.A02-2* homologs, even dozens of positive transgenic T_0 lines and plenty of T_2 plants had been performed by mutation screening. One of our hypotheses is that those four homologous copies probably have functional diversification in α-tocopherol biosynthesis or other key developmental processes such as seed vigor. Similar to this result, simultaneous mutation of five *BnJAG* homologs drastically affected the seed development, and its seeds are hard to survive, whereas the single mutation of *BnJAG.A08-NUB* homologous copy displays a pod-shattering resistance phenotype (Zaman et al., 2019).

To further evaluate the contribution of *BnVTE4* homologs in α-tocopherol biosynthesis, the mutation frequency of *VTE4.A02-1* and *VTE4.A02-2* homologs was quantified by the Hi-TOM high-throughput sequencing method. Hi-TOM data suggested that the mutation frequency of *VTE4.A02-1* and *VTE4.A02-2* homologous copies exhibited a significant difference in *bnvte4-1* and *bnvte4-5* mutation lines. In addition, sequencing data showed that the most common mutation types were –1/+1 bp indels, and the amino acid sequence was completely changed due to a frame shift (**Figures 3B–F**). Thus, the designed sgRNAs can effectively generate targeted mutagenesis in all *BnVTE4* homologous copies. This result was further verified by subsequence analysis of tocopherols content using HPLC, which demonstrated that the α- and γ-tocopherol contents and α-/γ-tocopherol ratios of these mutated lines showed significant alteration compared to WT control.

However, the effect of different mutation combinations on α-T content was a significant difference among five mutated lines. Similar mutation types (*bnvte4-3* and *bnvte4-5*) showed no significant difference in α-T content. There was no significant difference in the content of α-T when the mutation types of the other copies except *VTE4.A02-2* were similar (*bnvte4-1* and *bnvte4-4*), indicating that the *VTE4.A02-2* copy did not play a major role in α-tocopherol biosynthesis. This conclusion was supported by comparing *bnvte4-3* and *bnvte4-5*. The content of α-T was significantly different when the mutation types were similar for other copies except for *VTE4.A02-1* (*bnvte4-1* and *bnvte4-5*), indicating the *VTE4.A02-1* copy was important. *VTE4.C02-2* and *VTE4.A02-2* mutation types of *bnvte4-2* and *bnvte4-3* were different, and the content of α-T was significantly varied. The functionality of the *VTE4.C02-2* copy was revealed in *BnVTE4-2* by comparing it to other lines. The significant difference in the content of α-T when the mutation types of other copies except *VTE4.C02-2* are similar (*bnvte4-2* and *bnvte4-4*) indicates that *VTE4.C02-2* played an important role. Taken together, those data demonstrated that the four *BnVTE4* gene homologs might have functional differentiation in α-T biosynthesis, and their contribution was likely VTE4.C02-2 (BnaC02G0331100ZS) > VTE4.A02-1 (BnaA02G0247300ZS) > VTE4.A02-2 (BnaA02G0154300ZS). This knowledge will shed new light on the cultivation of high α-T-content oilseed rape.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

QH, X-RZ, CL, and DM conceived and revised the manuscript. CL designed the experiments. HZ, YQS, MS, XH, MH, and YS performed the experiments. HZ and CL analyzed the data. HZ drafted the manuscript. All authors reviewed and approved the manuscript.

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Development of a Genome-Edited Tomato With High Ascorbate Content During Later Stage of Fruit Ripening Through Mutation of *SlAPX4*

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Ascorbate is an essential antioxidant substance for humans. Due to the lack of ascorbate biosynthetic enzyme, a human must intake ascorbate from the food source. Tomato is one of the most widely consumed fruits, thus elevation of ascorbate content in tomato fruits will improve their nutritional value. Here we characterized *Solanum lycopersicum ASCORBATE PEROXIDASE 4* (*SlAPX4*) as a gene specifically induced during fruit ripening. In tomatoes, ascorbate accumulates in the yellow stage of fruits, then decreases during later stages of fruit ripening. To investigate whether *SlAPX* is involved in the decrease of ascorbate, the expression of *SlAPX*s was analyzed during fruit maturation. Among nine *SlAPX*s, *SlAPX4* is the only gene whose expression was induced during fruit ripening. Mutation of *SlAPX4* by the CRISPR/Cas9 system increased ascorbate content in ripened tomato fruits, while ascorbate content in leaves was not significantly changed by mutation of *SlAPX4*. Phenotype analysis revealed that mutation of *SlAPX4* did not induce an adverse effect on the growth of tomato plants. Collectively, we suggest that *SlAPX4* mediates a decrease of ascorbate content during the later stage of fruit ripening, and mutation of *SlAPX4* can be used for the development of genome-edited tomatoes with elevated ascorbate content in fruits.

Keywords: tomato, genome editing, *SlAPX*, CRISPR/Cas9, ascorbate

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INTRODUCTION

Ascorbate (L-ascorbic acid, L-threo-hex-2-enono-1,4-lactone) is one of the essential antioxidant molecules in plants and animals (Locato et al., 2013; Fenech et al., 2019). While many animals and plants can synthesize ascorbate, humans have lost the ability to synthesize ascorbate due to the mutations that occurred on the L-gulono-1,4-lactone oxidase, which is the last committed enzyme of the pathway (Drouin et al., 2011). Thus, humans must intake ascorbate through diets such as fresh vegetables and fruits. Tomato has been the most produced fruit in the world during the last 20 years. The total production of tomato fruits has been 182 million tons in 2018 and the annual production of tomatoes has increased during the last years (FAOSTAT., 2019). Even though the tomato is the most widely consumed fruit in the world, the tomato has been considered a moderate source of ascorbate due to its relatively low ascorbate content (Gest et al., 2013; Mellidou et al., 2021). Therefore, increasing ascorbate concentration in tomato fruits will have a great impact on human nutrition.

In plants, ascorbate can be synthesized through four different pathways, namely, the D-mannose/L-galactose pathway, the D-glucose pathway, the myo-inositol pathway, and the D-galacturonate pathway (Wheeler et al., 1998; Ishikawa et al., 2018; Paciolla et al., 2019). Among these four pathways, the D-mannose/L-galactose pathway has been recognized as a major ascorbate biosynthetic pathway in plants (Fenech et al., 2019). D-mannose/L-galactose pathway uses D-fructose-6-phosphate as a precursor for ascorbate production. Specifically, D-fructose-6-phosphate is transformed into D-mannose-1-phosphate by phosphomannose isomerase (PMI) and phosphmannomutase (PMN) (Qian et al., 2007; Maruta et al., 2008). GDP-D-mannose pyrophosphorylase (GMP) transfers guanosine monophosphate into D-mannose-1-phosphate to form GDP-D-mannose (Conklin et al., 1999). Then, GDP-D-mannose-3′,5′-epimerase (GME) converts GDP-D-mannose into GDP-L-galactose (Gilbert et al., 2009). GDP-L-galactose is converted into L-galactono-1,4-lactone by GDP-L-galactose-phosphorylase (GGP), L-galactose-1-phosphate phosphatase (GPP), and L-galactose dehydrogenase (L-GalDH) (Gatzek et al., 2002; Laing et al., 2004; Dowdle et al., 2007). The L-galactono-1,4-lactone is finally converted into ascorbate by L-galactono-1,4-lactone dehydrogenase (GLDH) (Wheeler et al., 2015). On the other hand, there exists an ascorbate degradation pathway in plants (Foyer and Noctor, 2011; Locato et al., 2013). Ascorbate is oxidized into monodehydroascorbate (MDHA) by ascorbate peroxidase (APX) and spontaneously converted into dehydroascorbate (DHA). DHA has then reduced again into ascorbate *via* dehydroascorbate reductase (DHAR) or degraded *via* DHA oxidation (Parsons et al., 2011; Caverzan et al., 2012), indicating that oxidation of ascorbate to DHA, which is committed by APX, is required for its degradation in tomato.

Increasing ascorbate content in plants can be achieved by either activation of its biosynthesis or inactivation of its degradation. Most of the attempts have been focused on ectopic expression of ascorbate biosynthetic genes in plants (Agius et al., 2003; Bulley et al., 2012; Macknight et al., 2017; Li et al., 2019). For example, heterogeneous expression of *GGP* significantly increases ascorbate content in potatoes, tomatoes, and strawberries (Bulley et al., 2012). Similarly, overexpression of other ascorbate biosynthetic genes such as *GMP*, *GME*, and *GPP* leads to a higher accumulation of ascorbate in rice plants (Zhang et al., 2015). Recently, it is also reported that pyramiding of transgenic tomato plants simultaneously overexpressing four ascorbate biosynthetic genes (*GME*, *GMP*, *GGP*, and *GPP*) increased ascorbate content up to 18% in tomato fruits (Li et al., 2019). In addition, activation of ascorbate recycling by overexpression of wheat *DHAR* increased ascorbate content in tobacco and maize plants (Chen et al., 2003). Even though activation of ascorbate biosynthesis or recycling greatly increases ascorbate content in plants, it often caused developmental defects due to metabolic perturbations (Bulley et al., 2012; Fenech et al., 2019). To minimize unintended effects by ectopic expression of ascorbate metabolic genes on plants development, suppression of ascorbate degradation genes, such as *APX*s, has been attempted in several plant species (Zhang et al., 2011; Chatzopoulou et al., 2020).

Ascorbate peroxidase (APX) plays an important role in protecting plants from oxidative stress conditions produced during normal developmental processes and stress responses through the enzymatic conversion of H_2O_2 into H_2O using ascorbate as an electron donor (Koussevitzky et al., 2008; Guo et al., 2020). Due to its importance for plant development and adaptation to the environment, the downregulation of *APX* also occasionally caused a negative effect on plants growth (Pnueli et al., 2003; Locato et al., 2013). For example, knockout mutation of Arabidopsis *AtAPX1* showed retarded growth and reduced stress tolerance with elevated hydrogen peroxide level (Pnueli et al., 2003). Plants possess multiple APX isozymes. They have localized either mitochondria, chloroplasts, peroxisomes, or cytosol (Shigeoka et al., 2002; Najami et al., 2008; Yin et al., 2019; Tyagi et al., 2020; Li et al., 2021), and *SlAPX* isozymes showed different kinetic properties such as optimal pH, Km, and Vmax (Marquez et al., 1996; Liu et al., 2019; Wu and Wang, 2019). In addition, their expression levels were differentially regulated in plants. These results imply that each *APX* might play distinguished functions during plant growth and stress responses. To improve ascorbate content in tomato plants with minimal alteration on plant growth, identification of the *SlAPX* specifically acting during fruit maturation is crucial. In tomatoes, there exist nine putative *SlAPX*s containing three cytosolic, two peroxisomal, two chloroplastic, and two uncharacterized *APX*s (Najami et al., 2008). Here, we aimed to develop tomato plants with elevated ascorbate content in fruits through mutation of the fruit-specific *SlAPX* by CRISPR/Cas9-mediated mutagenesis. Among nine *SlAPX*s, *SlAPX4* is the only gene showing fruit-specific expression patterns. Thus, we selected *SlAPX4* as a target for CRISPR/Cas9-mediated mutagenesis and analyzed the effect of *SlAPX4* mutation on ascorbate content, expression of ascorbate metabolic genes, and development of tomato plants.

MATERIALS AND METHODS

Plant Growth Conditions

Tomato seeds (*Solanum lycopersicum* cv. Micro-Tom) used in this study were kindly provided by prof. Sanghyeob Lee in the Sejong University in Korea. Tomato seeds were sown on a Murashige–Skoog (MS) solid medium [4.4 g/l of MS salt (Duchefa, Netherlands), 30 g/l of sucrose (Sigma-Aldrich, USA), and 0.5 g/l of 2-(N-morpholino) ethanesulfonic acid (MES) (Duchefa, Netherlands), pH 5.7] and incubated in a growth room with the light-dark cycle of 16 h light/8 h dark at 22 to 24°C for gene expression analysis and agrobacterium-mediated transformation. For growing tomato plants until fruit ripening stage, tomato seeds (*S. lycopersicum* cv. Micro-Tom) were sown on a soil pot supplemented with a complex fertilizer Green coat (DHC, Japan) and incubated in a growth room with the light-dark cycle of 16 h light/8 h dark at 22 to 24°C with 65% of relative humidity until maturity. Plants were fully watered at 2-day intervals. Each pot contained one tomato plant and the pots were randomly distributed in the growth room. An 8-week-old tomato plants were used for gene expression analysis and ascorbate quantification in leaves. A 10-week-old tomato plants were used for phenotypic analysis. For analyzing the expression

level of ascorbate metabolic genes and ascorbate content in fruits, three fruits per plant from three independent plants were collected at the stage of mature green, breaking, yellowing, and red ripening.

Plasmid Construction

To target the *SlAPX4* coding region for CRISPR/Cas9-mediated mutagenesis, four independent single-guide RNAs (sgRNAs) were cloned into the pAGM4723 vector using the Golden gate system as previously described (Engler et al., 2009). The *SlAPX4*-specific sgRNAs were amplified with a forward primer (sgRNA1; 5′-TGTGGTCTCAATTTTTGGAAATCGACGTT TGATGTTTTAGAGCTAGAAATAGCAAG-3′, sgRNA2; 5′-TGTGGTCTCAATTAAGCAGTTGAAAAATGTAAGGTTTTA GAGCTAGAAATAGCAAG-3′, sgRNA3; 5′-TGTGGTCTCAA TTGTTAAGAATTTTTTACATGAGTTTTAGAGCTAGAAA TAGCAAG-3′, and sgRNA4; 5′-TGTGGTCTCAATTGAT GTCAAAACCAAAACTGGGTTTTAGAGCTAGAAATAGC AAG-3′) and a reverse primer (CRISPR Universal, 5′-TGTGGTCTCAAGCGTAATGCCAACTTTGTAC-3′) using the pICH86966 plasmid as a template. The PCR products and *U6* promoter of pICSL01009 plasmids were cloned into the pICH47751 plasmid through BasI (NEB, USA) restriction site. The pICH47751 harboring sgRNA under the control of the *U6* promoter, pICH47732_NPTII, and pICH47742_35S:Cas9 plasmids were digested with BpiI (Thermo Scientific, USA), and integrated into pAGM4723 vector using T4 Ligase (NEB, USA). The resultant pAGM4723 harboring *SlAPX4*-specific sgRNA was used for transient expression analysis and agrobacterium-mediated transformation.

Transient Expression in Tomato Plants

The four plasmids harboring different sgRNAs were transformed into *Agrobacterium tumefaciens* strain GV3101. The transformed GV3101 were incubated in yeast extract peptone (YEP) liquid media [10 g/l of yeast extract (Sigma-Aldrich, USA), 10 g/l of peptone (Sigma-Aldrich, USA), and 5 g/l of NaCl (Sigma-Aldrich, USA), pH 7.2] supplemented with 50 mg/l rifampicin (Duchefa, Netherlands) and 50 mg/l kanamycin (Biosesang, Korea) for overnight at 28°C. In total, 1 ml of overnight culture was transferred into 20 ml fresh YEP media supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin and incubated for 16 h at 28°C. The bacterial solution was centrifuged at 3,500 rpm for 20 min, and the resultant bacterial pellet was resuspended in 10 mM $MgCl_2$ (Sigma-Aldrich, USA) solution supplemented with 200 μM acetosyringone (Sigma-Aldrich, USA) (final OD_{600} = 0.8–0.9) and incubated at room temperature for 3 h. The bacterial solution was infiltrated into tomato cotyledons using a needless syringe. The ∼50 infiltrated cotyledons were harvested 5 days after infiltration for genomic DNA extraction and further PCR analysis. The efficiency of four sgRNAs was evaluated by sequencing analysis of TA-cloned PCR products.

Agrobacterium-Mediated Tomato Transformation

The final construct harboring *SlAPX4*-specific sgRNA and SpCas9 was transformed into *A. tumefaciens* strain LBA4404 for agrobacterium-mediated transformation. The agrobacterium harboring the plasmids were incubated in YEP liquid media containing 50 mg/l rifampicin and 50 mg/l kanamycin overnight at 28°C. A total of 2 ml of the overnight culture solution was transferred into 3 ml of YEP media supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin and incubated at 28°C until final OD_{600} was between 0.7 and 0.8. The solution was centrifuged at 3,100 rpm for 20 min and the resultant pellet was resuspended in half strength of MS liquid media (2.2 g/l of MS salt, 20 g/l of sucrose, and 0.5 g/l of MES, pH 5.7) containing 200 μM acetosyringone for 4 h. The Agrobacterium solution was then cocultivated with tomato cotyledons for 10 min. After cocultivation, tomato cotyledons were placed upside down on coculture medium (4.4 g/l of MS salt, 30 g/l of sucrose, 8 g/l of plant agar, 0.1 mg/l of indole-3-acetic acid (IAA), 2 mg/l of zeatin, and 200 μM acetosyringone, pH 5.2) and incubated in the dark for 2 days. Then the tomato cotyledons were transferred into shoot induction medium (4.4 g/l of MS salt, 30 g/l of sucrose, 8 g/l of plant agar, 0.1 mg/l of IAA, 2 mg/l of zeatin, 50 mg/l of kanamycin, and 250 mg/l of cefotaxime, pH 6.0) and incubated until the shoot has appeared. After complete formation of the shoot, the callus was incubated on root induction medium (4.4 g/l of MS salt, 15 g/l of sucrose, 8 g/l of plant agar, 2 mg/l of indolebutyric acid, 50 mg/l of kanamycin, and 250 mg/l of cefotaxime, pH 6.0). The regenerated seedlings with shoots and roots were then transferred into the soil for further growth.

Analysis of Mutation Pattern in Transgenic Plants

To analyze the mutation patterns, the *SlAPX4* genomic region targeted by a sgRNA was amplified by PCR using the gene-specific primers (**Supplementary Table S1**) with genomic DNAs extracted from leaves of non-transgenic and transgenic plants. Plant genomic DNA preparation was carried out as follows. A total of 500 mg of tomato leaves were homogenized by a mortar and a pestle with liquid N_2. The ground sample was resuspended with plant genomic DNA extraction buffer [50 mM Tris-Cl (pH 7.6), 17 mM sodium dodecyl sulfate, 100 mM NaCl, 50 mM ethylenediaminetetraacetic acid (pH 7.6), 1% β-mercaptoethanol, and 42 mg/l RNase A] and incubated at 55°C for 10 min. A total of 500 μl of phenol: chloroform: isoamyl alcohol (25:24:1) was added into the extract and centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was transferred into a new microcentrifuge tube and one volume of isopropanol was added to the solution. The mixture was centrifuged at 13,000 rpm at 4°C for 10 min. The resultant pellet obtained after centrifugation was dissolved in 50 μl of sterilized distilled water and used as a template for a PCR reaction. PCR conditions were as follows: 95°C for 10 min and 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min. PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and used for further direct sequencing, targeted deep sequencing, and cloning into the pGEM-T Easy vector (Promega, USA).

Ribonucleic Acid Isolation and Quantitative Real-Time PCR Analysis

Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. A total of 1 μg of total RNA was treated with an RNase-free DNase I (Qiagen, Germany), and used for first-strand synthesis reactions using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instruction. The products were used for quantification of target transcripts through quantitative real-time PCR (qRT-PCR) analysis with gene-specific primers. The tomato *SlACTIN* (*SlACT*, Solyc03g078400) was used as an internal control for normalization. Gene expression and ascorbate content were analyzed from three biological replicates. The primer information used for qRT-PCR gene expression is given in **Supplementary Table S1**.

Ascorbate Quantification

Endogenous ascorbate content was quantified using the Ascorbic Acid Assay Kit (Abcam, UK) in fruits and high-performance liquid chromatography (HPLC) analysis in leaves. For ascorbate quantification in fruits, 40 mg of samples were rinsed with ice-cold phosphate-buffered saline (PBS) pH 7.4. The rinsed samples were homogenized with 400 μl of ddH_2O using TissueLyzer II (Qiagen, Germany). The crude extract was centrifuged at 13,000 rpm at 4°C for 5 min, and the supernatant was used for ascorbate quantification assay. Further analysis and quantification of ascorbate were performed according to manufacturer's instruction. Endogenous ascorbate content in leaves was determined by an HPLC analysis. A total of 0.01 g of plant powder ground with liquid nitrogen was homogenized in 3 ml of 5% meto-phosphate acid (MPA) (Sigma-Aldrich, USA). The homogenate was centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was filtered using a syringe filter (0.45 um, Biofact, Korea). The flow-through was incubated with 2 mg of dithiothreitol (DTT) in the dark for 25 min. Separation was achieved by isocratic elution with 0.1% trifluoroacetic acid (TFA) (pH 1.88). A total of 10 μl of the reaction mixture was injected into a Gemini C18 column (150 mm × 4.6 mm, particle size 5 μm; Phenomenex, USA) and flowed at a rate of 1 ml/min for 10 min. The column temperature was maintained at 30°C and the autosampler temperature was maintained at 8°C. Ascorbate was detected with photodiode array (PDA) at 244 nm. Quantification of ascorbate was performed using LabSolutions CS software (Shimadzu, Japan). The amount of ascorbate was determined from three biological replicates using the standard curve and normalized to the mass of fresh plant tissue.

Phenotype and Agronomic Trait Analysis

Ten mutant plants per line were used for phenotypic analysis of *slapx4* mutants. The height was measured from the bottom of the pot to the top of the plant. The number of buds, fruits, and seeds per was counted from each individual plant ($n = 10$).

Statistics

The statistical significance of gene expression and metabolite content between Micro-Tom wild-type (WT) plants and *slapx4* mutant plants with one-way ANOVA test using GraphPad Prism version 8.0.1 (https://www.graphpad.com/scientific-software/prism/). Three biological replicates were used for gene expression and ascorbate quantification. Ten independent tomato plants were used for phenotypic analysis (plant height, flower number, fruit number, and seed number).

RESULTS

Identification of the Fruit-Specific *SlAPX* in Tomato

To determine temporal regulation of ascorbate accumulation in tomato plants, we monitored changes in ascorbate content during fruit maturation. Ascorbate content was lower in the mature green (MG) and breaking (BK) stage than yellowing (YE) and red-ripening (RR) stages of tomato fruits (**Figure 1A**). The ascorbate content peaked in the YE stage then decreased in the RR stage. These results indicated that ascorbate is accumulated during the early stage of fruit maturation then decreased during the later stage of fruit maturation. To explain the observed changes through transcriptional regulation of the ascorbate metabolic pathway, we first analyzed the expression pattern of genes that are involved in ascorbate biosynthesis in tomatoes (**Supplementary Figure S1**). Gene expression analysis revealed that ascorbate biosynthetic genes (*SlGMP*, *SlGLDH*, and *SlGME2*), except *SlGME1*, showed the highest expression level in leaves. During fruit maturation, *SlGMP* and *SlGLDH* were highly expressed in mature green and breaking stages of fruit development, then the expression level was decreased in yellowing and red ripening stages of fruit development (**Supplementary Figure S1**). Expression of *SlGME1* was lower in the red ripening stage than other stages of fruit. The expression level of ascorbate recycling genes (*SlDHAR1*, *SlDHAR2*, and *SlMDHAR*) was higher in the early stage of fruit maturation than the later stage of fruit maturation (**Supplementary Figure S1**). We then checked the expression pattern of *SlAPX* genes during fruit maturation (**Figure 1B** and **Supplementary Figure S2**). Among the nine *SlAPX* genes, four *SlAPX*s (*SlAPX1*, *2*, *3*, and *4*) were mainly expressed in tomato plants (Sato et al., 2012) (**Supplementary Figure S2**). *SlAPX1*, *2*, and *3* were expressed in several tomato tissues, namely, leaves, roots, flowers, and fruits, while *SlAPX4* was predominantly expressed in fruits (**Figure 1B** and **Supplementary Figure S2**). Expression of *SlAPX1*, *2*, and *3* was generally higher during the early stage of fruit ripening (**Figure 1B**) while *SlAPX4* expression was gradually upregulated during fruit ripening (**Figure 1B**). Based on these data, we chose *SlAPX4* as a target of mutagenesis for fruit-specific accumulation of ascorbate in tomatoes.

Generation of CRISPR/Cas9-Mediated *slapx4* Mutants

Since *SlAPX4* is the only gene, whose expression is upregulated during the later stage of fruit ripening, mutation of *SlAPX4* might slow down the decrease of ascorbate content during the later stage of fruit ripening. To address the possibility, we generated *slapx4* mutant plants by applying clustered regularly

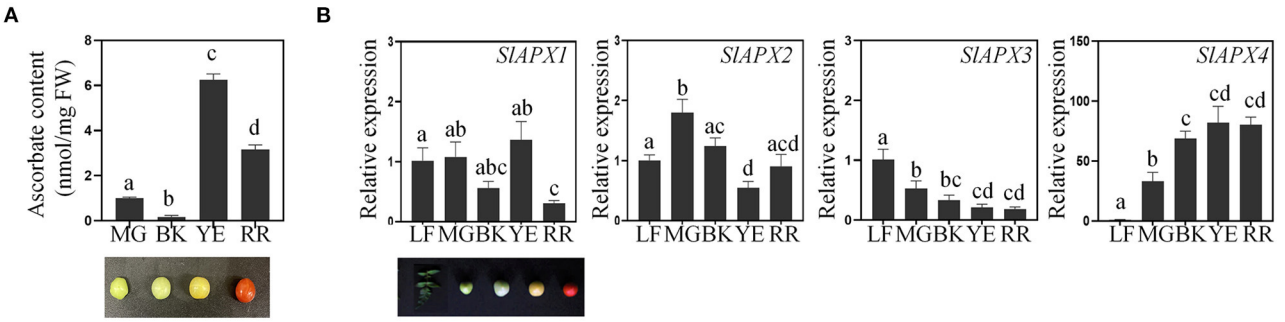


FIGURE 1 | Regulation of ascorbate accumulation during fruit development. **(A)** Change of ascorbate content during tomato fruit ripening. Ascorbate content (nmol per gram fresh weight) was determined from fruits at the indicated developmental stages. **(B)** The relative expression level of *SlAPX*s during fruit ripening. *SlACT* (Soly03g078400) was used as an internal control for normalization. Three tomato fruits from three individual plants at the indicated developmental stages were pooled for ascorbate quantification and gene expression analysis. Data represent the mean value + SD of three biological replicates. A significant difference was indicated with different letters above the bars (one-way ANOVA test). FW, fresh weight; MG, mature green; BK, breaking; YE, yellowing; RR, red ripening; LF, leaves.

TABLE 1 | Mutation efficiency of sgRNAs targeting *SlAPX4*.

sgRNA	Sequence	Total sequenced colonies (number)	Colonies with mutation on 3 bp upstream from PAM site (number)	Mutation efficiency (%)*
sgRNA1	**TTTGGAAATCGACGTTTGAT**CGG	442	0	0
sgRNA2	**AAGCAGTTGAAAAATGTAAG**AGG	273	0	0
sgRNA3	**GTTAAGAATTTTTTACATGA**TGG	548	2	0.36
sgRNA4	**GATGTCAAAACCAAAACTGG**TGG	561	5	0.89

*Mutation efficiency of sgRNAs determined by sequencing plasmids containing PCR fragments of genomic regions targeted by single-guide RNA (sgRNA). *Mutation efficiency= number of colonies with mutation on 3 bp upstream from PAM site/number of total sequenced colonies. Bold, sgRNA target site; underlined, PAM site.*

interspaced short palindromic repeats (CRIPSR)/CRISPR-associated protein 9 (Cas9) system in tomatoes. To screen a sgRNA that effectively induces mutation on the *SlAPX4* gene, we generated four plant expression vectors that encode independent single guide RNAs (sgRNAs) targeting the exon region of *SlAPX4*. The expression vectors were infiltrated into tomato cotyledons to determine the efficiency of each sgRNAs. The mutation efficiency of each sgRNA was determined by sequencing the genomic region targeted by a sgRNA. Among the tested four independent sgRNAs, the sgRNA4 that targets the first exon of *SlAPX4* showed the highest mutation efficiency (**Table 1**). Thus, the plant expression vector harboring SpCas9 and sgRNA4 was introduced into tomato plants to generate *slapx4* mutant plants. A total of 16 T_0 plants (CRISPR/Cas9^{SlAPX4}) were selected based on antibiotic resistance and PCR verification of *NPTII* and *Cas9* genes (**Figure 2A**). Among the 16 T_0 plants, four plants contained mutation on the *SlAPX4* genomic region targeted by sgRNA4. Specifically, one heteroallelic mutant (CRISPR/Cas9^{SlAPX4} #1, T insertion/C deletion), one monoallelic mutant (CRISPR/Cas9^{SlAPX4} #11, T insertion), and two mosaic mutants (CRISPR/Cas9^{SlAPX4} #7 and #12) were identified by sequencing analysis (**Figure 2B** and **Supplementary Figure S3**). Amino acid analysis revealed that either T insertion or C deletion caused a loss of multiple catalytic residues and cation-ligand residues with early termination (**Table 2**). Thus, CRISPR/Cas9^{SlAPX4} #1 and CRISPR/Cas9^{SlAPX4} #11 T_0 plants were further propagated into the T_1 stage, and three biallelic homozygous *SLAPX4* mutant plants with T base insertion (*SLAPX4* #1-T_ins and #11-T_ins) or C base deletion (*SLAPX4* #1-C_del) were selected for further analysis (**Figure 2C**).

Accumulation of Ascorbate in *slapx4* Mutant Plants

To investigate the effects of *SlAPX4* mutation on ascorbate accumulation in tomato plants, we analyzed ascorbate content in leaves and fruits of *slapx4* mutant plants (**Figure 3**). No significant difference was detected on ascorbate content in leaves between WT and *slapx4* mutant plants (**Figure 3A**). On the other hand, compared with WT control plants, all the selected *slapx4* mutant plants possessing either insertion or deletion mutation on *SlAPX4* accumulated significantly higher levels of ascorbate in fruits (**Figure 3B**). To investigate the possibility that the change of ascorbate content in *slapx4* mutant plants alters ascorbate metabolic gene expression, we analyzed the expression pattern of four ascorbate biosynthetic genes (*SlGMP*, *SlGLDH*, *SlGME*, and *SlGME2*) and three

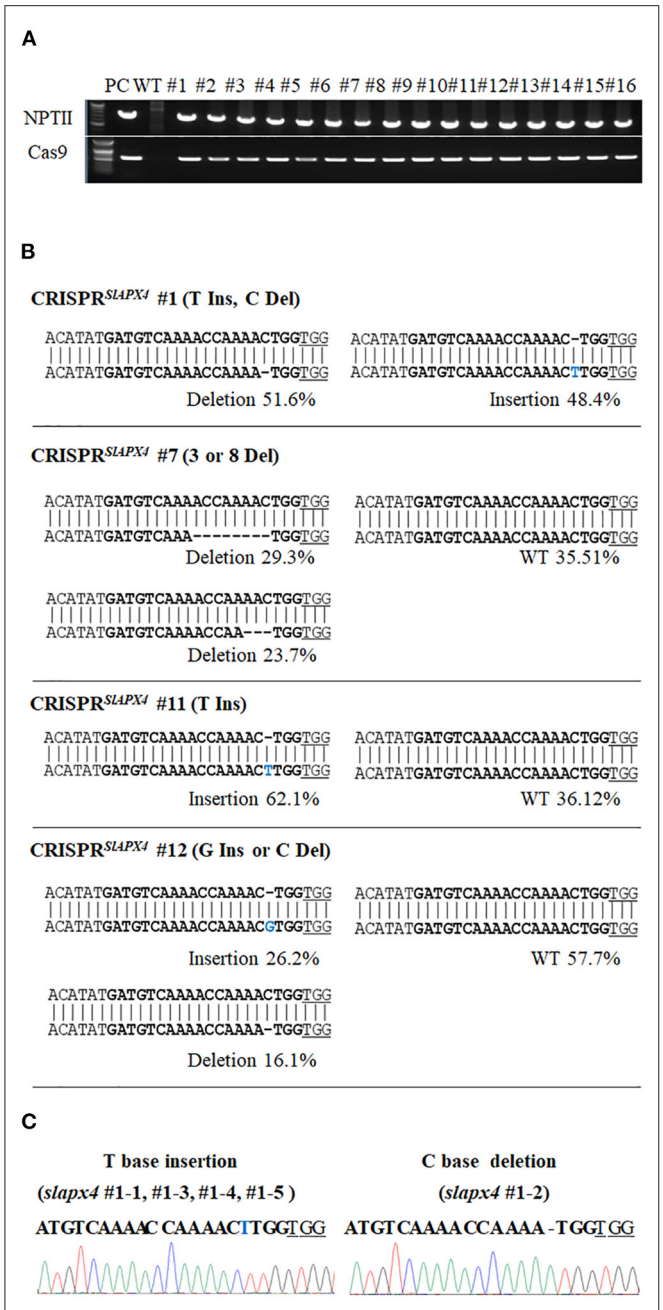


FIGURE 2 | Generation of *SLAPX4* mutant plants. **(A)** Validation of transgenic plants using *NPTII* and *Cas9*-specific PCR reactions. **(B)** Sequencing analysis of *SlAPX4* locus targeted by a single-guide RNA (sgRNA). Genomic DNA extracted from the T_0 stage of CRISPR^{SlAPX4} transgenic tomato plants was used for amplification of the *SlAPX4* genomic region targeted by a sgRNA through PCR reactions. The PCR products were then applied for targeted deep sequencing analysis. **(C)** A representative chromatograph of edited alleles in the T_1 stage of *SLAPX4* mutant plants was shown with the mutated target region. Bold, sgRNA target site; blue, base insertion; underlined, PAM site.

TABLE 2 | Amino acid sequence analysis of the target site in *slapx4* mutant plants.

Line	Genotype	Amino acid sequence
WT	WT	MVKCYPTVSEEYQKAVEKCKRKL RGLIAEKNCAPIMLRLAWHSAGTYDVKTK TGGPFGTIRHPNELKHGANNGLDI AVRLLEPIKEQFPILSYADFYQLAGVVAV EVTGGPDIPFHPGRQDKTEPPPEGRLPDA TKGSDHLREVFGHMGLSDKDIVALS GGHTLGRCHKERSGFEGAWTNNPLIFDN SYFKELLSGEKEGLLQLPSDKALLEDPVF RPLVEKYAADEDAFFADYAEAHLKLS ELGFADAE
slapx4 #1 T ins	T insertion	MVKCYPTVSEEYQKAVEKCKRKLRGLIAE KNCAPIMLRLAWHSA GTYDVKTKT**WWSIWNNQAPE***
slapx4 #1 C del	C deletion	MVKCYPTVSEEYQKAVEKCKRKLRGL IAEKNCAPIMLRLAWHSAGT YDVKTK**MVVHLEQSGTRMNLNMELTM VLILLFDFWSRSRNSSQ SYPTLTFISWLE***
slapx4 #11 T ins	T insertion	MVKCYPTVSEEYQKAVEKCKRKLRG LIAEKNCAPIMLRLAWHSAG TYDVKTKT**WWSIWNNQAPE***

WT, wild-type; asterisk, stop codon; Bold with underlined, mutated regions; Red, catalytic residues (Najami et al., 2008); Blue, cation-ligand residues (Najami et al., 2008); WT, Micro-Tom wild-type plants.

ascorbate recycling genes (*SlDHAR1*, *SlDHAR2*, and *SlMDHAR*) in leaves and fruits of *slapx4* mutant plants. The expression level of ascorbate biosynthesis and recycling genes in both leaves and fruits was similar between WT and *slapx4* mutant plants (**Figures 4A,B**). We then checked the expression pattern of *SlAPX*s in WT and *slapx4* mutant plants. Similar to ascorbate biosynthetic genes, the expression of *SlAPX1*, *2*, and *3* was similar between WT and *slapx4* mutant plants (**Figure 4C** and **Supplementary Figure S4**). On the other hand, compared with WT plants, *SlAPX4* expression was significantly reduced in *slapx4* mutant plants (**Figure 4C** and **Supplementary Figure S4**).

Phenotype Analysis of *slapx4* Mutant Plants

To investigate the potential effects of *SlAPX4* mutation on plant growth, we monitored the developmental phenotypes of *slapx4* mutants whose red ripening fruit showed higher ascorbate content (**Figure 5**). Overall, *slapx4* mutant plants did not show growth defects compared with WT control plants (**Figure 5A**). Total plant height determined at the breaking stage was similar between WT and two of three *slapx4* mutant plants (*slapx4* #1 C_del and #11 T_Ins) (**Figure 5B**). The number of flower buds was not significantly different between WT and *slapx4* mutant plants (**Figure 5C**). In addition, the fruit of WT and *slapx4* mutant plants showed similar morphology (**Supplementary Figure S5**). On the other hand, two of three *slapx4* mutant plants (*slapx4* #1 C_del and #11 T_Ins) produced more fruits (**Figure 5D**), and *slapx4* #1 C-del plants had a higher number of seeds than WT control plants (**Figure 5E**).

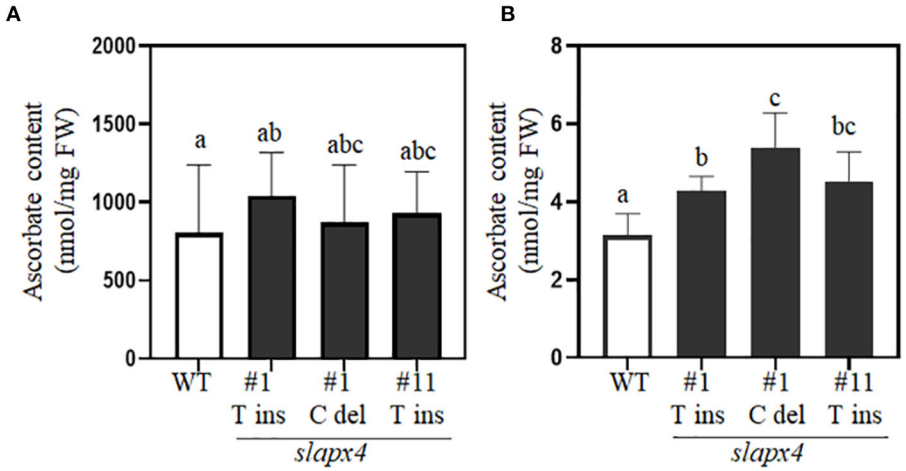


FIGURE 3 | Determination of ascorbate content in *SLAPX4* mutant plants. **(A,B)** Wild-type (WT) and *SLAPX4* mutant plants containing biallelic mutation (T base insertion; *SLAPX4* #1 T ins and #11 T ins, C base deletion; *SLAPX4* #1 C del) were grown in the soil. The plants were used for quantification of ascorbate content (nmol per fresh weight) in leaves **(A)** and red-ripening fruits **(B)**. Leaves and fruits from three independent plants were pooled for ascorbate quantification. Data represent the mean value + SD of three biological replicates. A significant difference was shown with different letters above the bars (one-way ANOVA test). FW, fresh weight.

DISCUSSION

Tomatoes have relatively low ascorbate content compared with other fruits, such as strawberry and grape (Beecher, 1998). Based on its importance as a human diet and its high raw intake, an increase of ascorbate content in tomato fruits will have far-reaching effects from a nutritional point of view. Here we have suggested the strategy to develop tomatoes with elevated ascorbate content through targeted mutagenesis of fruit-specific *SlAPX4* using the CRISPR/Cas9 system.

Ascorbate content analysis in tomato plants revealed that ascorbate levels increase during the early stages of fruit ripening and decrease during the later stage of fruit ripening (**Figure 1A**). Similar accumulation patterns were observed in various tomato cultivars originated from different continents (Abushita et al., 1997; Mellidou et al., 2012; Sacco et al., 2019), suggesting that developmental regulation of ascorbate content is well-conserved in many tomato cultivars. Ascorbate pool in fruits is known to be closely correlated with the oxidative status of tomato fruits (Imai et al., 2009; Li et al., 2010; Lima-Silva et al., 2012). The increase of ascorbate content would be beneficial to maintain redox balance during fruit development and ripening through the effective removal of reactive oxygen species (Jimenez et al., 2002; Gapper et al., 2013). However, little is known how ascorbate content increases during tomato fruit ripening. Gene expression analysis showed that expression of ascorbate biosynthetic genes was generally downregulated during fruit maturation (**Supplementary Figure S1**). These results suggest that accumulation of ascorbate during the early stage of fruit maturation could be accomplished by other mechanisms rather than transcriptional regulation of biosynthetic pathway (Imai et al., 2009; Li et al., 2010). One possible explanation for the phenomenon is the source to sink allocation of ascorbate in plants. It is predicted that translocation of ascorbate from leaves to developing fruits, rather than *de-novo* synthesis, provides an ascorbate pool in fruits at the early stage of fruit ripening (Badejo et al., 2012). It has also been suggested that enhanced rates of ascorbate recycling increased ascorbate content during fruit ripening (Mellidou et al., 2012). Another potential mechanism related to ascorbate accumulation during fruit ripening is a transcriptional regulation of *SlAPX* genes. We found that four *SlAPX*s (*SlAPX1, 2, 3*, and *4*) among nine *SlAPX*s were mainly expressed in tomato plants (**Figure 1B** and **Supplementary Figure S2**). Expression of *SlAPX1*, *2*, and *3* was gradually reduced during fruit ripening, while *SlAPX4* expression was induced during fruit ripening in tomato plants (**Figure 1B**). These temporal expressions of *SlAPXs* might be contributed to shaping ascorbate accumulation during fruit ripening.

Mutation of ascorbate peroxidase is a promising strategy for increasing ascorbate content in plants. It has been reported that the tomato cultivar with elaborated ascorbate content showed reduced expression of ascorbate peroxidase (Di Matteo et al., 2010). However, due to its importance for detoxification of hydrogen peroxide with ascorbate (Caverzan et al., 2012), precise functional characterization of ascorbate peroxidase is necessary to minimize potential negative effects caused by the mutation of *APX* on plant development. Expression characteristics of *SlAPX4* suggested it as an attractive target for developing genome-edited tomatoes with elevated ascorbate content in fruits. Unlike other *SlAPX* genes, *SlAPX4* was predominantly expressed in fruits than other tissues such as leaves, roots, and flowers (**Figure 1B** and **Supplementary Figure S2**). In addition, *SlAPX4* is the only gene whose expression was strongly expressed during the later stage of fruit ripening. On the other hand, other *SlAPX*s (*SlAPX1*, *SlAPX2*, and *SlAPX3*) were predominantly expressed during the early stage of fruit development (**Figure 1B** and **Supplementary Figure S2**). These results suggest that *SlAPX4* could be related to the decrease of ascorbate content during

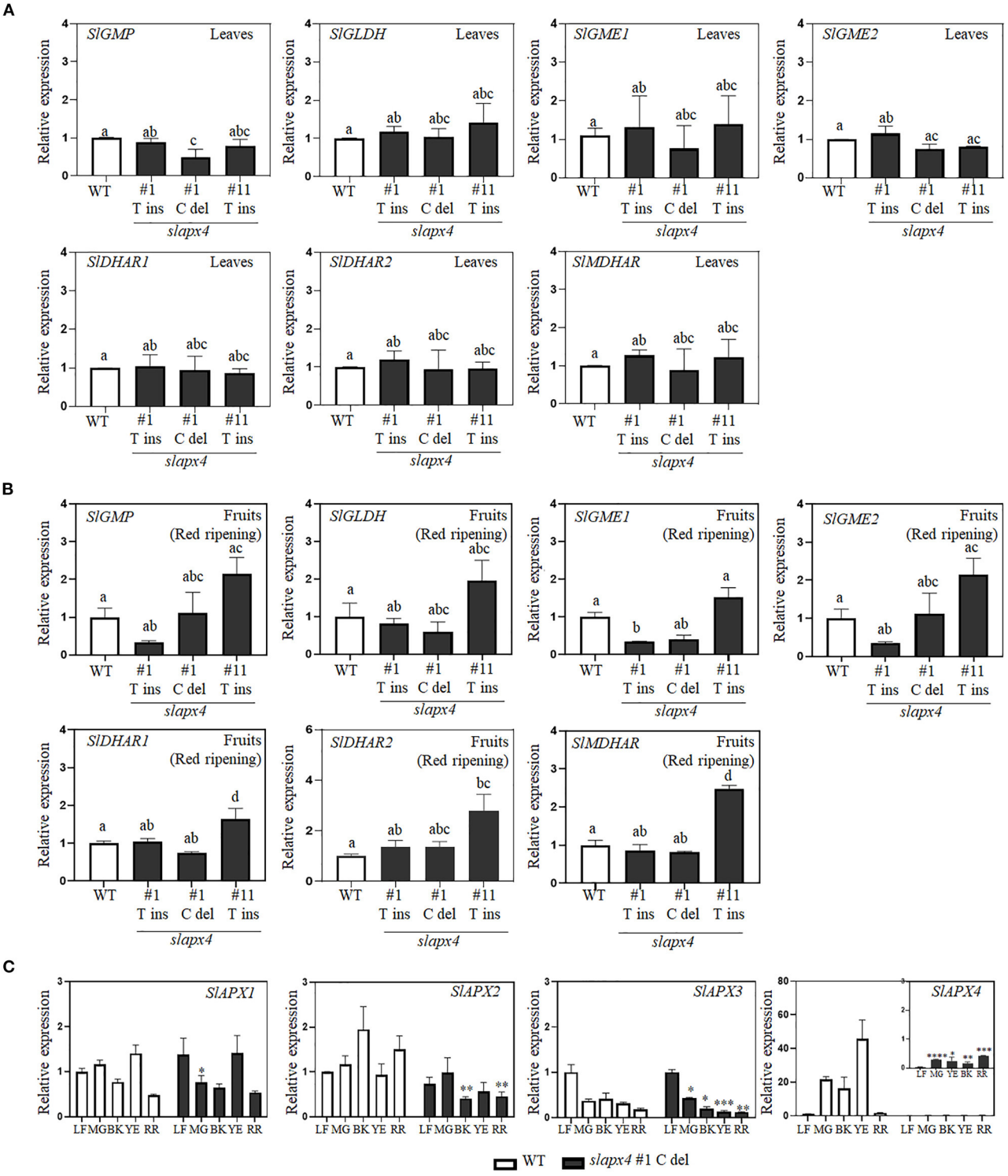


FIGURE 4 | Expression pattern of ascorbate metabolic genes in *SLAPX4* mutant plants. **(A,B)** Relative expression levels of ascorbate metabolic genes in leaves **(A)** and fruits **(B)** of wild-type (WT) and *SLAPX4* mutant plants (*SLAPX4* #1 T ins, #1 C del, and #11 T ins). **(C)** The relative expression of *SlAPX*s in WT and *SLAPX4* mutant plants during fruit maturation. Leaves or fruits from three individual tomato plants at the indicated stages of development were pooled for gene expression analysis. *SlACT* (Soly03g078400) was used as an internal control for normalization. Data represent the mean value + SD of three biological replicates. A significant difference was shown with different letters above the bars for **(A)** (one-way ANOVA test). Significant difference between WT and *SLAPX4* mutant plants was shown by asterisks for **(B)** (Student's *t*-test, $*p < 0.05$, $**p < 0.001$, and $***p < 0.0001$). LF, leaves; MG, mature green; BK, breaking; YE, yellowing; RR, red ripening.

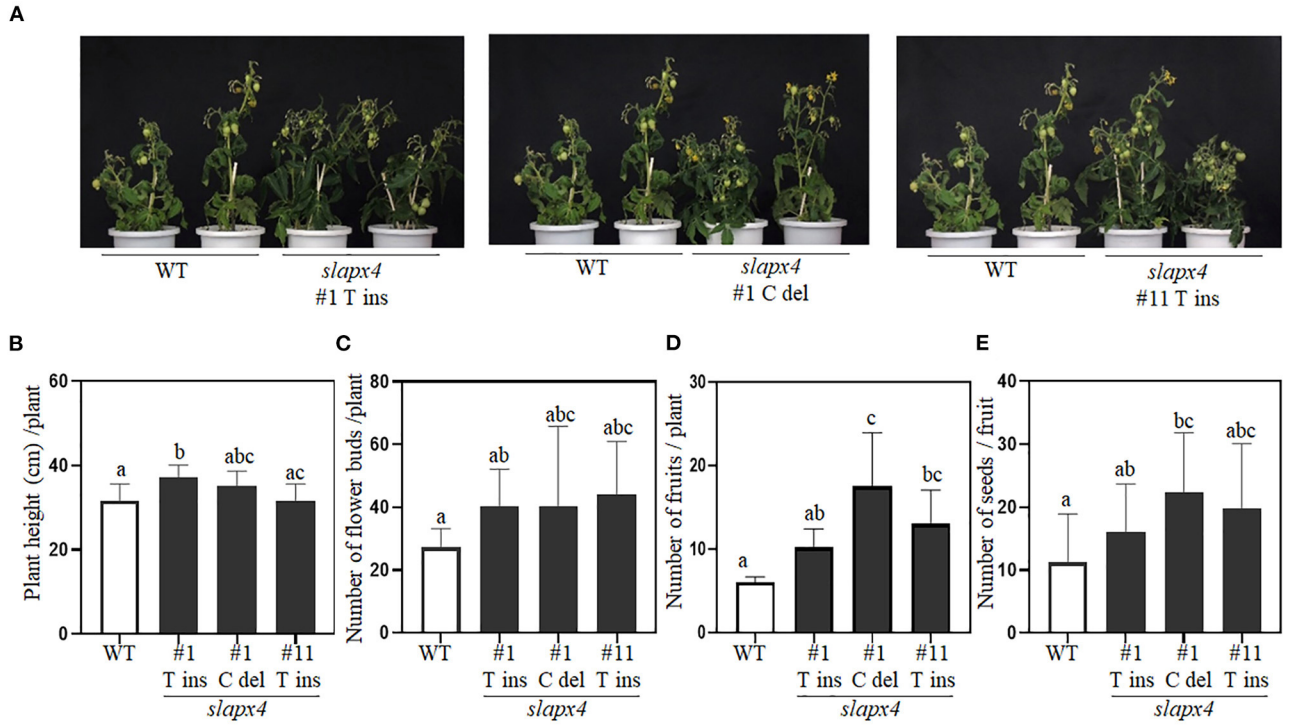


FIGURE 5 | Phenotypic analysis of *SLAPX4* mutant plants. **(A–E)** Wild-type (WT) and *SLAPX4* mutant plants (*SLAPX4* #1 T ins, #1 C del, and #11 T ins) were grown on the soil until maturity. **(A)** The phenotype of the representative plants was visualized by taking a picture at the mature green stage. Same WT plants were used for three independent pictures. Plant height **(B)**, flower bud number **(C)**, fruit number **(D)**, and seed number **(E)** per plant were measured from ten independent plants. Data represent the mean value + SD ($n = 10$). A significant difference was shown with different letters above the bars (one-way ANOVA test).

the later stage of fruit ripening. Ascorbate content analysis revealed that the mutation of *SlAPX4* significantly increased ascorbate content up to 2-fold in red ripening fruits (**Figure 3B**). Similar to our result, it has been reported that suppression of mitochondrial *APX* expression increases ascorbate content in plants due to lower degradation of ascorbate (Zhang et al., 2011; Chatzopoulou et al., 2020). However, suppression of *APX* expression often caused abnormal growth patterns (Pnueli et al., 2003; Chatzopoulou et al., 2020). These growth alterations could be caused by disruption of the ascorbate metabolic pathway during plant development. Gene expression analysis revealed that the expression level of ascorbate metabolic genes in *slapx4* mutant plants was similar to that of WT control plants in leaves (**Figure 4A**). In addition, the leaf ascorbate content was not significantly affected by the *SlAPX4* mutation (**Figure 3A**). Moreover, phenotype analysis of *slapx4* mutant plants revealed that all the generated *slapx4* mutant plants grew normally without growth retardation (**Figure 5**). These results suggest that *SlAPX4* is mainly involved in ascorbate metabolism during fruit maturation. Interestingly, *slapx4* mutant plants produced slightly more flowers and fruits compared with WT control plants (**Figures 5C,D**). A similar result was obtained from tomato plants with elevated ascorbate by suppression of ascorbate oxidase (Abdelgawad et al., 2019). These observed growth changes support the idea that the increase of ascorbate content affects plant development through alteration of hormonal signal transduction (Lima-Silva et al., 2012; Akram et al., 2017).

Even though the increase of ascorbate content in fruits is beneficial for human nutrition, the excessive increase of ascorbate content causes developmental defects in fruit shape and seed production (Bulley et al., 2012). Overexpression of the ascorbate biosynthetic gene increases metabolic flux to ascorbate biosynthesis, resulting in the decrease of metabolites required for cell wall biosynthesis (Dumville and Fry, 2003; Fenech et al., 2019). Our data showed that fruit shape and number of seeds of *slapx4* mutant plants were similar to those of wild type plants (**Figure 5E**), suggesting that targeted mutagenesis of specific ascorbate degradation pathway would be less destructive than ectopic activation of the biosynthetic pathway in plants. In conclusion, the data presented here suggest that *SlAPX4* is a valuable candidate gene to engineer tomato plants for increasing ascorbate content in mature fruits without growth defects.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JHD and YHJ planned and designed all the aspects of the study. JHD and SYP generated gene editing vectors and tomato transgenic plants. JHD, SYP, SHP, HMK, TDM, and SHM participated in the phenotypical analysis of the plant. JHD, JSS, and YHJ performed the data interpretation and wrote the manuscript. All authors contributed to the article and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.836916/full#supplementary-material

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ORIGINAL RESEARCH
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Expanding the Editing Window of Cytidine Base Editors With the Rad51 DNA-Binding Domain in Rice

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Recently developed base editors provide a powerful tool for plant research and crop improvement. Although a number of different deaminases and Cas proteins have been used to improve base editors the editing efficiency, and editing window are still not optimal. Fusion of a non-sequence-specific single-stranded DNA-binding domain (DBD) from the human Rad51 protein between Cas9 nickase and the deaminase has been reported to dramatically increase the editing efficiency and expand the editing window of base editors in the mammalian cell lines and mouse embryos. We report the use of this strategy in rice, by fusing a rice codon-optimized human Rad51 DBD to the cytidine base editors AncBE4max, AncBE4max-NG, and evoFERNY. Our results show that the addition of Rad51 DBD did not increase editing efficiency in the major editing window but the editing range was expanded in all the three systems. Replacing the human Rad51 DBD with the rice Rad51 DBD homolog also expanded the editing window effectively.

Keywords: ssDBD, Rad51 DBD, CBEs, editing window, rice

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INTRODUCTION

Many important agronomic traits are controlled by single nucleotide polymorphisms (SNPs) and incorporation of beneficial SNPs into elite lines by traditional breeding is a long and tedious process. In recent years, CRISPR-based genome editing technologies have provided revolutionary tools allowing to incorporate SNPs in a fast and efficient way. The "basic" CRISPR/Cas9 system produces double-stranded breaks (DSBs) with high-target precision, which mainly leads to either small base insertions, deletions (indels), or substitutions although the nature of the changes are random (Hilton and Gersbach, 2015; Zhang et al., 2017). The development of base editors (BEs) by fusing a deaminase to a Cas9 nickase, has provided a more reliable and efficient method to generate SNPs. According to the nature of the deaminase, BEs are classified into adenosine base editors (ABEs) and cytidine base editors (CBEs). The ABEs can generate site-specific A·T to G·C substitutions while the CBEs can produce site-specific C·G to T·A substitutions (Komor et al., 2016; Gaudelli et al., 2017; Rees and Liu, 2018).

From the initial, relatively inefficient BEs, several developments have increased the editing efficiency, expanded the targeting scope, and improved the editing specificity (Anzalone et al., 2020). CBEs have undergone improvements through several generations, from BE1 to BE4max, with average editing efficiencies increasing from 0.8 ∼ 7.7 to 65% (Komor et al., 2016, 2017;

Koblan et al., 2018). Similarly, ABEs have evolved to increase editing efficiency (Gaudelli et al., 2017; Koblan et al., 2018; Richter et al., 2020). Most improvement approaches have focused on the improvement of deaminases (such as evoFERNY and TadA8e) and optimization of system components, such as the domain-linking sequences and nuclear localization signals (NLSs), achieving important gains in editing efficiency (Koblan et al., 2018; Thuronyi et al., 2019; Wei et al., 2021). However, the editing window of the base editors is still relatively narrow, limiting their usefulness in functional genomics research and crop improvement. The nature of the deaminase is the main contributor to the size of the editing window. For most cytidine deaminases, such as rAPOBEC1, hAID, hAPOBEC3A, and PmCDA1, the editing window spans the protospacer positions 4 (C4) to 8 (C8) (counting the PAM as positions 21–23). The A3A base editor, using the human APOBEC3A cytidine deaminase, exhibited an expanded editing window, from C1 to C17, in plants (Zong et al., 2018). The evolved deaminases evorAPOBEC1, evoFERNY, and evoCDA1 also expanded the editing window from positions C1 to C15 (Zeng et al., 2020b). Although some of the newly developed CBEs have a relatively large editing window, the editing efficiency for PAM-proximal sequences (protospacer positions 12–20) is still very low (Anzalone et al., 2020).

Fusion of a single-stranded DNA binding domain (DBD) from the Rad51 protein with CBEs has also been proven to increase the editing efficiency and broaden the editing window in animal and plant systems (Zhang et al., 2020; Xu et al., 2022). The Rad51 protein plays significant roles in eukaryotic DNA recombination, including homology search and DNA-strand exchange (Conway et al., 2004; Heyer et al., 2010). Fusion of the human Rad51 with the Cas9 (D10A) nickase successfully mediated recombination by the homology-directed repair (HDR) pathway (Rees et al., 2019). The Rad51 N-terminal domain contains 114 residues involved in DNA binding (Aihara et al., 1999) and several groups have successfully used this domain with CBEs and prime editors (PEs) to improve editing efficiency and expand the editing window (Zhang et al., 2020; Song et al., 2021). Fusion of the Rad51 DBD with the Nmcas9-based CBE, termed hyper CBE (hyCBE), has proven successful in rice. While no base editing was achieved using the Nm1-BE3 and Nm2-BE3 systems, the C-to-T conversion frequency for Nm1-hyBE3 was 12.5 and 10.4% at the NAL1-5 and PDS-6 targets respectively and 14.6% at the GRF6-1 target for Nm2-hyBE3 (Xu et al., 2022).

In this work, we fused the human Rad51 DBD to several CBEs including AncBE4max, AncBE4max-NG, and evoFERNY, and also fused the rice Rad51 DBD to AncBE4max and evoFERNY in an attempt to produce improved CBE systems for rice. Although the editing efficiency did not increase as expected, the editing window was expanded to C16. These new systems will be useful for the generation of SNPs, especially in saturation mutagenesis.

MATERIALS AND METHODS

Materials and Growth Conditions

The *Oryza sativa* L. japonica varieties Zhonghua11 and Xiushui134 were grown in the paddy field in Shanghai, China, during the normal rice-growing season, and seeds were collected for callus production. All transgenic rice was grown in a rice greenhouse (12-h light 28°C and 12-h darkness at 22°C).

Vector Constructions

Rice codon-optimized human Rad51 DBD (342-bp) and 96-bp linker (Wei et al., 2021) containing *BamH* I restriction sites at both ends were synthesized (TSINGKE, Shanghai, China) and cloned into the *BamH* I site of AncBE4max (Wang et al., 2019) between Anc689APOBEC and nCas9, to produce hyAncBE4max. For the construction of hyAncBE4max-NG, hyAncBE4max was digested with *BamH* I, and the human Rad51 DBD-linker fragment cloned into *BamH* I-digested AncBE4max-NG vector between Anc689APOBEC and nCas9-NG (Wang et al., 2019). The rice Rad51 DBD (342-bp) with a 96-bp linker containing *BamH* I restriction sites at both ends were synthesized (TSINGKE, Shanghai, China) and cloned into the *BamH* I site of AncBE4max between Anc689APOBEC and nCas9, to produce hyriceAncBE4max.

For the construction of the evoFERNY vector, the rice codon-optimized evoFERNY and the bipartite NLS (bpNLS) sequence were synthesized (TSINGKE, Shanghai, China) and cloned into the *Kpn* I/*BamH* I sites of the PUC57 vector, to produce PUC57-evoFERNY. PUC57-evoFERNY was digested with *Kpn* I/*BamH* I and the resultant bpNLS-evoFERNY fragment was cloned into the *Kpn* I/*BamH* I-predigested AncBE4max vector between the maize *Ubiquitin* (*ZmUbi*) promoter and nCas9(D10A). To produce the hyevoFERNY and hyriceevoFERNY vectors, the human Rad51 DBD-linker or rice Rad51 DBD-linker sequences were amplified using the specific primers (**Supplementary Table 5**) and cloned into the *BamH* I site of the binary vector evoFERNY using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China).

For the construction of the target-specific base editing vectors, oligos containing the target-specific sequences and the adaptor sequences were synthesized and ligated to *Bsa* I-digested base editors, including AncBE4max, hyAncBE4max hyriceAncBE4max, AncBE4max-NG, hyAncBE4max-NG, evoFERNY, hyevoFERNY, and hyriceevoFERNY respectively. The final vectors were verified by Sanger sequencing using M13F as a primer. Primers used for vector construction are listed in **Supplementary Table 5**.

Rice Transformation

Rice calli derived from seeds of Zhonghua11 and Xiushui134 were transformed by *Agrobacterium tumefaciens*-mediated transformation (Nishimura et al., 2006). Briefly, binary vectors were transferred into *Agrobacterium tumefaciens* EHA105 and were used to inoculate rice calli. After 48–60 h of co-cultivation with *Agrobacterium* at 25°C, the calli were moved to selection media containing 50 mg L^{-1} hygromycin for 1 month. Resistant calli were moved to medium containing 2 mg L^{-1} Kinetin (KT), 0.2 mg L^{-1} naphthalene acetic acid (NAA), and 50 mg L^{-1} hygromycin for regeneration. Regenerated shoots were moved to a medium containing 50 mg L^{-1} hygromycin for rooting.

Genotyping of Transgenic Rice Lines

Genomic DNA was isolated from T0 generation rice leaves, derived from different tillers, using the cetyltrimethylammonium bromide (CTAB) method (Porebski et al., 1997). The M13F and sg-target-R primers were first used to identify positive transgenic lines and then the genomic regions surrounding the target sites were amplified using specific primers (**Supplementary Table 5**). PCR products were subjected to Sanger sequencing and subsequently analyzed using Geneious software to identify genotyping.

RESULTS

Fusion of the Human Rad51 DNA-Binding Domain Expands the Editing Window of AncBE4max in Rice

The human Rad51 DBD was codon-optimized for rice using the online GenScript Codon Optimization Tool[1] and fused to the AncBE4max to generate hyAncBE4max (**Figure 1A**). The AncBE4max chosen for our work contained the key elements of the Anc689 APOBEC deaminase, nickase Cas9 (nCas9) which recognizes NGG protospacer adjacent motif (PAM) sequences, two copies of the uracil glycosylase inhibitor (UGI), and two bipartite nuclear localization signals (bpNLS) at the N- and C-termini respectively (**Figure 1A**; Wang et al., 2019). The human Rad51 DBD was fused between the Anc689 APOBEC and nCas9 (D10A) with 32-amino acid linkers on each side (**Figure 1A**). Expression of the Anc689APOBEC/human Rad51 DBD/nCas9 cassette was driven by the maize *Ubiquitin* (*ZmUbi*) promoter while the rice *U6* promoter was used to drive the expression of the sgRNA (**Figure 1A**). Five target sites in three different genes were selected to analyze editing efficiency and editing window using the AncBE4max and hyAncBE4max systems (**Supplementary Table 1**). The binary vectors were used to transform rice callus *via Agrobacterium*-mediated infection and independent T0 transgenic lines genotyped by amplification of the target sequences followed by Sanger sequencing.

At the *OsRbcS2*-TS1 site, both AncBE4max and hyAncBE4max exhibited editing activity at positions C5 and C7 (**Figure 1B** and **Supplementary Table 2**). Although the C-to-T efficiency at position C7 was quite similar for both systems (AncBE4max 36.4 vs. hyAncBE4max 35%); contrary to our expectations, the efficiency of AncBE4max (36.4%) at position C5 was more than twice higher than that of hyAncBE4max (15%) (**Figure 1B** and **Supplementary Table 2**). At the *OsWaxy*-TS2 site, AncBE4max showed 10% editing efficiency at positions C2 and C3, while we failed to detect any editing activity for hyAncBE4max. At position C6 of the same target, both systems showed similar high C-to-T efficiency (50–55%), while at position C10, the C-to-T substitution efficiency was 10% for AncBE4max and 25% for hyAncBE4max (**Figure 1C** and **Supplementary Table 2**). At the *OsWaxy*-TS3 site, the substitution efficiency for hyAncBE4max and AncBE4max at the C5 and C6 positions were comparable (45 vs. 60% at C5, and 50 vs. 60% at C6) (**Figure 1D** and **Supplementary Table 2**). Nevertheless, the hyAncBE4max editing window was expanded to positions C14 and C16, with substitution efficiencies of 30.0% at position C14 and 10.0% at position 16 (**Figure 1D**). For the *OsWaxy*-TS4 site, AncBE4max produced 4.5% editing while hyAncBE4max showed no editing at position C4, while at positions C6 and C7, the C-to-T substitution efficiency was 18.8% for AncBE4max and 37.5% for hyAncBE4max (**Figure 1E** and **Supplementary Table 2**). At the *OsALS*-TS5 site, both AncBE4max and hyAncBE4max exhibited high editing efficiency at positions C6 (87.5 vs. 77.8%, respectively), C7 (87.5 vs. 55.6%), and C8 (37.5 vs. 55.6%) (**Figure 1F** and **Supplementary Table 2**).

The aforementioned results suggest that the editing window for hyAncBE4max was C5-C16, compared to C2-C10 for AncBE4max. Within the C4-C8 editing window, the average C-to-T efficiency for hyAncBE4max was similar or lower than AncBE4max (**Figure 1G**). The hyAncBE4max performed better at positions C10-C16, especially close to the PAM (**Figure 1G**). The homozygous rate, percentage of edited plants with homozygous mutations, was similar between AncBE4max and hyAncBE4max at the five target sites studied (**Supplementary Table 3**). Both systems performed better for TC motifs and poorly for GC motifs, in accordance with previous reports, due to the context preference of the APOBEC1 deaminase (**Figures 1B–F**; Komor et al., 2016; Gehrke et al., 2018; Thuronyi et al., 2019).

Unexpectedly, the incidence of indels was high for both AncBE4max and hyAncBE4max (24.7 and 35.0% respectively) (**Supplementary Figure 1A**). Both systems also generated C-to-G substitution byproducts at a relatively high rate (6.14% for AncBE4max and 7.72% for hyAncBE4max) (**Supplementary Figure 2A**).

The Addition of the Human Rad51 DNA-Binding Domain Expands the Editing Window of AncBE4max-NG

SpCas9-NG can recognize NG PAMs expanding the number of target sites compared to the canonical NGG PAM but their C-to-T editing efficiency is much lower than SpCas9-NGG (Nishimasu et al., 2018; Zeng et al., 2020a). We attempted to increase the editing efficiency of SpCas9-NG by fusing the human Rad51 DBD to AncBE4max-NG (**Figure 2A**). Four target sites from three genes were used to test the editing characteristics of the hyAncBE4max-NG system (**Figures 2B–E** and **Supplementary Table 2**).

At the *OsRbcS2*-TS6 site, AncBE4max-NG and hyAncBE4max-NG exhibited similar low editing efficiency at positions C6 (10.0 vs. 7.4%) and C7 (6.7 vs. 7.4%) (**Figure 2B**). AncBE4max-NG editing efficiency was also low at C4 (3.3%) and C10 (6.7%) while hyAncBE4max-NG displayed no edit activity at either position (**Figure 2B**). At the *OsRbcS2*-TS7 site, hyAncBE4max-NG exhibited higher efficiency than AncBE4max-NG at position C9 (30 vs. 5%) (**Figure 2C**); however, only AncBE4max-NG displayed activity at position C6 (5%) (**Figure 2C**). At the *OsRCA*-TS8 and *OsRCA*-TS9

[1]https://www.genscript.com.cn/gensmart-free-gene-codon-optimization.html

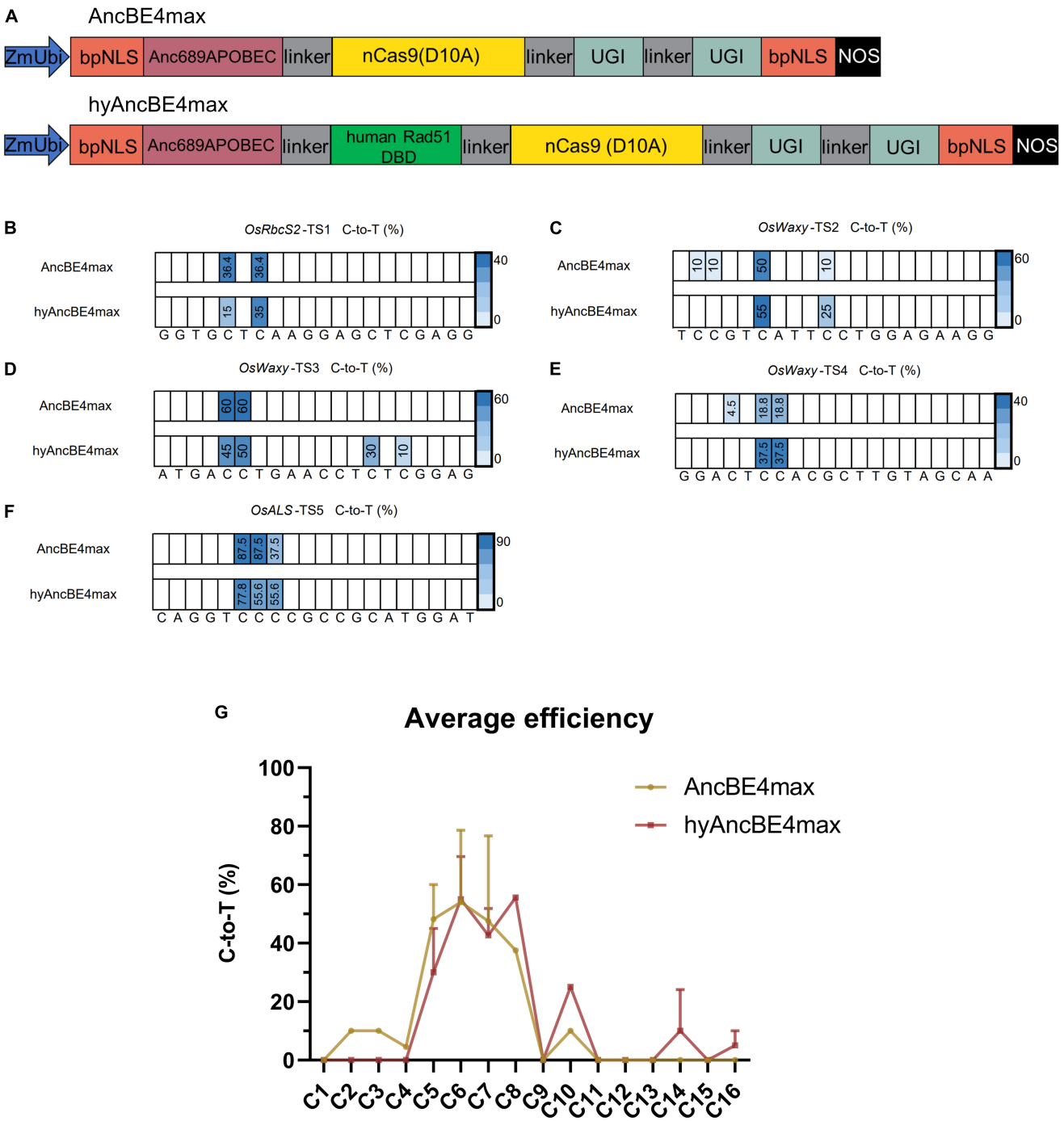


FIGURE 1 | Characterization of AncBE4max and hyAncBE4max in rice. **(A)** Schematic structures of AncBE4max and hyAncBE4max. The human Rad51 DBD was synthesized using a plant optimized code and cloned into AncBE4max (Wang et al., 2019) to form hyAncBE4max. ZmUbi, maize *ubiquitin-1* promoter; bpNLS, bipartite nuclear localization signal; nCas9(D10A), nickase Cas9 with D10A substitution; UGI, uracil glycosylase inhibitor; NOS, NOS terminator. **(B–F)** C-to-T conversions in five target sites using AncBE4max or hyAncBE4max. **(G)** Average C-to-T editing efficiency for AncBE4max and hyAncBE4max based on all five target sites. Data are means ± SD.

sites, hyAncBE4max-NG exhibited higher efficiency than AncBE4max-NG at position C5 (70 vs. 50% for *OsRCA*-TS8 and 80 vs. 60% for *OsRCA*-TS9) (**Figures 2D,E**). As for the AncBE4max system, the average C-to-T efficiencies of hyAncBE4max-NG and AncBE4max-NG in the center of the editing window (C4-C8) were similar (**Figure 2F**). But, contrary to our expectations, the hyAncBE4max-NG editing window (C5-C9) was narrower than BE4max-NG (C4-C10) (**Figure 2F**).

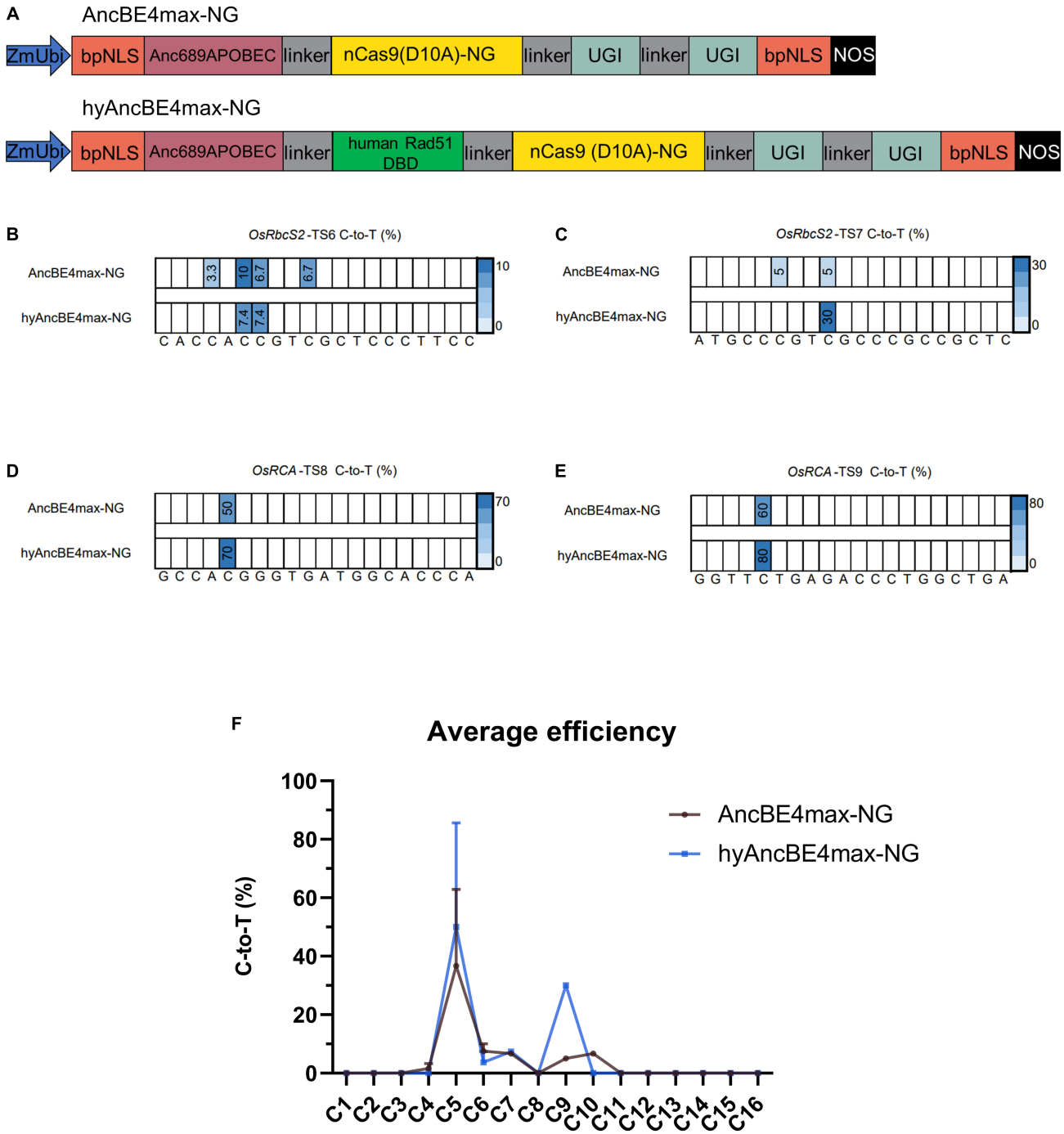


FIGURE 2 | Characterization of AncBE4max-NG and hyAncBE4max-NG in rice. **(A)** Schematic structures of AncBE4max-NG and hyAncBE4max-NG. The human Rad51 DBD was synthesized using a plant optimized code and cloned into AncBE4max-NG (Wang et al., 2019) to form hyAncBE4max-NG. ZmUbi, maize *ubiquitin-1* promoter; bpNLS, bipartite nuclear localization signal; nCas9(D10A)-NG, nickase Cas9-NG with D10A substitution; UGI, uracil glycosylase inhibitor; NOS, NOS terminator. **(B–E)** C-to-T conversions of four target sites using AncBE4max-NG or hyAncBE4max-NG. **(F)** Average C-to-T editing efficiency for AncBE4max-NG and hyAncBE4max-NG based on all four target sites. Data are means ± SD.

The average incidence of indels for hyAncBE4max-NG and AncBE4max-NG was much lower than that observed in the AncBE4max and hyAncBE4max system, with 2.3% for AncBE4max-NG and 0.93% for hyAncBE4max-NG (**Supplementary Figure 1B**). Interestingly, both hyAncBE4max-NG and AncBE4max-NG exhibited a 30% C-to-G rate at the *OsRCA*-TS9 site, the highest C-to-G rate among all nine target sites (**Supplementary Figure 2B**).

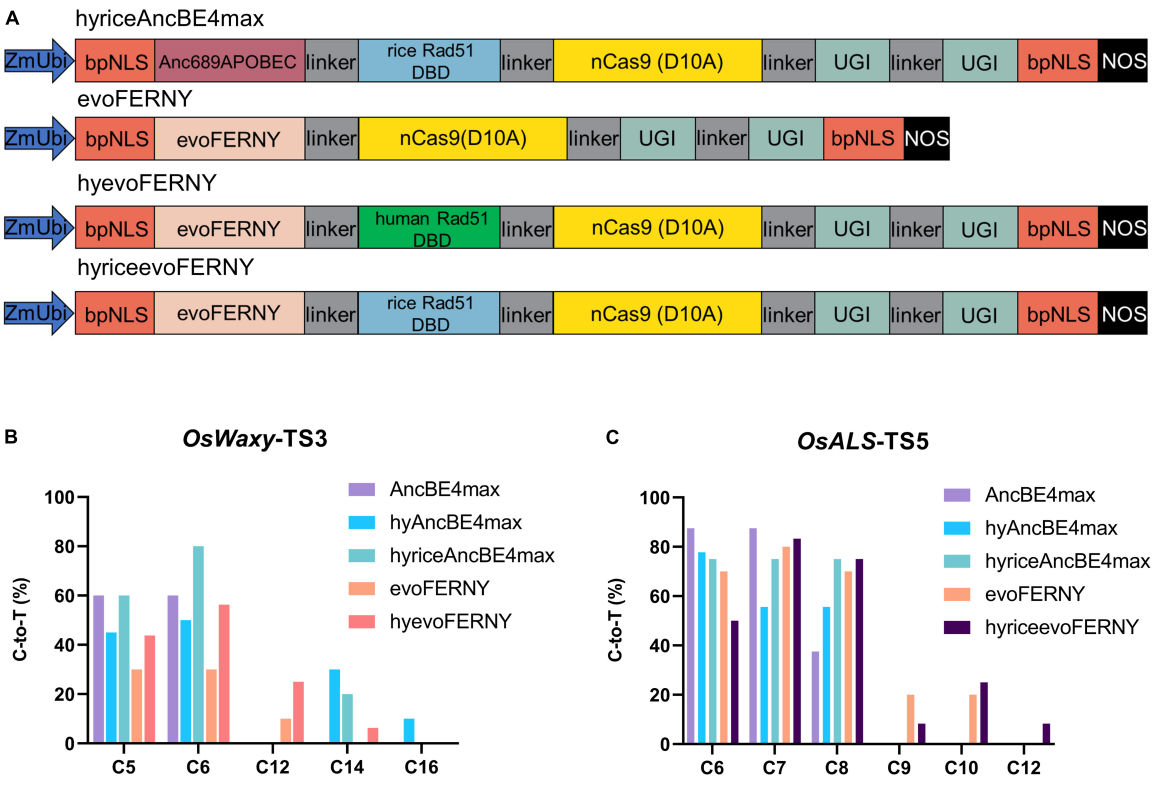


FIGURE 3 | Characterization of hyriceAncBE4max, evoFERNY, hyevoFERNY, and hyriceevoFERNY in rice. **(A)** Schematic structures of hyriceAncBE4max, evoFERNY, hyevoFERNY, and hyriceevoFERNY. Rice Rad51 DBD was synthesized and cloned into AncBE4max to form hyriceAncBE4max. The cytosine deaminase evoFERNY was synthesized using a plant optimized code and cloned replacing Anc689APOBEC in AncBE4max to generate evoFERNY. HyevoFERNY and hyriceevoFERNY were produced by introducing the human Rad51 DBD or rice Rad51 DBD with evoFERNY respectively. **(B)** C-to-T editing efficiency at different positions for *OsWaxy*-TS3 using different base editors. **(C)** C-to-T editing efficiency at different positions for *OsALS*-TS5 using different base editors.

Testing the Effect of the Rice Rad51 DNA-Binding Domain on the AncBE4max and evoFERNY Cytidine Deaminases

Since the human Rad51 DBD did not have the desired effect on the hyAncBE4max and hyAncBE4max-NG systems, we replaced it with the rice Rad51 DBD (**Supplementary Figure 3**), to generate hyriceAncBE4max (**Figure 3A**). Recently, the cytidine deaminase evoFERNY was produced using the phage-assisted continuous evolution system and exhibited superior performance in editing efficiency, target scope, and target context compatibility in mammalian cells and rice (Thuronyi et al., 2019; Zeng et al., 2020b). We combined evoFERNY with either human Rad51 DBD or rice Rad51 DBD to generate hyevoFERNY and hyriceevoFERNY, respectively (**Figure 3A**).

The editing characteristics for the aforementioned hyCBE base editors were tested at *OsWaxy*-TS3 and *OsALS*-TS5 sites. The editing efficiency of hyriceAncBE4max (60%) was equal to that of AncBE4max (60%) and better than hyAncBE4max (45%) at position C5 of *OsWaxy*-TS3 (**Figure 3B** and **Supplementary Table 2**). At position C6, hyriceAncBE4max exhibited higher efficiency (80%) than both AncBE4max (60%) and hyAncBE4max (50%) (**Figure 3B**). Similar to hyAncBE4max, hyriceAncBE4max also expanded the editing window to C14 at this target (**Figure 3B** and **Supplementary Table 2**). At the *OsALS*-TS5 site, hyriceAncBE4max (75%) displayed similar high editing efficiency to hyAncBE4max (77.8%) and AncBE4max (87.5%) at position C6 and outperformed hyAncBE4max (75 vs. 55.6%) at position C7 (**Figure 3C** and **Supplementary Table 2**). At position C8, hyriceAncBE4max exhibited higher C to T editing efficiency (75%) than hyAncBE4max (55.6%) and AncBE4max (37.5%) (**Figure 3C**). Overall, hyriceAncBE4max showed similar or better editing efficiency than hyAncBE4max at these two target sites and it also expanded the editing window.

Compared with AncBE4max, the evoFERNY system produced lower editing efficiencies at positions C5 and C6 of *OsWaxy*-TS3 and positions C6 and C7 of *OsALS*-TS5 (**Figures 3B,C**). HyevoFERNY displayed higher editing efficiencies than evoFERNY at positions C5, C6, and C12 (43.8, 56.3, 25% for hyevoFERNY and 30, 30, 10% for evoFERNY) and expanded the editing window from C12 to C14 at the *OsWaxy*-TS3 site (**Figure 3B**). Compared with evoFERNY, hyriceevoFERNY, containing the rice Rad51 DBD, exhibited lower editing efficiencies at positions C6 (50 vs. 70%) and C9 (8.3 vs. 20%) of the *OsALS*-TS5 target site, and similar editing efficiencies at positions C7 (83.3 vs. 80%), C8 (75 vs. 70%), and C10 (25 vs. 20%). The editing window of hyriceevoFERNY also expanded to C12 compared with C10 of evoFERNY (**Figure 3C**).

Results of this study show that fusion of a human Rad51 DBD or rice Rad51 DBD to either AncBE4max or evoFERNY can broaden the editing window, but the effect on editing efficiency was not satisfactory. Overall, the rice Rad51 DBD outperformed its human counterpart in rice.

DISCUSSION

Compared to the development of new deaminase variants, which mostly improves base editing efficiency, a fusion of base editors with ssDNA binding protein domains (DBD) has the potential to expand the editing window and improve the editing efficiency given that the DBD can stabilize the Cas9-nickase-generated ssDNA. This strategy has been successfully used in both mammalian cell lines and mouse embryos (Zhang et al., 2020). In addition, a fusion of the Rad51 DBD allowed editing of target sites that could not be edited using conventional base editors in rice (Xu et al., 2022). Since the Rad51 DBD is a non-sequence-specific protein, it has also been used to stabilize the ssDNA and improve the efficiency in the Primer Editor (PE) system (Song et al., 2021).

In this study, the effect of adding DBDs to CBE systems in rice, by fusing the plant optimized-code human Rad51 DBD to the CBE base editors AncBE4max, AncBE4max-NG, and evoFERNY, and the rice Rad51 DBD to AncBE4max and evoFERNY was studied. The results show that the addition of Rad51 DBD had different effects when fused to different Cas9 variants. The human Rad51 DBD improved editing efficiency over the original system in 3 out of 4 target sites when fused to the Cas9-NG AncBE4max-NG, while fusion to the Cas9-NGG AncBE4max resulted in similar or lower editing efficiencies in 4 out of 5 target sites (**Figures 1**, **2**). The moderate or lack of increase in editing efficiency observed in our experiments is in open contrast with the results from Zhang et al. (2020), which reported up to 257-fold increases in editing activity in mammalian systems. Xu et al. (2022) recently tested the effect of the Rad51 DBD in the editing efficiency of a CBE system (Nm1-enBE3) containing the *Neisseria meningitidis* Cas9 and the rat APOBEC1 deaminase in rice. Nm1-enBE3 failed to produce base editing in any of the six targets used in the study while the addition of the Rad51 DBD resulted in low editing efficiencies in 5 of the six targets and failed to produce any editing in the sixth target. Combined, these results and our results suggest that the Rad51 DBD effect on editing efficiency is more accentuated in mammalian systems compared to plant systems, although it could improve plant CBEs with very low efficiency.

Recently, the addition of the human Rad51 DBD to ABE systems produced diverse results in editing efficiency when fused to different Cas9 variants (Tan et al., 2022).

Consistent with previous reports, it was observed that fusion of the Rad51 DBD expanded the editing window to nucleotides close to PAM. The editing window was expanded from position C10 to C16 in hyAncBE4max and from the position, C12 to C14 in hyevoFERNY (**Figures 1G**, **3C**). This study results suggest that hyCBEs performed better at sequences proximal to the PAM. At position C9 of *OsRbcS2*-TS7, the hyAncBE4max-NG exhibited an editing efficiency of 30% compared to 5% for AncBE4max-NG (**Figure 2C**). Similarly, at position C6 of *OsWaxy*-TS2, hyAncBE4max and AncBE4max exhibited comparable editing efficiencies (55 vs. 50%) but hyAncBE4max outperformed AncBE4max at position C10 (25 vs. 10%) (**Figure 3C**). At the *OsALS*-TS5 target, the editing efficiency for hyAncBE4max was lower than AncBE4max at positions C6 (77.8 vs. 87.5%) and C7 (55.6 vs. 87.5%). However, hyAncBE4max (55.6%) performed better than AncBE4max (37.5%) at position C8 (**Figure 1F**). Interestingly, Rad51 DBD narrowed the editing windows for PAM-distal nucleotides. For AncBE4max, at the *OsWaxy*-TS2 and *OsWaxy*-TS4 target sites, the editing window started at C2 and C4, respectively, while AncBE4max-NG displayed activity at position C4 for the *OsRbcS2*-TS6 and *OsRbcS2*-TS7 (**Figures 1C,E**, **2B,C**). However, the corresponding hyCBEs failed to edit at any of the sites. These results suggest that the Rad51 DBD may have adverse effects on the binding or the activity of the cytidine deaminase for PAM-distal sequences.

It has been reported that the DBD had different effects when fused to the rat APOBEC1 and the human APOBEC3A cytidine deaminases in HEK293T cells (Zhang et al., 2020). Fusion of the human Rad51 DBD to the cytidine deaminases Anc689APOBEC and evoFERNY in this study had a similar effect at the *OsWaxy*-TS3 and *OsALS*-TS5 sites (**Figures 3B,C**). The rice Rad51 DBD performed better than its human homolog when fused to AncBE4max and evoFERNY.

Since the human Rad51 did not increase the off-target effects in mammalian cell lines and mouse embryos, to evaluate the effect of Rad51 on off-targeting in rice, we used CRISPR-GE to search for the homologous sequences within two nucleotides mismatch relative to the sgRNAs and found four putative off-target sites (Xie et al., 2017; Zhang et al., 2020). The editing events, including C to T substitution, C to G substitution, and indels, were undetected at one putative off-target site which contained two nucleotides mismatch within the seed region relative to *OsRbcS2*-TS1 (**Supplementary Table 4**). For the other three putative off-target sites, generally, the hyCBEs system exhibited similar or even lower off-target efficiency than the CBE system, which indicated that Rad51 also did not increase the off-target effects in rice (**Supplementary Figure 4** and **Supplementary Table 4**).

For the *OsRbcS2*-TS1, *OsWaxy*-TS2 and *OsWaxy*-TS3 targets, both AncCBEmax and hyAncCBEmax showed higher indels (40.1, 40, and 30% for AncCBEmax; 50, 35, and 40% for hyAncCBEmax) (**Supplementary Figure 1A**). For the *OsWaxy*-TS4 target, hyAncCBEmax contained the indels with 50%, while no indels occurred for AncCBEmax at this target. Conversely, at target *OsALS*-TS5, the indels were 12.5% for hyAncCBEmax, while no indels occurred for hyAncCBEmax (**Supplementary Figure 1A**). For the NG system, the indels only occurred at *OsRbcS2*-TS6 (3.7%) for hyAncCBEmax-NG and at *OsRCA*-TS9 (30%) for AncCBEmax (**Supplementary Figure 1B**). In the evoFERNY and hyevoFERNY systems, no indels occurred. Our results suggest that the incidence of indels is related to the nature of the cytidine deaminase, but further research is needed to draw this conclusion.

In summary, we have tested the effects of Rad51 DBD in CBE systems in rice and proved that it can extend the editing window for PAM-proximal sequences, providing advantages for gene

functional research and precision molecular breeding, especially for gene saturation mutagenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HZ and CWe designed the work. HZ and CWa supervised the work and analyzed the data. CWe, HL, and PL generated all constructs. CWe, HL, WW, QC, and RL performed the genotyping analysis. HZ and CWe analyzed the data. HZ, CWe, CWa, and JB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.865848/full#supplementary-material

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REVIEW
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Advance of Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 System and Its Application in Crop Improvement

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Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 is the third generation of novel targeted genome editing technology after zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs). It is also one of the most promising techniques for mutating and modifying genes. The CRISPR-Cas9 system has the advantages of simplicity, high efficiency, high specificity, and low production cost, thus greatly promoting the study of gene function. Meanwhile, it has attracted the attention of biologists. After the development and improvement in recent years, CRISPR-Cas9 system has become increasingly mature and has been widely used in crop improvement. Firstly, this review systematically summarizes the generation and advantages of CRISPR-Cas9 system. Secondly, three derivative technologies of the CRISPR-Cas9 system are introduced. Thirdly, this review focuses on the application of CRISPR-Cas9 system in gene knockout, gene knock-in, and gene regulation, as well as the improvement of yield, quality, and biological resistance of important crops such as rice, wheat, soybean, corn, and potato. Finally, this review proposes the potential challenges of CRISPR-Cas9 system, and discusses the future development of CRISPR-Cas9 system.

Keywords: CRISPR-Cas9 system, gene knockout, application, crop improvement, future development

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THE PRODUCTION AND ADVANTAGES OF CRISPR-Cas9 TECHNOLOGY

Ishino et al. (1987) first discovered the existence of special repeat sequences separated by spacer sequences of similar size in the *Escherichia coli* genome when he was studying the *E. coli*. Jansen et al. (2002) named it as the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and at the same time found CRISPR-associated (*Cas*) gene, near the CRISPR sequence, and analyzed its function. Barrangou et al. (2007) discovered an acquired immunity against phage and other exogenous genes mediated by CRISPR sequence and Cas protein in prokaryotes. Jinek et al. (2012) elucidated the mechanism of action of CRISPR-Cas. Based on the acquired immune system, Church's laboratory modified the system to construct a type II CRISPR/Cas system, namely the CRISPR-Cas9 system, which has become a technology that can edit DNA in animal and plant cells (Mali et al., 2013). The two main elements of the

CRISPR-Cas9 system are guide RNA (sgRNA) and Cas9 protein. The target genes of the CRISPR-Cas9 system usually contain a short Protospacer adjacent motif (PAM), and the PAM sequence is usually NRG (R = G/A). SgRNA is an RNA sequence designed according to the target gene and has the function of guiding the Cas9 protein to cut the target gene. Cas9 protein is an endonuclease containing RuvC and HNH domains, which are responsible for cleaving the target strand and non-target strand of the target gene, respectively. The cleavage site is located 3 ~ 8 nt upstream of PAM (Friedland et al., 2013). The working mechanism of the CRISPR-Cas9 system is that the Cas9 protein is guided by sgRNA to introduce a DNA double-strand break (DSB) into a specific target gene. Once this break occurs in the cell, it will trigger homologous recombination (HR) or non-homologous end joining (NHEJ) two automatic repair mechanisms, resulting in the insertion, deletion, and replacement of target gene sequences, to achieve directional modification of the genome (Jinek et al., 2012; Li et al., 2013). Genome editing technology is a technology for studying gene function and targeted modification of the genome. CRISPR-Cas is the third generation of new targeted genome editing technology after zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs). CRISPR-Cas9 technology is the most widely used type of CRISPR-Cas system. Both ZFNs and TALENs are composed of a specific DNA binding domain and a non-specific cleavage domain (endonuclease FokI). CRISPR-Cas9 is also composed of a specific sgRNA and a non-specific endonuclease Cas9. In terms of specificity and off-target effects, CRISPR-Cas9 is stronger than ZFN, but slightly worse than TALEN. In terms of design and construction system, CRISPR-Cas9 is simpler in design than ZFN and TALEN. In addition, CRISPR-Cas9 has low production cost, not restricted by methylation, and can edit multiple genes at the same time (Li et al., 2013; Kumar et al., 2016; Liu et al., 2016). All things considered, CRISPR-Cas9 is the first choice when using sequence-specific nucleases for gene editing.

DERIVATIVE TECHNOLOGY OF CRISPR-Cas9 TECHNOLOGY

CRISPR-dCas9 System

The CRISPR-dCas9 system was born in 2013. Qi et al. (2013) mutated the two conserved endonuclease domains of the Cas9 protein in the CRISPR-Cas9 system, namely the RuvC domain and the HNH domain. Cas9 protein loses endonuclease activity and cannot cut DNA, but still retains the ability to bind to DNA sequences. The CRISPR-dCas9 system usually plays a role in the process of gene regulation. The properties of dCas that can bind to double-stranded target genes are similar to transcription factors that regulate gene expression, but dCas cannot function alone. The purpose of activating or inhibiting gene transcription can be achieved by combining dCas with transcriptional activator protein/transcriptional repressor protein. In addition, although dCas cannot function alone, it can cause steric hindrance and prevent gene transcription.

Base Editor

Base editors (BEs) include single base editors and double base editors.

Single base editors include cytidine base editors (CBEs) and adenine base editors (ABEs). The basic components of the CBE system include: sgRNA, nicking Cas9 (nCas9) protein with single-strand cleavage activity, cytosine deaminase, and uracil glycosylase inhibitor (UGI). The specific working mechanism is: nCas9 cleaves the non-target strand under the guidance of sgRNA, and cytosine (C) in the editing window is catalyzed by cytosine deaminase to remove an amino group and mutate to uracil (U), and UGI prevent the base U from being excised. During DNA replication, U is recognized as thymine (T), which is complementary to adenine (A), and in the next round of DNA replication, A and T normal pairing to achieve transformation of C to T (Komor et al., 2016). The ABE system is composed of sgRNA, nCas9 protein with single-strand cleavage activity and adenine deaminase fused. The working principle is: nCas9 cuts the non-target strand under the guidance of sgRNA, and adenine deaminase catalyzes the removal of an amino group from A in the target gene editing window to become inosine (I). It is recognized as guanine (G) during DNA replication, which is complementary to C. In the next round of DNA replication, G is paired with C, which finally completes the A to G transition (Gaudelli et al., 2017).

Double base editor is the Saturated Targeted Endogenous Mutagenesis Editor (STEME); this double base editor can induce C to T and A to G mutations at the target site at the same time under the guidance of a sgRNA, significantly increasing the saturation and diversity of base mutations (Li et al., 2020a).

Prime Editor

The prime editor (PE) was developed in 2019 and theoretically enables 12 substitutions of four types of bases. The main elements include prime editing extended guide RNA (pegRNA), nSpCas9 (H840A), and moloney murine leukemia virus (M-MLV) reverse transcriptase. Among them, pegRNA is a modification of gRNA, adding a primer-binding sequence (PBS) and a reverse transcription template (RT) to its 3′ end, and adding editing information to the reverse transcription template, and nSpCas9 is a modification of Cas9 so that it only has cleavage single-stranded DNA activity. The PE is that the gRNA is paired with the target gene, instructing nSpCas9 to cut the target strand, generating a single-strand break nick, PBS binds to the 3′ end of the break nick, bringing RT to the nick. And then, under the action of MMLV, using RT as a template, a single-stranded DNA sequence is synthesized from the 3′ end of the broken nick. The automatic repair mechanism of the organism is triggered, and the newly synthesized DNA sequence is used as a template to repair the other DNA chain, thereby in any base substitution is introduced on the double strand of DNA (Anzalone et al., 2019).

CRISPR-Cas9 APPLICATION

In recent years, the research on the CRISPR-Cas9 system has been applied in various forms, including applications in gene

knockout, gene knock-in, and gene regulation. Among them, gene knockout is the most widely used (Zhao et al., 2019). **Table 1** summarizes the applications of CRISPR-Cas9 in these three aspects.

Application in Gene Knockout

Gene knockout is a key technology to study gene function and permanently change cell phenotype. CRISPR-Cas9 is applied to crops in a variety of ways. The traditional method is to construct a knockout vector, which is transferred into plants through agrobacterium-mediated transformation and other methods. The target gene knockout mutant can be obtained after 1–2 generations (Peng, 2017; Jiang et al., 2020). The target gene for gene knockout can be single or multiple. For each target gene, one or more sgRNAs can be designed to locate different target sites. Multiple sgRNAs can be assembled into a binary carrier at the same time through Golden Gate, Gibson, or isocaudomer technology (Arturo et al., 2015; Ma, 2015). Generally speaking, designing multiple targets for a gene will increase the efficiency of gene editing (Shi et al., 2018). It is relatively difficult to obtain co-knockout mutants of multiple genes in theory, but studies have shown that the tandem of multiple sgRNAs does not seem to affect editing efficiency (Shen et al., 2017).

Single-Gene One-Site Knockout

Liu et al. (2020b) used CRISPR-Cas9 to target and mutate the transcription factor *SlLBD40* in the border domain of tomato lateral organs to enhance the drought tolerance of tomato. Feng et al. (2016) used CRISPR-Cas9 technology for targeted knockout of maize *Zmzb7* gene, and successfully obtained the albino phenotype mutant material. In addition, based on the CRISPR-Cas9 system, they compared the gene mutation efficiency of the heterochromatic region and the homochromatin region of maize, and found that the mutation efficiency is around 50%. It shows that the gene targeting of the CRISPR-Cas9 system has nothing to do with chromatin state. Using CRISPR-Cas9 technology, Cai (2019) constructed knockout vector targeting at maize grain development gene *Emp10*. The mutant exhibited stagnant embryo and endosperm development, empty and deflated kernels after maturity, embryo lethality, and inability to germinate. Researchers analyzed the mechanism of the *Emp10* gene and found that the deletion of this gene affects the assembly and activity of mitochondrial electron transport chain complex I, impairs the oxidative phosphorylation pathway, affects ATP synthesis after fertilization, and leads to poor grain development.

Single-Gene Multiple-Site Knockout

Liu et al. (2019) constructed CRISPR-Cas9 expression vector targeting *SlAP3* gene. Sequencing results showed that deletion mutations occurred at 1–9 bp bases upstream of two target sites of *SlAP3* gene, resulting in early termination of *SlAP3* protein sequence translation. Thereby obtain male sterile tomato lines, homozygous mutants all showed phenotype of reduced number of flower organs such as petals and stamens. Cai et al. (2018) used CRISPR-Cas9 to induce targeted mutations of *GmFT2a* gene in soybean photoperiodic flowering pathway, and designed a total of three target sites, resulting in six frameshift mutations that all lead to early termination of translation, and all of the mutants showed late flowering phenotypes. Aiming at four target sites of *GmSPL3* gene, Wu et al. (2019) constructed CRISPR-Cas9 four-site knockout vector and successfully obtained spl3abcd mutants. Under short-day conditions, *spl3 abcd* mutants showed phenotypes of smaller leaves, fewer node, shortened polarity, and reduced height, indicating that *GmSPL3* plays an important role in regulating soybean plant morphology.

Multiple-Gene Co-knockout

In order to figure out the relevancy between different genes or obtain a plurality of excellent traits coexisting strains, the CRISPR-Cas9 polygenic knockout system is used to simultaneously knock out multiple genes. This makes it possible for obtaining strains with different combinations of good traits, providing new materials for crop breeding.

Shen et al. (2017) successfully constructed co-knockout vectors of eight genes related to agronomic traits in rice using CRISPR-Cas9 technology, and found that five of them (*EP3*, *GS3*, *GW2*, *DEP1*, and *Gn1a*) were related to yield, while the other three were related to flavor (*BADH2*), photoperiod (*Hd1*), and plant shape (*LPA1*). By screening the transgenic plants, various combinations of mutant strains are obtained, including homozygous mutants with a common mutation in six, seven, and eight genes, providing strategies for the rapid introduction of genetic diversity in crop breeding. The mutation efficiency of eight genes was found to be almost the same in the transient expression system with single-gene and multiple-gene knockout, suggesting that the number of sgRNAs in tandem may not affect the editing efficiency. Zheng et al. (2020) knocked out two *BnaMAX1* homologs in rapeseed by using targeted mutagenesis of CRISPR-Cas9 and obtain semi-dwarfed rapeseed mutants with increased branching and yield, the mutation rate of which was 56.3–67.38%. Two branching-related genes *BnaBRC1* and *BnaBRC2* in *Brassica napus* were mutated by using CRISPR-Cas9 technique, and the functional deletion mutant brc1 displayed an obvious multi-branch phenotype, indicating that BnaBRC1 is involved in the regulation of *Brassica napus* branch (Amoo, 2020). Liu et al. (2020a) used CRISPR-SpCas9 to edit *TaAQ* and *TaDq* genes in wheat. In T1 generation, *TaDq* deletion plants and wild-type (WT) plants were only different in plant height. In addition, *TaAQ* knockout plants or *TaAQ* and *TaDq* co-knockout plants were more vulnerable than WT plants.

Application of Gene Knock-in

The most traditional application of CRISPR-Cas9 gene knockout can only reduce the expression level of genes, but a large number of genes can be improved by increasing their expression. To achieve the aim of it, gene knock-in or knock-up might be an efficient approach.

A general technical route for knock-up is to insert regulatory elements such as promoters or enhancers near the target gene. The herbicide resistance of rice can be improved by transgenic

TABLE 1 | Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 application.

Species	Target gene(s)	Gene function	CRISPR-Cas9 technical principle	Mutation mode	Mutant features	References
Single gene one site knockout						
Tomato	*SlLBD40*	Lateral organ boundaries domain transcription factor	CRISPR-Cas9 knockout vector	Gene knockout	Strong drought resistance	Liu et al., 2020b
Maize	*Zmzb7*	Encodes an IspH protein that plays an important role in the methyl-D-erythritol-4-phosphate (MEP) pathway	CRISPR-Cas9 knockout vector	Insertion and deletion	Albino	Feng et al., 2016
Maize	*Emp10*	Related to rice grain development	CRISPR-Cas9 knockout vector	Gene knockout	The grains are poorly developed	Cai, 2019
Single gene multiple site knockout						
Tomato	*SlAP3*	A direct homolog of *Arabidopsis* floral organ development gene (*AP3*)	CRISPR-Cas9 double site knockout vector	Deletion	Male sterility	Liu et al., 2019
Soybean	*GmFT2a*	Integrons in the photoperiodic flowering pathway	CRISPR-Cas9 knockout vector	Insertion and deletion	Late flowering	Cai et al., 2018
Soybean	*GmSPL3*	Regulate soybean plant morphology	CRISPR-Cas9 four site knockout vector	Insertion or deletion causes an early termination of the translation	Leaf size smaller, node number reduced, node spacing shortened, and plant height decreased	Wu et al., 2019
Multiple gene co-knockout						
Rice	*DEP1*	Rice spike-type-related gene	CRISPR-Cas9 co-knockout vector	Gene co-knockout (mutants with various combinations of mutations)	Erect spike, increase or decrease in grain size of spike	Shen et al., 2017
	EP3				Large spike, Grain number per spike increased	
	Gn1a				Grain number per spike increased	
	GS3	Rice grain-type-related gene			Increase in grain length and 1,000-grain weight	
	GW2				Grain width and yield increased	
	LPA1	Rice plant-type-related gene			Plant type loose	
	BADH2	Rice fragrance gene			The grain has a unique fragrance	
	Hd1	Rice photoperiod-related gene			Show a delayed heading stage under short-day conditions	
Rapeseed	Two *BnaMAX1* homologous genes	Regulation of plant height and axillary bud growth	CRISPR-Cas9 co-knockout vector	Insertion, deletion, or substitution	Semi-dwarfing, branching increase and yield increase	Zheng et al., 2020
Brassica napus	*BnaBRC1*, *BnaBRC2*	Related to rice branch	CRISPR-Cas9 co-knockout vector	Gene knockout	Distinct multiple branches	Amoo, 2020
Wheat	*TaAQ*, *T aDq*	Not given	CRISPR-Cas9 co-knockout vector	Deletion	Changes in plant height of *TaDq* deficient plants, *TaAQ* knockout plants were more fragile, and *TaAQ* and *TaDq* co-knockout plants were more brittle	Liu et al., 2020a

(Continued)

TABLE 1 | Continued

Species	Target gene(s)	Gene function	CRISPR-Cas9 technical principle	Mutation mode	Mutant features	References
Gene knock-in						
Rice	*PPO1*	Herbicide resistance gene	CRISPR-Cas9 co-knock-up vector	Gene knock-up (chromosome fragment inversion)	Anti-herbicide	Lu et al., 2021
	HPPD	Herbicide resistance gene		Gene knock-up (chromosomal fragment duplication)		
Rice	Two genomic safe harbors	Synthetic carotenoids	CRISPR-Cas9 knock-in vector	Gene knock-in	Increased content of carotenoids	Dong et al., 2020
Gene regulation						
Arabidopsis	*AtFIS2*	*Arabidopsis* fertilization-independent seed2	CRISPR-dCas9-VP64 (transcription activator) vector	Gene silencing by demethylation of site C in the promoter CpG is lifted	Activated gene expression	Lowder et al., 2015
Strawberry	The uORF of *FvebZIPs1.1*	Encoding the basic (region) leucine zipper proteins	Base editor A3A-PBE-expression vector	Substitution	Strawberry sweetness increased	Xing et al., 2020
Rice	The 5′UTR intronic splicing site (5′UISS) of *Wx*^{a}	*Wx*^{a} encoding a granule bound NDP-glucose-starch glucosyltransferase	CRISPR-dCas9 vector	Insertion and deletion	Different starch content	Zeng et al., 2020

overexpression of *PPO1* and *HPPD*, which are two herbicide resistance genes in rice. However, there is randomness in the integration of target genes into the genome in traditional transgenic technology. Linjian Jiang's team improved the herbicide resistance of rice through CRISPR-Cas9 double-target editing. The first target is the highly expressed gene *CP12* near *PPO1* and its promoter, and *PPO1* gene was driven by the promoter of *CP12* by fragment inversion. The second target is the highly expressed gene *Ubiquitin2* near *HPPD* and its promoter, and the high expression of *HPPD* could be drove by the promoter of Ubiquitin2 through segment repetition (Lu et al., 2021).

Inserting the target gene into the appropriate genome site is an effective approach to get gene knocked-in. The traditional random integration of vectors into plant genome based on agrobacterium or particle bombardment sometimes reduces the crop yield. But CRISPR-Cas9 technology can be used to achieve targeted insertion at genome safe harbor (GSHs), thus avoiding the impact on crop yield. GSH is the region in chromosome that contains transgenes, which will not adversely affect the host organism due to genome destruction. Dong et al. (2020) used CRISPR-Cas9 technology to insert a 5.2 kb carotenoid biosynthetic cassette in rice genome safe harbor, and successfully obtained unmarked rice plants with high carotenoid content in seeds.

Applications in Gene Regulation

According to the process of gene expression, gene regulation can be divided into transcriptional regulation and post-transcriptional regulation, and transcriptional regulation can be divided into DNA regulation at the genetic level and chromatin regulation at the epigenetic level (Zhu et al., 2019). As one of the most flexible systems in genome regulation technology, CRISPR-Cas9 functions mainly through DNA regulation at genetic level.

By modifying the Cas protein, a protein with loss of nuclease activity and retention of DNA sequence recognition, namely dead Cas9 (dCas), was obtained (Liu et al., 2020c). The binding characteristics of dCas with double-strand target genes were similar to those of transcription factors regulating gene expression, but dCas cannot function alone. Therefore, researchers combined dCas with transcriptional activator protein/transcriptional suppressor protein to activate or inhibit gene transcription (Luo and Gu, 2016). In *Nicotiana benthamiana* leaves, dCas9 fused to the transcriptional activator EDLL controls the expression of different genes in leaves (Piatek et al., 2015).

Targeting sgRNA in promoter region can regulate gene expression more efficiently (Moradpour and Abdulah, 2020). For example, the transcriptional activator VP64 was fused with dCas9 and bound to C of CpG methylation site in *Arabidopsis thaliana* promoter region, which relieved the inhibition of CpG methylation on *AtFIS2* gene transcription (Lowder et al., 2015). In addition, alleles with different transcriptional and phenotypic characteristics were obtained by using eight gRNAs targeting cis-regulatory elements (CREs) in the CLV3 promoter region of tomato (Rodríguez-Leal et al., 2017).

The researchers also targeted sgRNA to open reading frames and splicing-related sites. The uORF of diploid strawberry

bZIP1.1 was edited by cytosine base editor (A3A-PBE), which improved the translation of primary ORF and increased sweetness in strawberries (Xing et al., 2020). Zeng et al. (2020) used CRISPR-dCas9 system to target the 5′ UTR intron splicing site (5′ UISS) of Wx^a gene, which controls starch synthesis, for post-transcriptional regulation to obtain mutants with different starch content, providing a new method for cultivating high-quality rice.

In addition, although dCas cannot play a role alone, it can cause steric hindrance. When the DNA sequence bound by dCas is the promoter or transcription start site of the target gene, it can prevent the initiation of transcription; when dCas binds to the reading frame of the target gene, it can prevent RNA polymerase binding, transcription factor binding, and transcription extension (Moradpour and Abdulah, 2020; Guan et al., 2021).

Besides, CRISPR-Cas9 also plays a role through epigenetic chromatin regulation, using dCas9 to bind DNA methylase and acetylase to modify genes, or changing chromatin structure to regulate gene expression by changing the interaction between enhancer and promoter (Moradpour and Abdulah, 2020; Zhu et al., 2020).

THE APPLICATIONS OF CRISPR-Cas9 TECHNOLOGY IN CROPS

Researchers are paying attention to CRISPR-Cas9 because of its high efficiency. A large number of experiments have proved that this technology is suitable for a variety of crops, such as rice, wheat, maize, soybean, and potato, and can achieve the expected experimental results, among which, rice is the most widely used. Crop improvement is usually carried out by increasing crop yield, improving crop quality, obtaining biological and abiotic resistance, and obtaining male sterile materials. **Table 2** summarizes the application of CRISPR-Cas9 in the crop improvement of rice, wheat, maize, soybean, and potato.

Rice

As one of the most important food crops, rice is the staple food for more than half of the world's population. It is expected that by 2030, Chinese population will reach 1.65 billion, with a food deficit of 140 million tons. It is urgent to improve rice yield and crop quality (Guo et al., 2019). As an efficient genome editing technology, CRISPR-Cas9 has made great contributions to rice improvement in recent years.

Rice germination rate, tiller number, panicle number per plant, grain number per panicle, and thousand grain weight are the key indicators to determine rice yield, which can be improved by editing-related genes. Gibberellin (GA) is a promoter of seed germination, which can relieve seed dormancy and stimulate germination, while DELLA protein is a key protein that inhibits the signaling pathway of GA. SLR1 is the DELLA protein in rice. Geng et al. (2021) used CRISPR-Cas9 gene editing technology to construct a rice *OsSLR1* knockout vector. Compared with wild-type Nipponbare rice, the *slr1* mutant has a significantly higher germination rate and faster growth rate 2 days after sowing. In order to solve the problem of uneven germination rate during rice planting, grain weight of rice is one of the three major factors affecting rice yield. Grain weight is determined by the synthesis and accumulation of starch in grains and the size of grains. It is a typical quantitative trait. *TGW6* gene is one of rice grain weight-related genes, encoding an indoleacetic acid-glucose hydrolase. The loss-of-function mutant can cause a decrease in the content of indoleacetic acid in the endosperm, thereby increasing the cell number, grain length, and grain weight, and carbohydrate accumulation before heading, thereby increasing rice yield. Wang et al. (2016a) took rice *TGW6* as the target gene and carried out three-point mutation through CRISPR-Cas9 technology. The mutation rate of T0 generation was 90%, and the thousand-grain weight of mutant offspring increased by 5%, laying the foundation for the cultivation of high-yield rice. The *FWL2* gene is widespread in plants and controls organ and fruit size by inhibiting cell division. Wang (2018) used CRISPR-Cas9 technology to design two target sites for *OsFWL2*, a gene that regulates grain number per panicle in Zhonghua 11, to construct knockout vectors, respectively, creating lines with significantly increased grain number per panicle and yield per plant, providing new germplasm for rice yield improvement.

Clustered regularly interspaced short palindromic repeats-Cas9 technology is used to increase the aroma of rice and control the synthesis of amylose and glutamate to improve the edible quality of crops. The main component of rice aroma is 2-acetyl-1-pyrroline (2-AP). *Badh2*, a recessive gene that controls aroma in rice, encodes an aldehyde dehydrogenase that oxidatively inactivates 2-AP synthesis precursors (Ren et al., 2021). Qi et al. (2020) used the CRISPR-Cas9 system to edit *OsBadh2* of Nipponbare, and successfully obtained rice mutants with increased 2-AP content and significantly improved aroma traits. Amylose content in rice is a key component affecting edible quality of rice. Rice *Waxy* genes (*Wx*) are the main genes controlling amylose content in rice. Granule-bound starch synthaseI (GBSSI) encoded by *Wx* gene directly controls synthesis of grain amylose. Feng et al. (2018) used Liu Yaoguang's multi-target intelligent editing system to edit the Wx locus in the rice excellent maintainer line 209B, and obtained the glutinous rice maintainer line WX209B with an amylose content of 1%, which was then transformed into the glutinous rice sterile line WX209A. The homozygous deletion mutation rate in the T0 generation was 26.9%, proving that the multi-target gene editing system can increase the homozygous mutation rate, and homozygous mutants can be stably inherited to offspring. The *AAP* gene family encodes amino acid permease. *AAP6* gene can not only enhance the expression of key genes of storage proteins, but also promote the absorption of amino acids by plants and increase the protein content. High grain protein content (GPC) decreases eating and cooking quality (ECQ) of rice. Using the CRISPR-Cas9 system to mutagenize the amino acid transporter genes *OsAAP6* and *OsAAP10*, in four varieties with different genetic backgrounds, the content of glutamate protein can be reduced, thereby improving the quality of rice. The GPC of T1 generation decreased by 2.9–19.1%,

TABLE 2 | Applications of CRISPR-Cas9 technology in crops.

Species	Target gene	Gene function	CRISPR-Cas technical principles	Mutation mode	Mutant features	References
Rice						
	OsSLR1	Encoding DELLA protein, inhibit gibberellin synthesis and seed germination	CRISPR-Cas9 three-site knockout vector	Gene knockout	The germination rate was increased	Geng et al., 2021
	TGW6	1,000-grain weight related gene in rice	CRISPR-Cas9 three-site knockout vector	Insertion or deletion	1,000 grain weight increased by 5%	Wang et al., 2016a
	OsFWL2	Gene regulating grain number per spike in rice	CRISPR-Cas9 double-site knockout vector	Insertion or deletion	Spike number and single-strain yield were increased	Wang, 2018
	Hd2/Os PRR37 Hd5/DTH8/Ghd8 Hd4/Ghd7	Main effect gene in rice heading stage	CRISPR-Cas9 co-knockout vector	Co-knockout (base substitution, insertion or deletion)	Precocious, scented rice	Zhou, 2017
	Badh2	Rice fragrance gene				
	Wx	Negative regulation of amylose content	CRISPR-Cas9 knockout vector	Gene knock-out	Amylose content was significantly reduced in non-waxy sterile lines	Feng et al., 2018
	OsAAP6, *OsAAP10*	Amino acid transporter genes	Construct CRISPR-Cas9 knockout vectors respectively	Single-base insertions or deletions, and large fragment were absent	Glutamate content, high grain protein content were all decreased, and amylose content were significantly reduced	Wang et al., 2020c
	Pita	Negative regulating the resistance of rice blast	CRISPR-Cas9 co-knockout vector	Insertion and deletion	The resistance to rice blast was increased, and the expression of genes associated with the signal transduction pathway such as salicylic acid, jasmonic acid, and ethylene was upregulated in the homozygous mutant strains	Xu et al., 2019
	Pi21	Recessive rice blast resistance gene		Insertion, deletion, and substitution		
	ERF922	Negative regulating the resistance of rice blast		Insertion, deletion, and substitution		
	HW3	Susceptibility gene of bacterial blight of rice	CRISPR-Cas9 knockout vector	Gene knockout	Enhanced resistance to bacterial blight of rice	Hao, 2016
	Os8N3	Encodes a member of the sugar transporter family, and is a Xanthomonas susceptibility gene	CRISPR-Cas9 knockout vector	Gene knock-out	Sucrose concentration in embryo sac decreased, grain filling defects, resistance to Xanthomonas	Kim et al., 2019
	AFP1	Abiotic stress related gene in rice	CRISPR-Cas9 double-site knockout vector	Small fragments deletion, and substitution	The ability of cold resistance, heat resistance, and osmotic stress was improved	Zhou et al., 2021b
	OsALS	Encodes acetolactate synthase	CRISPR-Cas9 knockout vector	Substitution	Plant height became shorter and herbicide resistance increased	Wang et al., 2020a
			CBE	Base substitution (C-T)	Tolerant to IMI herbicide	Wang et al., 2019
	OsNramp5	Key ion transporters of Mn, Cd, and Fe absorption in rice roots	CRISPR-Cas9 knockout vector	Gene knock-out	Low cadmium	Long et al., 2019
	PTGMS2-1	Male fertility genes	CRISPR-Cas9 knockout vector	Insertion and deletion	Photoperiod/heat sensitive male sterility	Lan et al., 2019
	TMS5	Temperature sensitive male sterility gene	CRISPR-Cas9 knockout vector	Insertion and deletion	Thermosensitive male sterile rice, the starting temperature of thermosensitive sterility of T2 generation TMS lines was about 24°C	Wu et al., 2018
	ZEP1	Encoding the central element of the meiotic association complex	CRISPR-Cas9 knockout vector	Gene knockout	Male sterility, female fertility is normal, genetic recombination efficiency was greatly increased, and genetic interference was completely eliminated	Liu et al., 2021a
Wheat						
	TaGASR7	Negative regulation of grain length and grain weight genes	Transient expression of CRISPR-Cas9 DNA or RNA	Insertion and deletion	1,000 grain weight increased	Zhang et al., 2016

(Continued)

TABLE 2 | Continued

Species	Target gene	Gene function	CRISPR-Cas technical principles	Mutation mode	Mutant features	References
	Wheat-glial gene	Genes associated with glutelin synthesis	CRISPR-Cas9 knockout vector	Insertion and deletion	Low in glutelin, non-transgenic wheat	Sánchez-León et al., 2017
	Ta SBEIIa	Genes related to starch synthesis in wheat	CBE based on CRISPR-Cas9	C-T single base substitution	Nutrition content such as amylose, resistant starch, protein, and soluble pentosan were significantly improved	Li, 2021a
	MLO	The powdery mildew susceptibility gene	CRISPR-Cas9 knockout vector	Insertion	Resistance to powdery mildew	Wang et al., 2014
	Three homologs of *EDR1*	A negative role in the defense response to powdery mildew	CRISPR-Cas9 knockout vector	Gene knock-out	Resistance to powdery mildew	Zhang et al., 2017
	Three *TaNP1* homologous alleles	Probably encoding a glucose-methanol-choline oxidoreductase, it is necessary for male sterility	CRISPR-Cas9 knockout vector	Triple gene knockout	Triple homozygous mutants were completely male sterile	Li, et al., 2020b
Soybean						
	GmNARK	A receptor kinase that inhibits the expression of nodule formation related genes	CRISPR-Cas9 three-site knockout vector	Insertion and deletion, resulting in early termination of translation	Supernodules, short plants, and dark green leaves	Bai et al., 2019
	GmFAD2-1A	Key enzyme that catalyze the conversion of oleic acid to linoleic acid	CRISPR-Cas9 three-site knockout vector	Deletion and substitution	The oleic acid content was significantly improved	Hou et al., 2019
	GmFATB1a, *GmFATB1b*	Encoding a thioesterase, is a key enzyme for fatty acid synthesis	CRISPR-Cas9 co-knockout vector	Deletion	The content of saturated fatty acid was reduced and the double mutants showed male sterility	Jing et al., 2021
	GmSnRK1.1, *GmSnRK1.2*	Participate in anti-stress pathways	CRISPR-Cas9 co-knockout vector	Deletion	Reduced sensitivity to abscisic acid	Li et al., 2018
Maize						
	LIG	Liguleless1	(1) CRISPR-Cas9 knockout vector	Insertion	Not given	Svitashev et al., 2015, 2016
	MS26	Male fertility genes		Insertion and deletion	Not given	
	MS45		(2) SNP based on CRISPR-Cas9	Insertion and deletion	Not given	
	ALS1	Encodes acetolactate synthase		Insertion and deletion	Not given	
	ALS2			Insertion and deletion	Resistant to chlorsulfuron	
	The 5′ untranslated section of *ARGOS8*	Negative regulator of ethylene reaction	CRISPR-Cas9 knock-in vector	Promoter insertion	Yields increase under drought conditions	Shi et al., 2017
	ZmBADH2-1, *ZmBADH2-2*	Homologous genes that control fragrance genes	CRISPR-Cas9 co-knockout vector	Insertion, deletion, and substitution	The grains have a fragrant rice flavor	Zhang et al., 2017
	MS8	Male fertility genes	CRISPR-Cas9 knockout vector	Substitution	Male sterile	Chen et al., 2018
Potato						
	GBSS	Encoding granular bound starch synthase	CRISPR-Cas9 four-site knockout vector	Insertion and deletion	Amylose content increased significantly in homozygous mutant plants	Andersson et al., 2017
	StSSR2	Encoding the sterol side chain reductase 2 genes, a key gene for the synthesis of solanine	CRISPR-Cas9 co-knockout vector	Insertion, deletion, and substitution	Low solanine, low temperature glycosylation resistance, and high amylopectin	Zhao, 2019
	VInv	Acid vacuolar invertase that plays a key role in low temperature saccharification				
	GBSSI	Grain binding starch synthase gene associated with amylose synthesis				
	S-RNase	Participates in the control of gametophyte self-incompatibility	CRISPR-Cas9 knockout vector	Early termination of translation	Self-compatible diploid lines of potato	Enciso-Rodriguez et al., 2019

and the GPC of T2 generation decreased by 1.5–10.4% (Wang et al., 2020c).

Rice blast and bacterial blight of rice are two major diseases leading to rice yield reduction, and CRISPR-Cas9 was used to obtain disease-resistant rice by editing susceptible genes. The *OsERF922* gene encodes an AP2/ERF-like transcription factor that is strongly induced by both pathogenic and non-pathogenic rice blast fungi and negatively regulates rice blast resistance. Wang et al. (2016b) used CRISPR-Cas9 to target and mutate the rice ERF transcription factor gene *OsERF922* to enhance rice blast resistance and obtain genetic materials without changes in main agronomic traits, providing new germplasm resources for rice breeding. The *Pi21* gene is an invisible rice blast resistance locus, and its expression product is rich in proline. The reason of disease resistance is that there is a small fragment deletion in the proline region. The substitution of 918 amino acids of the protein encoded by the *Pita* gene leads to rice blast resistance. Xu et al. (2019) used CRISPR-Cas9 technology to construct a co-editing vector with *Pita*, *Pi21*, and *ERF922* as target genes. The triple mutant homozygous line with enhanced rice blast resistance was successfully obtained, which has high utilization value. Rice bacterial blight caused by Xanthomonas oryzae pv. Oryzae (Xoo) leads to a 50% reduction in rice yield. Hao (2016) used CRISPR-Cas9 technology to identify the bacterial blight susceptibility-related gene *HW3* in rice. The first exon was modified by site-directed modification, and rice blast resistant plants were successfully obtained. The results of field inoculation phenotype showed that it inhibited the early development of pathogen Xoo, but after inoculation with pathogen 15, the lesions appeared to grow, so it was not the main susceptibility genes. The pathogenicity of Xoo relies on a class of effectors called transcription activator-like (TAL) effectors, *Os8N3* gene belongs to the sugar final export sugar transporter (sweet) family and is one of the susceptibility genes induced by TAL effectors. Os8N3 can scavenge toxic copper in xylem vessels where Xoo proliferates and spread, and makes Xoo readily available nutrients for its growth and toxicity to cause disease. Kim et al. (2019) used CRISPR-Cas9 technology to target mutant rice *Os8N3*. A mutant strain resistant to Xanthomonas oryzae was obtained, and agronomic traits including pollen fertility were hardly affected, indicating that this method can be successfully used for crop breeding.

Drought, salt, weeds, and heavy metals have always been the resistance to the growth and development of rice. CRISPR-Cas9 has great significance in improving the abiotic resistance of rice. Due to the global climate change, the frequency of drought is increasing, and drought has become an important abiotic stress of rice yield fluctuation (Rao et al., 2020). Zhou et al. (2021b) used CRISPR-Cas9 technology to knock out the *AFP1* gene to improve the drought tolerance, heat tolerance, and osmotic stress ability of rice. On the one hand, the root length was significantly increased, and on the other hand, the sensitivity to abscisic acid and the water loss rate of leaves were reduced. Other agronomic traits changed, plant height and seed setting rate decreased, effective tillering increased, panicle length increased significantly, and yield per plant varied between 4.06 and 11.75%. The *OsALS* gene encodes acetolactate synthase, and existing studies have shown that mutations in the *ALS* gene are tolerant to the herbicide imidazolinone (IMI). Current studies have shown that loss-of-function mutations in *ALS* genes can be lethal, so it is very important to obtain non-function-free genes with *als* mutations. Wang et al. (2019) optimized the CBE system to obtain base editors with improved editing efficiency. Successful substitution of C-T at position 1882 of the *ALS* gene, the mutation efficiency is as high as 71.4%, and the mutant strain is tolerant to IMI herbicide. Wang et al. (2020a) took the acetolactate synthase (*OsALS*) gene as the target and used CRISPR-Cas9 technology to mutate *OsALS*, the C-T substitution at nucleotide 1882 to form a new rice herbicide resistance allele *G628W*, increased the herbicide resistance of rice, and the feasibility of creating new genetic variation for crop breeding. The non-transgenic progeny with the homologous *G628W* allele exhibited similar agronomic characteristics to wild-type plants in addition to reduced plant height. Rice is a cadmium-rich plant. Excessive intake of cadmium will seriously endanger human health. The *OsNramp5* gene encodes a manganese and iron transporter, which is the main protein for cadmium absorption in roots, and its loss can significantly reduce the content of cadmium in grains. Long et al. (2019) constructed a CRISPR-Cas9 knockout vector for *OsNramp5* gene, and successfully created low-cadmium indica rice. The cadmium content of four *OsNramp5* gene knockout lines grown under non-cadmium pollution conditions was lower than 0.02 mg/kg, which was on average higher than that of wild-type. When planted in cadmium-contaminated soil, the grain cadmium content of *OsNramp5* knockout lines of different varieties was lower than 0.1 mg/kg, an average reduction of 94.8% compared with wild-type.

Male sterile lines are the core of two-line hybrid rice breeding. Traditional hybrid breeding methods have long breeding cycle and high labor intensity. Non-transgenic male sterile lines can be obtained by using CRISPR-Cas9 technology to knock out rice fertility genes and after 1–2 generations of breeding, providing excellent materials for rice breeding (Wu et al., 2018). Lan et al. (2019) took 93–11 and Huazhan's male fertility gene *PTGMS2-1* gene as the target gene, used CRISPR-Cas9 technology to construct an expression vector, and successfully obtained photoperiod/heat-sensitive gene male sterile rice. Almost completely sterile, with increased tiller number and significantly lower plant height compared to WT, with basically normal agronomic traits. Using rice *TMS5* as the target gene, Wu et al. (2018) constructed the CRISPR-Cas9 gene editing vector, transformed the excellent intermediate material GH89, and successfully obtained thermo-sensitive two-line sterile plants. Liu et al. (2021a) constructed the *ZEP1* gene knockout vector (encoding the central element of meiosis synapsis complex) in rice using CRISPR-Cas9 technology, and obtained mutant zep1 with the male sterile and female fertile. Genetic analysis showed that in the absence of *ZEP1*, the efficiency of genetic recombination was greatly increased, and the genetic interference was completely eliminated. These results indicated that synapsis played a role in regulating rice disturbance. At the same time, the successful acquisition of this mutant provides a new method for increasing genetic diversity in crop breeding.

Wheat

Wheat is the second largest food crop in the world. Increasing wheat production is also an important way to alleviate population growth and environmental degradation. Wheat is allohexaploid with a huge genome, and this characteristic increases the difficulty of breeding. But the high specificity and high efficiency of the CRISPR-Cas9 system reduces the difficulty of this. In recent years, researchers have obtained stable inherited mutants with excellent traits, applying CRISPR-Cas9 technology to wheat (Wang et al., 2014).

Similar to cereal crop rice, genes related to thousand-grain weight of wheat are the key to affecting wheat yield. Zhang et al. (2016) used *TaGASR7* as the target gene to bombard the callus of hexaploid bread wheat and tetraploid durum wheat through transient expression of CRISPR-Cas9 DNA or RNA, and obtained T0 mutants with increased thousand-grain weight.

High-glutelin wheat can cause diseases such as celiac disease in susceptible people. Editing genes related to glutelin synthesis is an effective way to reduce glutelin content. Based on CRISPR-Cas9 technology, Sánchez-León et al. (2017) constructed pANIC-CR-*Alpha1* and pANIC-CR-*Alpha2* expression vectors by using the conserved region near the coding sequence of the immune dominant epitope of wheat glutelin as target sites, they successfully obtained low-protein, non-transgenic wheat through transformation. Resistant starch is a kind of starch that is difficult to digest, absorb, and enter the bloodstream. It has the functions of controlling weight, lowering blood sugar, and promoting the absorption of vitamins and minerals (Yang et al., 2020). *SBE* is a key gene for amylopectin synthesis. The synthetic pathway of resistant starch is similar to that of amylose, and there is a significant positive correlation. Inhibiting the expression of *SBE* gene will increase the synthesis of resistant starch. Using CRISPR-Cas9 technology to construct rice gene C-T single base editing vector, the starch branching enzyme gene *TaSBEIIa* was knocked out, and a new high-resistant starch winter wheat germplasm was obtained (Li, 2021a).

Powdery mildew is a major fungal disease of wheat crops, causing severe yield losses in wheat. Mildew resistance locus O (*MLO*) genes are plant-specific family genes, and *mlo* mutants have broad resistance to powdery mildew fungi. Wang et al. (2014) used the CRISPR-Cas9 system to realize the directed mutation of the wheat powdery mildew susceptibility gene *MLO*, and successfully created wheat with long-lasting and broad-spectrum resistance to powdery mildew, which will provide an important starting material for the disease resistance breeding of wheat powdery mildew. Zhang et al. (2017) used CRISPR-Cas9 technology to simultaneously modify three homologs of the wheat *EDR1* (enhanced disease resistance1) gene to obtain a mutant strain resistant to powdery mildew without mold-induced cell death. Therefore, this potentially valuable traits generated by CRISPR-Cas9 technology may provide new germplasm for disease resistance breeding.

In hybrid seed production, the male sterile line of wheat can be used as the female parent to improve the seed purity of excellent offspring and overcome the difficulty of artificial pollination. Wheat is allohexaploid, so it is difficult to obtain nuclear recessive male sterile mutants with traditional methods. The CRISPR-Cas9 system can realize the simultaneous mutation of multiple homologous alleles and obtain male sterile plants (Sun, 2019; Li et al., 2020b). The homologous genes of *TaNP1* gene are *OsNP1* gene and *ZmIPE1* gene in rice and maize, respectively, both of which encode a glucose-methanol-choline oxidoreductase. The *osnp1* mutant and *zmipe1* mutant showed complete male sterility. Li et al. (2020b) used CRISPR-Cas9 technology to edit three *TaNP1* homologous alleles to obtain a completely male sterile *TaNP1* triple homozygous mutant wheat.

Soybean

Soybean is an important oil crop and the main source of human plant protein, which has important economic value. With the increase in the world's population and the improvement of human living standards, it is of great significance to use CRISPR-Cas9 technology to quickly cultivate high-yield and high-quality soybean varieties.

Rhizobium is a symbiosis of legumes and rhizobia, which can fix nitrogen in the atmosphere and provide nitrogen nutrition for plant growth. The biological nitrogen fixation of soybeans provides an important guarantee for the sustainable development of agriculture. However, nitrogen fixation is an energy-consuming process, too many nodules will become a burden on soybeans, so the number of nodules is regulated by the nodulation autonomous regulatory mechanism. *GmNARK* gene encodes a receptor kinase in the regulatory pathway. Using CRISPR-Cas9 technology, three sgRNAs were designed to knock out the *GmNARK* gene, and the mutants with super nodulation, short plant and dark green leaf phenotype were successfully obtained, which provided genetic material for studying the mechanism of nodulation and nitrogen fixation in soybean, and provide new germplasm resources for soybean molecular breeding (Bai et al., 2019).

Using CRISPR-Cas9 technology to knock out genes related to the synthesis of soybean protein, oleic acid, and saturated fatty acids is the key to obtaining high-quality soybean oil (Cai, 2016). Oleic acid is an unsaturated fatty acid, which has the effect of lowering cholesterol, slowing down atherosclerosis and preventing cardiovascular disease, so cultivating soybeans with high oleic acid content has a very important use value. Delta-twelve fatty acid desaturase 2 enzyme (FAD2) is a key enzyme that catalyzes the formation of linoleic acid from oleic acid. Inhibiting the *FAD2-1A* gene can increase the ratio of oleic acid/linoleic acid in soybean seeds. Targeting the soybean oleic acid gene *GmFAD2-1A*, Hou et al. (2019) constructed a CRISPR-Cas9 editing vector fused with three gRNAs, and increased the oleic acid content in soybean seeds of Huaxia 3 from 20 to 23%, without significantly affecting agronomic traits including total protein and total fat content in soybean seeds. Excessive intake of saturated fatty acids in humans can cause elevated cholesterol levels and increase the risk of atherosclerosis. FATB protein is a thioesterase that has the function of releasing free fatty acids and ACP, and the released fatty acids are involved in fatty acid chain elongation. Jing et al. (2021) used CRISPR-Cas9 technology to knock out the *GmFATB1* gene encoding the FATB protein, which significantly reduced the content of two saturated fatty acids in soybean seeds, providing excellent materials for soybean breeding.

Abiotic stress is an important factor affecting soybean yield, and it is of great significance to knock out soybean abiotic stress-sensitive genes. Sucrose non-fermenting related protein kinases (SnRKs) are a class of Ser/Thr protein kinases that are widely present in plants and play important regulatory roles in plant growth, development, metabolism, and stress resistance. *GmSnRK1.1* and *GmSnRK1.2* are two homologous genes of *SnRK1*. Li et al. (2018) used *GmSnRK1.1* and *GmSnRK1.2* as targets to construct a CRISPR-Cas9 double gene knockout vector. The double gene mutation rate was 48.6%, and mutants have reduced sensitivity to abscisic acid.

Maize

Maize is the third largest cereal crop after rice and wheat with a wide planting area. New varieties of maize accounted for more than 35% in various factors of increasing yield. Compared with traditional breeding, CRISPR-Cas9 technology has strong specificity, short breeding cycle and other advantages, which greatly promotes the creation of excellent maize varieties (Zhu, 2015).

Clustered regularly interspaced short palindromic repeats-Cas9 technology is used to improve the abiotic stress resistance of maize. Svitashev et al. (2015) used CRISPR technology to construct an editing vector for the maize *ALS2* gene encoding acetolactate synthase and successfully obtained chlorsulfuron-resistant plants. Drought is an important factor affecting the yield of maize. Shi et al. (2017) used the CRISPR-Cas9 system to insert the GOS2 promoter into the 5′untranslated region of *ARGOS8* gene by targeting the promoter of *ARGOS8* gene, a negative regulator of ethylene reaction in maize, and obtained drought-resistant maize varieties with improved yield.

In the face of human demand for maize edible quality, CRISPR-Cas9 technology is used to edit maize flavor-related genes to obtain fragrance maize. *BADH2* gene encodes betaine aldehyde dehydrogenase. The mutation of *BADH2* can inhibit the synthesis of aroma substance 2-AP. *ZmBADH2-1* and *ZmBADH2-2* genes are the homologous genes of *BADH2* gene in maize. Zhang et al. (2021) constructed CRISPR-Cas9 co-knockout vectors of *ZmBADH2-1* and *ZmBADH2-2* genes, and successfully created maize varieties with fragrant rice flavor in grains. Whether the mutation of this gene affects other agronomic traits remains to be further studied.

Male sterile lines are important materials for the breeding of many crops. CRISPR-Cas9 technology has shown great potential in breeding male sterile lines. Svitashev et al. (2016) designed specific gRNAs based on the CRISPR-Cas9 system for maize liguleless1 (*LIG*), acetolactate synthase gene (*ALS2*), and two male fertility genes (*MS26* and *MS45*). Then, maize embryo cells were bombarded with particles by ribonucleo protein complex, which assembled from Cas protein and gRNA. Compared with DNA delivery experiments, it was found that the mutation frequency and mutation types of the two were similar, and the off-target rate of RNP delivery and the homozygous mutation rate was also significantly reduced. Chen et al. (2018) constructed a CRISPR-Cas9 vector for the maize MS8 gene and transformed it. The *MS8* gene was not detected in the T0 transgenic lines obtained, but the *MS8* gene could be detected in the F1 and F2 generations. They screened the male sterile plants. In the F2 generation, a transgenic-free *ms8* male sterile plant was successfully obtained, which can be used in a maize hybrid production system without being restricted by the regulatory framework of genetically modified organisms (GMO). In addition, there was an off-target site in this study, but it did not cause adverse effects on plants.

Potato

Potato is an important food crop in China, not only as a vegetable consumption, but also an important raw material for starch production. However, due to the complex potato genetic background, self-incompatibility characteristics, and long traditional hybrid breeding cycle, low predictability, new variety breeding process is slow, using CRISPR-Cas9 technology can effectively accelerate the breeding process (Li, 2019).

Changes in the ratio of amylose and amylopectin in potatoes will greatly change the properties of starch. Changing the ratio of potato starch or containing only one starch will gain advantages in many applications (Zeeman et al., 2010). Andersson et al. (2017) targeted potato grain binding starch synthase (*GBSS*) gene and constructed targeted knockout vector using CRISPR-Cas9 system. The results showed that only when all four alleles of *GBSS* were mutated, the amylose content in the corresponding positive plants decreased significantly compared with the wild-type, while the amylopectin content increased. However, the homozygous mutant with increased amylopectin content also has the problem of unstable DNA integration and needs to obtain stable genetic offspring through hybridization. Zhao (2019) targeted three genes in potatoes: Sterol side chain reductase 2 gene (*St SSR2*), acid vacuole invertase gene (*VInv*), and grain binding starch synthetase gene (*GBSSI*), were simultaneously knocked out by CRISPR-Cas9 technique. A new potato strain with low solanine resistance, low-temperature saccharification resistance, and high amylopectin resistance were developed. The knockout of *St SSR2* has no effect on the phenotype and physiological indexes of potato. After the complete knockout of *VInv*, the reducing sugar content of potato decreases. After the knockout of *GBSSI* gene, the amylose content is completely suppressed, there was no obvious effect on the type, physiological index and tuber yield, and it was basically suitable for field cultivation.

The tetraploid character of potato can hinder the breeding process, so researchers tried to invent diploid inbred lines, but due to the diploid gametophyte self-incompatibility, prevented the production of diploid homozygous lines. The S locus RNase (*S-RNase*) gene is associated with gametophyte self-incompatibility, Enciso-Rodriguez et al. (2019) successfully obtained a stable self-compatible potato diploid lines by targeting *S-RNase* gene with CRISPR-Cas9 system, which greatly promoted potato breeding.

PROBLEMS WITH CRISPR-Cas9

Technical Problems

Off-Target Effect

The off-target effect of CRISPR-Cas9 technology is caused by random cutting of non-target sites in the genome by Cas9. There are two main reasons for the off-target effect.

Firstly, sgRNA may bind to untargeted sequences. The CRISPR-Cas9 gene editing system relies on the specific binding of the recognition sequence of sgRNA with the targeted sequence. However, due to the extreme complexity of the genome, sgRNA designed according to the target site may match with other sequences similar to the target sequence. This local match also activates Cas9 endonuclease activity, resulting in off-target effect.

Secondly, Cas9 may recognize non-standard PAM. CRISPR-Cas9 gene editing system relies on Cas9 to cut three bases upstream of PAM site. However, the actual situation is that Cas9 can not only recognize the standard PAM near the target site, but also recognize the non-standard PAM. If non-standard PAM is ignored when designing the targeted sequence, it may also cause a certain degree of off-target (Yuan et al., 2017).

Exogenous Genes

Editing vectors carrying Cas9 and sgRNA usually infect plants through agrobacterium-mediated transformation. Some vector fragments may be randomly integrated into the plant genome, thus introducing foreign genes. The existence of foreign components may cause unexpected biosafety problems (Bao et al., 2019). For sexually reproducing crops like rice, plants without exogenous components can be screened from progeny by genetic separation of sexual generations. However, crops that do not undergo sexual reproduction, such as potato, cannot isolate and remove foreign genes through genetic separation, so it is impossible to avoid the residue of foreign gene components (Yao et al., 2017).

Therefore, the elements of editing vector can be loaded in the form of ribonucleoprotein, which can be applied to crops without genetic separation of sexual generations to avoid the introduction of foreign genes at the beginning (Svitashev et al., 2016; Zhen et al., 2017). However, this method is difficult to construct and operate, so it needs to be optimized to improve its efficiency and adapt to more crops. He and Zhao (2020) introduced several methods for obtaining transgenic-free plants for asexual plants, such as nbonucleoprotem transfection, transient expression of transgenes without DNA integration, and nano-biotechnology.

Recently, Liu et al. (2021b) developed a web tool for foreign element detection (FED) of genome-edited organism. It can complete the sequence detection of 46,695 exogenous components at one time according to the whole genome sequencing data when the information of exogenous vector components is unknown. Meanwhile, the FED can accurately identify the fragment length and insertion position of exogenous components in the genome. It provides an efficient tool for biosafety detection of genome editing products.

Regulatory Issues

At present, the crop regulation problem caused by CRISPR-Cas9 technology is due to the introduction of foreign genes when CRISPR-Cas9 technology is applied in crops, resulting in unpredictable biosafety. From a scientific point of view, CRISPR-Cas9 technology realizes mutation through cell self-repair, which is very similar to natural mutation, physical mutagenesis, and chemical mutagenesis of crops, but the essence cannot be judged (Wang and Zhao, 2018). There are differences in the regulatory policies of CRISPR-Cas9 edited plants around the world.

Compared with the strict and clear regulatory policies for GMO, countries around the world lack clear and consistent supervision policies for CRISPR-Cas9 edited plants, and there are also disputes over regulatory standards. The United States government takes the final product as the regulatory object and follows the "principle of case analysis" for the supervision of CRISPR-Cas9 editing crop products, which is cooperatively regulated by the United States Department of Agriculture, the Environmental Protection Agency and the Food and Drug Administration. At present, a variety of CRISPR-Cas9 editing crops have passed the review of the three departments, exempting from the regulation of GMO (Pan et al., 2021).

EU countries proposed to take process as the supervision object for CRISPR-Cas9 products. As long as transgenesis is involved in the development process, they should be strictly regulated regardless of the existence of the exogenous components in the final plants. In 2018, the European Court of Justice ruled that Gene-edited crops should follow GMO regulatory procedures (Wang et al., 2020b).

Regulation three of the Regulations on the Safety Management of Agricultural Gmos, promulgated in 2001 and revised in 2017, stipulates that "agricultural genetically modified organisms as mentioned in this regulation refer to the animals, plants, microorganisms, and their products used in agricultural production or agricultural products processing, which has been changed in the composition of the genome by using the genetic engineering technology." Gene editing technology is one of the genetic engineering technologies. Therefore, it seems that gene editing crops should be supervised like the genetically modified organisms, but there is no explicit stipulation on how to supervise gene editing crops. Moreover, some Chinese scholars believe that the regulation of CRISPR-Cas9 editing crops without exogenous DNA such as SDN-1 according to traditional genetically modified organisms is likely to hinder the development of this technology (Fan et al., 2020). In recent years, Chinese scientists Gao Caixia, Academician Cao Xiaofeng, Academician Liu Yaoguang, Academician Zhu Health, and Li Jiayang have put forward reasonable suggestions on the supervision of gene editing crops through the media or papers (Wang et al., 2020b).

THE PROSPECT OF CRISPR-Cas9

Since the discovery of regularly spaced repeats in 1978, the mechanism of action of the CRISPPR/Cas9 system has been elaborated in 2012, and CRISPPR/Cas9 has gradually penetrated into the field of biology. As a sequence-specific nuclease, after continuous development and improvement by scientists, it basically surpasses ZFNs and TALENs in terms of application efficiency and extensiveness. The main advantages of the CRISPPR-Cas9 system are its strong specificity, high efficiency,

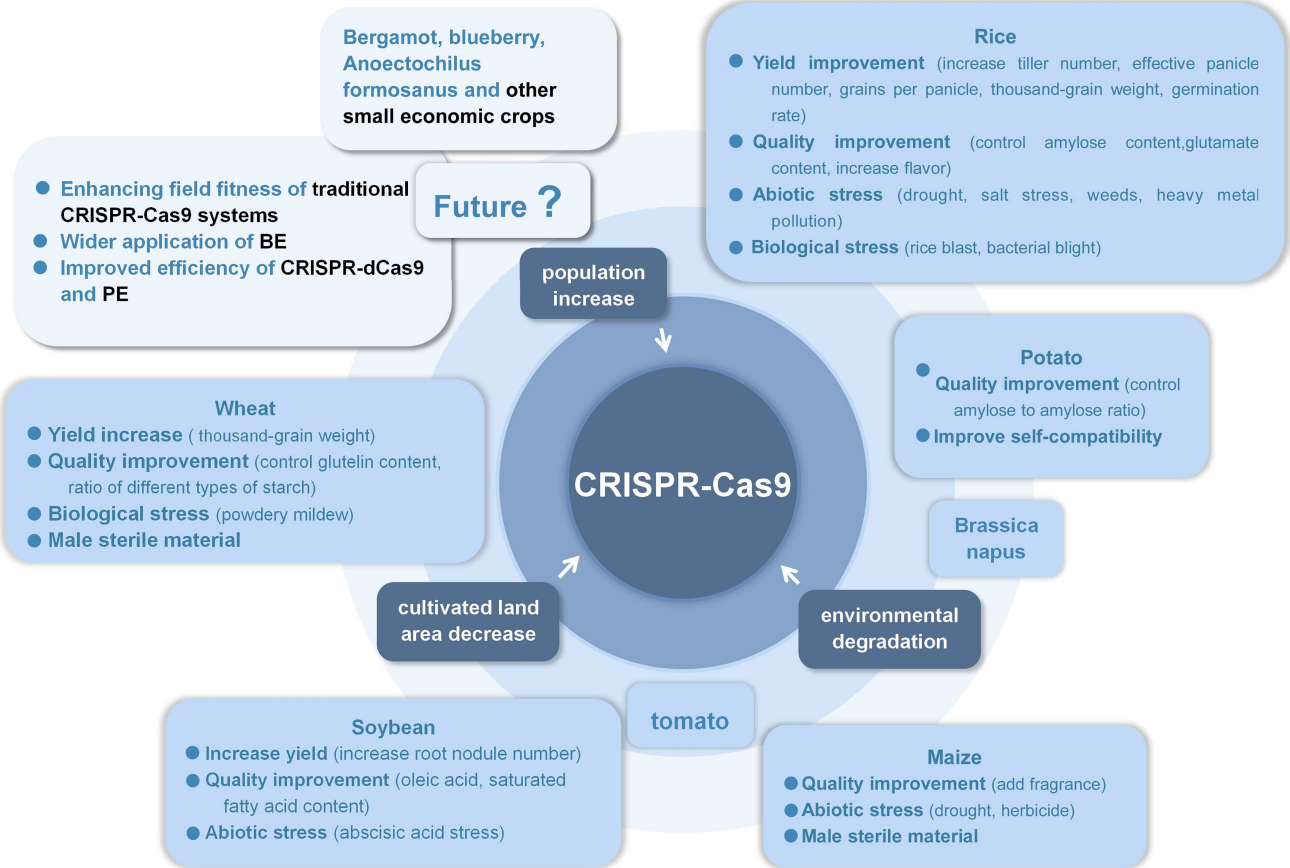


FIGURE 1 | The prospects of CRISPR-Cas9 in crop improvement.

simple design, low cost, and the ability to edit multiple genes simultaneously. It is precisely because of the powerful advantages of CRISPPR-Cas9 that the system has attracted widespread attention from biological researchers around the world as soon as it came out. Therefore, in the 10 years from 2012 to 2022, the CRISPPR-Cas9 system has been rapidly developed and expanded. In addition to the traditional CRISPR-Cas9 system based on DNA double-strand breaks, there are base editors and prime editors based on single-strand breaks, as well as CRISPR-dCas9 systems that do not cause DNA breaks.

Traditional CRISPPR-Cas9 system has been successfully used in tens of crop improvement: (1) model plant *Arabidopsis thaliana* and tobacco, (2) food crops like rice, wheat, maize, sorghum, potato, and cassava, (3) oil crops like soybean, peanut, rapeseed, and sesame, (4) vegetable crops like tomato, cabbage, carrot, mushroom, and luffa, (5) fruit crops like apple, banana, pear, grape, strawberry, citrus, sweet orange, and litchi, (6) tree crops like cotton, poplar, larch, tea, rubber tree, coffee, and cocoa, and (7) flowers and herbs like petunia, chrysanthemum, lily, phalaenopsis, torenia fournieri, hemerocallis, liverwort, alfalfa, and panax, which reflects the pretty wide applicability of this technique (Liu et al., 2017, 2021c; Yao et al., 2017; Shi et al., 2018; Chen, 2020; Huang et al., 2020; Li and Xiao, 2020; Liu, 2020; Lv et al., 2020; Situ et al., 2020; Cao, 2021; Chen et al., 2021; Huang, 2021; Kan et al., 2021; Li, 2021b; Liao et al., 2021; Wang et al., 2021; Yang, 2021; Zhou et al., 2021a). **Figure 1** summarizes the past applications of CRISPR-Cas9 technology and prospects its potential applications in the future.

Especially in the application of important crops such as rice, wheat, corn, soybean, and potato, it has been widely used in increasing yield, improving crop quality, obtaining biological resistance and abiotic resistance, and obtaining male sterile materials, etc. The traditional double-strand break-dependent CRISPR-Cas9 system significantly improved most traits. On the one hand, the application of these excellent varieties created in crop breeding should be strengthened to make them suitable for field planting and effectively reduce the burden on farmers. On the other hand, applying the huge application potential of CRISPR-Cas9 technology to more crops, such as bergamot, clematis, blueberry, and other small commercial crops, first, it can increase the yield and meet the greater market demand. Second, it can improve the taste and nutritional value to meet the quality requirements of human beings. In addition, it can improve the abiotic resistance of crops and cultivate different varieties for the same crop to adapt to various

climatic conditions, so as to reduce transportation costs and reduce the price of rare crops.

As a tool for accurate gene regulation, the CRISPR-dCas9 system has great potential for development, and has already been used in *Arabidopsis*, rice, and tobacco. However, there is still a problem of low efficiency, which needs to be further optimized by researchers.

As a precise base-changing system, BE have created varieties with improved traits in crops such as rice, wheat, *Arabidopsis*, and rapeseed. The application of herbicide resistance is particularly prominent. *ALS* genes in rice, wheat, tomato, potato, and watermelon were edited to make crops tolerant to IMI.

As PE can perform any kind of substitution to the four bases, it has great application potential. At present, guided editing has been completed in rice and wheat, but faced with the challenge of low editing efficiency. At present, researchers are constantly optimizing the guided editing system in order to achieve higher editing efficiency in the future.

Although CRISPR-Cas9 technology faces challenges such as introducing foreign genes and causing off-target effects, these challenges have been gradually overcome through improvements by researchers. In view of the introduction of foreign genes by CRISPR-Cas9 technology, the study found that the foreign genes can be lost through progeny separation, so as to obtain non-transgenic offspring, or by assembling sgRNA and Cas9 into a ribonucleoprotein complex, the source of foreign genes can be avoided. For off-target effects caused by CRISPR-Cas9 technology, it could be reduced by optimizing sgRNA and modifying Cas protein. It is believed that more and more efficient optimization measures will be applied to crop improvement in the near future.

AUTHOR CONTRIBUTIONS

YR and KW are the content designers. XY wrote the manuscript. YR and CW revised the manuscript. CP looking up references. All authors contributed to the article and approved the submitted version.

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CRISRP/Cas9-Mediated Targeted Mutagenesis of Tomato Polygalacturonase Gene (*SlPG*) Delays Fruit Softening

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Polygalacturonase (PG) gene has been documented as a key candidate for the improvement of fruit firmness, which is a target trait for tomato production because it facilitates transportation and storage. To reduce the expression of the *PG* gene, most of the elite commercial tomato varieties were obtained by RNA interference technology. However, this approach of producing commercialized tomatoes by integration of the exogenous gene is controversial. In this work, CRISPR/Cas9 technology was used to induce the targeted mutagenesis of the *SlPG* gene to delay the softening of tomato fruit. Results showed that the *SlPG* gene was frameshift mutated by 4 bp deletion, 10 bp deletion, and 1 bp insertion, which generated premature translation termination codons. Compared with wild-type (WT), homozygous T_1-generation tomato plants exhibited late fruit softening under natural conditions. Consistent with this phenomenon, the firmness value of WT fruit was lower in *slpg* mutant fruit, and the physiological loss of water was higher. Collectively, these data demonstrate that the mutation of the *SlPG* gene delays tomato fruit softening. More importantly, 8 out of 20 transgene-free tomato plants, which were homozygous for null alleles of *SlPG,* were separated in the T_3-generation of line *slpg*T_2-#2. This transgene-free *slpg* may provide materials for more in-depth research of *SlPG* functions and the molecular mechanism of fruit softening in tomatoes.

Keywords: tomato, polygalacturonase, CRISPR/Cas9, genome editing, fruit softening

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INTRODUCTION

Tomato (*Solanum lycopersicum*), one of the most significant commercial vegetable crops grown in the world, has abundant nutritive compounds and elements (e.g., lycopene, zeaxanthin, vitamin C, and potassium) for human health (Vincent et al., 2013). Fruit softening is the most common defect consideration of commercial importance and has tremendous associated costs in terms of fruit shelf life, frequency of harvest, transportation, and storage (Garcia-Gago et al., 2009). During fruit ripening, the biochemical and physiological processes are sophisticated, and the solubilization

of the cell wall is caused by a group of enzymes (Payasi et al., 2009). One of the key enzymes is PG (EC 3.2.1.15), which functions in the breakdown of pectin, a polymer of galacturonic acid that forms part of the structural support of cell wall (Bird et al., 1988). In 1965, the PG enzyme was first reported in relation to the firmness of the tomato fruit (Hobson, 1965), and the *PG* gene sequence was first cloned from tomatoes in 1986 (Grierson et al., 1986). In 1988, with the application of molecular genetics, PG gene expression was first manipulated with transgenic approaches in tomatoes, where the suppression of *PG* gene expression resulted in significantly firmer fruit (Bird et al., 1988; Sheehy et al., 1988; Smith et al., 1988). Since then, the suppression of *PG* gene expression in other fruits, including strawberries (Quesada et al., 2009; Pose et al., 2013), pear (Zhang S. et al., 2019), and kiwifruit (Huang et al., 2020), have been reported and the desired traits were also obtained. Mostly, these fruits are reformed using antisense RNA (Smith et al., 1990; Ju et al., 1994; Kramer and Redenbaugh, 1994) or site-selected insertion (Cooley and Yoder, 1998) through transgene in elite commercial background varieties. Similar to other transgenic plants, the random integration of exogenous transgenes into the tomato genome may cause unstable and/or off-target effects, thereby causing public concern for human consumption and the commercialization of these tomatoes is restricted by complicated safety evaluation. Therefore, delayed tomato fruit softening that does not compromise exogenous genes is a key characteristic in modern tomato breeding.

In 2014, a developed technology, clustered regularly interspaced short palindromic repeat (CRISPR)/Cas (CRISPR-associated) (Doudna and Charpentier, 2014), as a robust and effective tool for the targeted gene of interest, has rapidly emerged in plant breeding with simple design and flexibility of operation (Xie et al., 2014; Zhang Y. et al., 2019). Since then, this system has been applied to generate and estimate targeted mutations in many representative plants, such as Arabidopsis (Li et al., 2013), tobacco (Nekrasov et al., 2013), rice (Shan et al., 2013), soybean (Michno et al., 2015), wheat (Upadhyay et al., 2013), and maize (Svitashev et al., 2015). CRISPR/Cas9-mediated genome editing in tomatoes was first successfully achieved in 2014 (Brooks et al., 2014) by designing two single guide RNAs (sgRNAs) to create mutagenesis in the *SlAGO7* gene. In 2017, the high rates of homozygous and biallelic mutants of *SlPIF4* were generated in the first generation by applying CRISPR/Cas9 technology, and the gene modifications were stably transmitted to the next generation (Pan et al., 2017). In 2018, the promoter of sgRNA, which provides a strong technical basis for creating mutagenesis in tomato, was optimized for this technology (Yan et al., 2018). In 2019, cytidine base editors, CRISPR/Cas9 derived tools, were applied in tomato successfully (Veillet et al., 2019), and the acetolactate synthase (ALS) gene was edited with the rate of 12.9%. These works have shown that CRISPR/Cas9 is a simple, efficient, and powerful gene editing tool in tomato molecular breeding.

In this study, CRISPR/Cas9 technology was used to induce the targeted mutagenesis of the *SlPG* gene in tomatoes. The use of the transient assay to select the high editing rate of sgRNA has not been reported previously. Out of 10 stable transgenic tomato events, 7 *slpg* mutants were obtained *via Agrobacterium*-mediated transformation. In T_1-generation tomato plants, three homozygous for null alleles of *SlPG* frameshift mutated by short deletions (4 and 10 bp) and insertion (1 bp) exhibited late fruit softening under greenhouse conditions. The homozygous T_2 *slpg* mutants, which have mutation types that were consistent with T_1 generation, also exhibited late fruit softening. More importantly, in T_3-generation tomato plants, "transgene-free" *slpg* mutants without any transgenic element were separated successfully. Such an approach is expected to reduce the deleterious effects caused by the random integration of the transgene into the tomato genome. These *slpg* mutants will provide materials for more in-depth research on improving tomato quality.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The tomato cultivar Micro-Tom was used for transformation in this study. Micro-Tom refers to a miniature dwarf tomato that can grow at a high density, has a short life cycle, and can be transformed efficiently. It became a new model plant for functional genomic study. The origin, background, and biological characteristics of Micro-Tom were clear (Liu et al., 2008). WT Micro-tom (as a control) and all seeds collected from T_0 mutant plants were sown under greenhouse conditions (16 h light/8 h dark and 22°C/18°C of day/night temperature, 80% humidity, and 10,000 lux light intensity).

SgRNA Design and the CRISPR/Cas9 Vector Construction

The tomato gene *SlPG* sequence (GenBank Accession No.: NM_001247092) was first cloned and published in 1986 (Grierson et al., 1986) and its sequence information (solyc10g080210) was downloaded from *Solanum lycopersicum* ITAG3.2 of the Phytozome website[1] (Goodstein et al., 2011). For the CRISPR/Cas9 vector construction, the sgRNAs used for genome editing were designed by using the website tool CRISPOR[2] (Concordet and Haeussler, 2018). In this study, five sgRNAs that target the *SlPG* gene were selected and named SP1, SP2, SP3, SP4, and SP5. All the sgRNA sites were located at the front exons of the *SlPG* gene (**Figure 1A**). The sgRNA sequences were synthesized by Genewiz (Suzhou). For each sgRNA, a pair of DNA oligonucleotides was annealed to generate dimers, which were subsequently integrated upstream of the sgRNA scaffolds in the plasmid vector while expressing Cas9 and sgRNA. The sequence of Cas9 was codon-optimized for maize and assembled downstream of the double cauliflower mosaic virus (CaMV) 35S promoter together with a customized sgRNA driven by the AtU6-26 promoter. The kanamycin resistance gene, neomycin phosphotransferase II (*nptII*), driven by a CaMV 35S promoter, was selected as a plant screening marker. The schematic of the *SlPG*-CRISPR/Cas9 vector is shown in **Figure 1B**. In this study,

[1] www.phytozome.net/

[2] http://crispor.tefor.net/

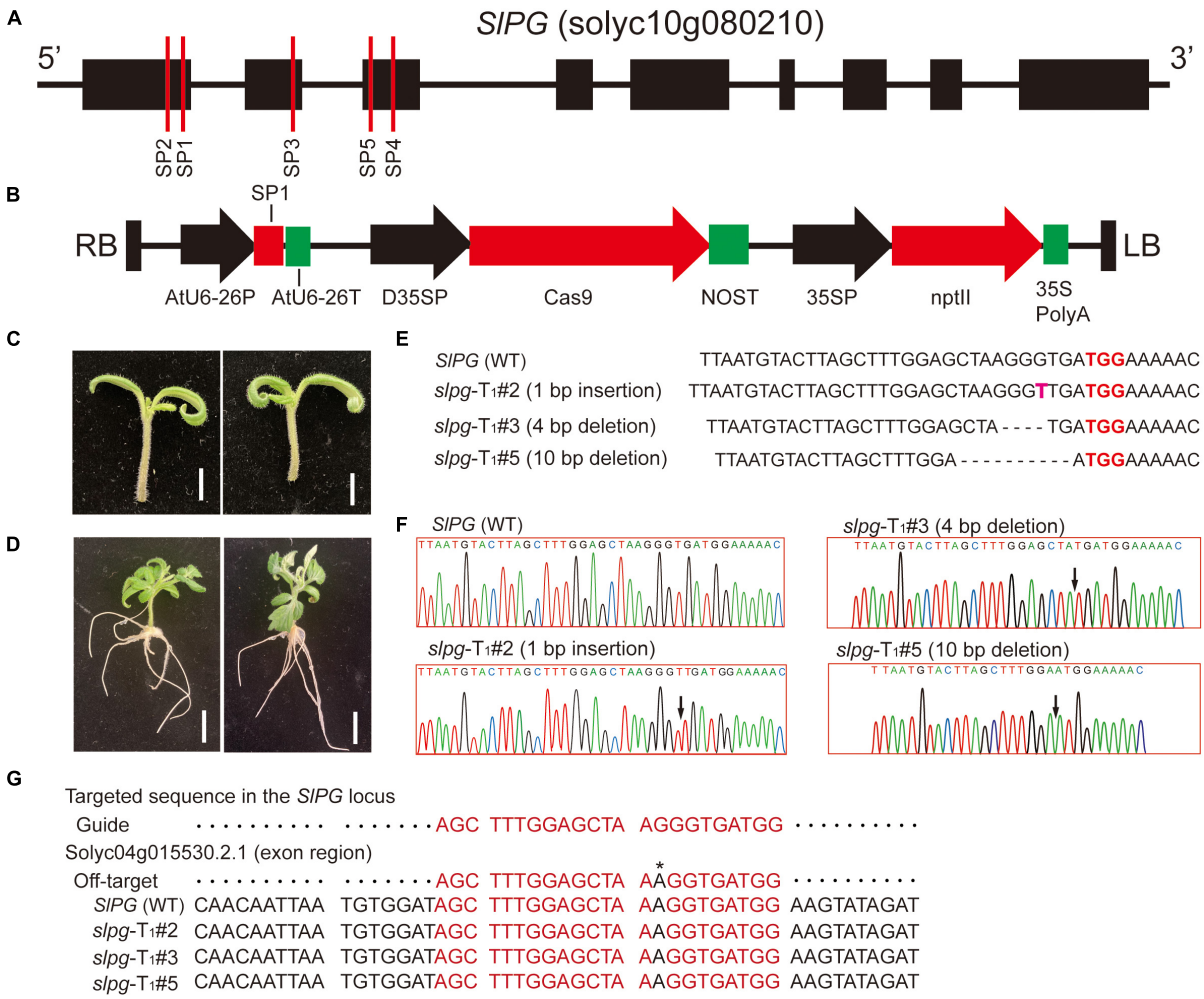


FIGURE 1 | CRISPR/Cas9 technology was used to induce the targeted mutagenesis of the *SlPG* gene in tomatoes. **(A)** Schematic representation of tomato *SlPG* gene and target sites used in this study. Black boxes and lines represent exons and non-coding regions, respectively. Red vertical lines indicate the target site position and name of designed sgRNAs. **(B)** Schematic illustrating the pg1 vector. RB/LB, right/left border of T-DNA; AtU6-26P, *Arabidopsis* U6-26 promoter; SP1, sgRNA-SP1; AtU6-26T, *Arabidopsis* U6-26 terminator; D35SP, double cauliflower mosaic virus (CaMV) 35S promoter; Cas9, coding region of Cas9; NOST, Nos terminator; 35SP, 35S promoter; nptII, kanamycin resistance gene; 35S PolyA, 35S PolyA terminator. **(C)** Tomato seedlings were selected as explants used for transient transformation. Scale bar, 1 cm. **(D)** Well-developed hairy roots. Scale bar, 1 cm. **(E)** Sequences of WT plant and T1 homozygous *slpg* mutants (*slpg*-T_1#2, *slpg*-T_1#3, and *slpg*-T_1#5) and representative mutation types induced at target site SP1 are presented, respectively. Nucleotides in red represent PAM sequences. Nucleotide in pink represents insertions and dashes between nucleotides represent deletions. **(F)** Sequence peaks of WT plant and T1 homozygous *slpg* mutants (*slpg*-T_1#2, *slpg*-T_1#3, and *slpg*-T_1#5). The black arrowheads indicate the location of mutations. **(G)** Representative putative off-target site recognized by the gRNA designed in this study. One representative site was predicted by the website tool CRISOR. The sequence at the top of the alignment is predicted off-targeted sequence. Red sequences, nucleotides corresponding to the site recognized by the gRNA designed in this study; Black nucleotide with * on top, nucleotide mismatch in the sgRNA region.

SlPG-CRISPR/Cas9 vector containing sgRNA SP1, SP2, SP3, SP4, and SP5 was named pg1, pg2, pg3, pg4, and pg5, respectively. The schematic of pg1 vector is shown in **Figure 1B**.

Transient Transformation of CRISPR/Cas9 in Tomato and Validating sgRNA Editing Activity

To test sgRNA editing activity, the constructed vectors (pg1, pg2, pg3, pg4, and pg5) were mobilized into *A. rhizogenes* K599 *via* electroporation. Hairy root transformation was performed following the reference (Pavli and Skaracis, 2010) with minor revision. The healthy and well-developed seedlings were selected as explants for root transformation. The hypocotyl was cut with the sterile blade at the bottom of the seedling (**Figure 1C**) and immersed the whole seedling into the K599 suspension for 10 min. Inoculated seedlings (5–7 seedlings per petri dish) were placed on square dishes that contained a cocultivation medium used in stable transformation and placed vertically in the greenhouse for 2 days. The seedlings were transferred into growth boxes that contained seedling germination medium, supplemented with appropriate concentrations of cefotaxime

and antibiotics to eliminate bacteria and select transformed roots. After 15 days, seedlings with well-developed hairy roots emerged (**Figure 1D**) and could be directly used for the next analysis. To evaluate the ratio of mutagenesis of *SlPG*, genomic DNA was extracted from the hairy roots of each plant, and then the regions spanning the target sites were amplified by PCR. The PCR products were purified and sequenced. Target mutagenesis can be viewed *via* sequence peaks overlapping at the target site.

Stable Transformation of CRISPR/Cas9 in Tomato and Screening for Mutations by Sequencing Analysis

CRISPR/Cas9 expression vectors were individually transformed into *A. tumefaciens* EHA105 *via* electroporation. The tomato cotyledon was used for tissue culture and transformation explant according to the protocol previously reported (Mallikarjunaiah and Moudgalya, 2017). Genomic DNA was extracted from the leaves of each plant in the T_0 generation, and transgenic plants were identified by PCR primer Cas9-F/R and then used for subsequent gene editing analysis using primer SP1-F/R (**Supplementary Table 1**). Different types of gene editing can be identified *via* sequence peaks. Subsequently, the homozygous mutant types were identified by sequence alignment with the WT plant sequence. This method was also used in the T_1 and T_2 generations.

Potential Off-Target Analysis

Genomic DNA of three T_1 homozygous *slpg* mutants and WT tomato plants was used to evaluate the off-target effect of sgRNA SP1 used in this study. Off-target sites were estimated by the web tool CRISOR (see footnote 2) (Concordet and Haeussler, 2018). PCR analysis was performed using primers specific to the targeted gene (**Supplementary Table 1**). The amplified products were sequenced and evaluated the off-target effect of the sgRNA SP1.

RNA Isolation and qPCR Analysis of Gene Expression

The WT plants and T_2 homozygous *slpg* mutants grown in a greenhouse were used to compare the expression levels of *SlPG*. Total RNAs were extracted from frozen tissue using *TransZol* by following the protocol provided by the product supplier (TransGen Biotech, Beijing, China). For reverse transcription, 2 μg of total RNA was used to synthesize first-strand cDNA using *TransScript*® II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). For qRT-PCR, the reactions were performed using *TransStart*® Green qPCR SuperMix (TransGen Biotech, Beijing, China). The PCR amplification conditions begin with a denaturing step for 20 s at 95°C, followed by 40 cycles of 95°C for 5 s and a primer extension reaction at 60°C for 30 s. PCR products were monitored using Bio-Rad CFX connect (Bio-Rad). All PCR reactions were run with three biological replicates each. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. *SlActin* (solyc03g078400) was selected as the internal reference.

Fruit Firmness Measures and Softening Analysis

Fruit firmness was measured using a GY-4 digital fruit hardness tester fruit sclerometer (ALIYIQI, China) according to a previously reported method (Yang et al., 2017). Fresh intact fruits from different developmental stages, including breaker stage (BR), pink stage (PK), light red stage (LR), and red ripe stage (RR), were collected. A total of 20 fruits from different plants were taken at each stage, and each stage fruit was tested twice at equidistant points along the equatorial plane of the fruit. The fresh weight and size of each fruit were also measured to calculate the physiological loss of water. For the fruit softening analysis, more than 20 fruits at the RR stage were collected, the surface was sterilized first, and then stored at room temperature (23–25°C and 55–60% relative humidity). Every 10 days, fruit firmness was measured, and visual softening and collapse of fruit were assessed (Nambeesan et al., 2010). A total of 20 fruits were taken from different plants at each stage for analysis.

Statistical Analysis

All experiments were repeated three times independently, and all results are reproducible. Statistical analyses were performed using SPSS 19.0 software. Two-tailed Student's *t*-test was applied to compare the significance of differences between different groups. The *P*-values less than 0.05 were recognized as significant. OriginPro8 was used for drawing box plots and histograms. Values represent mean ± standard error (SE; $n = 20$).

Primer Sequences Used in This Study

The primer sequences used for screening the transgenic positive transformants in transient and stable transformation, detecting the mutation induced by CRISPR/Cas9, potential off-target analysis, identifying transgene-free *slpg* mutant lines, and qRT-PCR analysis are listed in **Supplementary Table 1**.

RESULTS

Evaluation of the Genome Editing Efficiency of SgRNAs by Transient Transformation

Hairy roots that contain different sgRNAs (SP1, SP2, SP3, SP4, and SP5) grown to a length of 5–6 cm (**Figure 1D**) were harvested individually and mutations at the targeted sites were examined. According to the results (**Table 1**), 15 out of 20 hairy roots show gene editing by the sgRNA SP1, which was predicted to target the first exon of the *SlPG* gene. In terms of editing efficiency, the ratio of SP1 gene editing is relatively high and reached 75%. Subsequently, the vector pg1 was chosen for further stable transformation.

Targeted Mutagenesis of *SlPG* Induced by CRISPR/Cas9

The mutants of *SlPG* at the target sites were named *slpg*. The T_0 transgenic line was identified, and 7 out of 10 had

TABLE 1 | Summary of mutations generated for each sgRNA.

Name for sgRNA	gRNA-PAM[a]	No. hairy roots sampled	No. hairy roots mutation detected	% mutations
SP1	5′-AGCTTTGGAGCTAAG GGTGA**TGG**-3′	20	15	75
SP2	5′-TGTACTTAGCTTTGGA GCTA**AGG**-3′	19	3	15.8
SP3	5′-ACTGAAATAGAAGATC TGCA**TGG**-3′	18	4	22.2
SP4	5′-GGAGGAACTATCAATG GCAA**TGG**-3′	16	6	37.5
SP5	5′-TTTCAGACTACAAAGA TAGA**AGG**-3′	18	0	0

[a] *The PAM sequences are highlighted in bold.*

heterozygous-targeted mutations in *SlPG*. The site-directed mutagenesis of *SlPG* was also observed at the target site in the T_1 generation. A total of five T_1 plants that were homozygous of *SlPG* induced by CRISPR/Cas9 were identified and three types of mutations for null alleles of *SlPG* were founded at target site SP1 [1 bp insertion (*slpg*-T_1#2), 4 bp deletion (*slpg*-T_1#3) and 10 bp deletion (*slpg*-T_1#5)] (**Figure 1E**). DNA sequencing peaks showing successful gene editing at the target site are shown in **Figure 1F**. All types of frameshift mutations induced by CRISPR/Cas9 at the target site generated premature translation termination codons (PTCs) (**Supplementary Figure 1**). These results demonstrated that CRISRP/Cas9 generates the targeted mutations and loss of function in the tomato *SlPG* gene.

Potential Off-Target Analysis

According to the predicted result, the one most likely to be the off-target site of SP1 target sites of *SlPG* was found in an exon of Solyc04g015530.2.1 (**Figure 1G**). They examined potential off-target site only possessed mismatches of 1 bp compared with the on-target guide sequences (**Figure 1G**). Sequencing analysis demonstrated that sequences of the off-target site in all three homozygous *slpg* lines completely matched the WT sequence in tomato. This result indicates that no mutations were induced at the off-target site.

Stable Inheritance of Induced Mutations of the Mutants and Morphological Characteristics

To determine whether homozygous *slpg* can transmit the induced mutations and phenotypes to their progenies, a total of 20 T_2-generation plants of each mutation type deriving from three individuals of corresponding T_1 *slpg* lines were selected randomly, respectively. The result demonstrated that the targeted mutagenesis of *SlPG* was stably inherited and had maintained consistent mutation types from the T_1 generation. To assess the consequences of the site-directed mutagenesis in the targeted loci, we examined the morphological characteristics of the plant body and fruits in all three *slpg* mutants and WT plants and detected no morphological differences (**Supplementary Figure 2**). In addition, some of the agronomic characteristics of all *slpg* mutants and WT plants were also evaluated (**Supplementary Table 2**). Results indicate that the growth and development of all *slpg* mutants and WT plants are consistent and are not affected by gene editing.

Expression Patterns of *SlPG* in WT Plants and *slpg*-T_2 Homozygous Mutants

To assess the potential roles of *SlPG* throughout the development of the tomato fruit, we conducted detailed quantitative real-time PCR (qRT-PCR) to examine its transcription in five different fruit development sections [e.g., mature green (MG), BR, PK, LR, and RR]. In WT plants, the expression of *SlPG* was relatively low and negligible in MG fruit. However, the expression level was drastically enhanced from the PK section and reached a maximum in the LR section (**Figure 2A**). The result showed that the *SlPG* expression level was relatively high

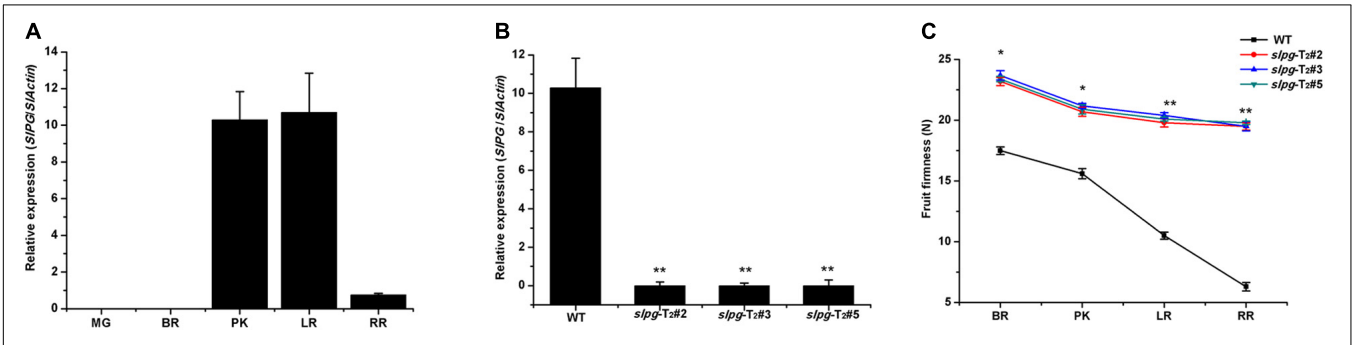


FIGURE 2 | Expression patterns of *SlPG* and phenotype identification of tomato *slpg* mutant. **(A)** Expression of *SlPG* in fruit at different developmental stages: MG, BR, PK, LR, and RR; **(B)** Expression of *SlPG* in WT plants and T_2 homozygous *slpg* mutants (*slpg*-T_2#2, *slpg*-T_2#3, and *slpg*-T_2#5). Relative transcript levels of *SlPG* were analyzed by qRT-PCR and normalized to *SlActin*. Quantitative PCR data represent means values for three independent biological replicates ($n = 3$); **(C)** Phenotype of enhanced fruit firmness at different fruit development stages in *slpg* mutants. The fruit firmness values represent the means ± standard error (SE) of 20 individual fruit per line at each stage. * and ** indicate significant differences between WT plants and T_2 homozygous *slpg* mutants (*slpg*-T_2#2, *slpg*-T_2#3, and *slpg*-T_2#5) with $p < 0.05$ and $p < 0.01$, respectively, as determined by *t*-test.

in the PK and LR sections while relatively low in the RR section, indicating that *SlPG* functioned mainly in the fruit ripening section and secondary in the fruit softening. In *slpg* mutants, real-time PCR results showed that *SlPG* transcripts were significantly decreased in the PK section when compared with WT plants (**Figure 2B**).

Targeted Mutagenesis of *SlPG* Enhances Fruit Firmness and Exhibits Delayed Fruit Softening

The suitable values for the weight and size of fresh tomato fruit were 4.5 ± 0.2 g and 1.2 ± 0.1 cm, respectively. We next measured the fruit firmness at four different ripening stages (e.g., BR, PK, LR, and RR). The firmness in the WT fruit decreased gradually with fruit ripening, with values of 17.5 ± 0.31, 15.6 ± 0.42, 10.5 ± 0.29, and 6.3 ± 0.34 newton (N) for each stage. In line *slpg*-T_2#2, the values of fruit firmness for each stage were 23.2 ± 0.34, 20.7 ± 0.38, 19.8 ± 0.29, and 19.5 ± 0.27 N. In line *slpg*-T_2#3, the values of fruit firmness for each stage were 23.7 ± 0.39, 21.2 ± 0.21, 20.4 ± 0.23, and 19.5 ± 0.38 N. In line *slpg*-T_2#5, the values of fruit firmness for each stage were 23.4 ± 0.22, 20.9 ± 0.38, 20.1 ± 0.17, and 19.8 ± 0.14 N. All *slpg* mutant lines showed similar symptoms of firmness, implying that the targeted mutagenesis of *SlPG*-enhanced fruit firmness (**Figure 2C**). The WT and *slpg* mutant fruits were harvested at the RR stage and stored at the greenhouse. WT fruits were wrinkled after 10 days of storage, whereas *slpg* mutant fruits showed similar symptoms of senescence after 20 days of storage. Comparative analysis of images of fruit after storage of 0, 10, and 20 days showed fewer wrinkles for *slpg* mutant than WT fruit (**Figure 3A**). During storage, fruit firmness was much higher in *slpg* mutant than in WT fruits (**Figure 3B**). In addition, the *slpg* mutant fruits exhibited lower physiological loss of water than WT fruits (**Figure 3C**). We compared the fruit softening time between all *slpg* mutants with WT plants and found that *slpg* mutants delayed fruit softening.

Generation of Transgene-Free Mutant Tomato Lines

To obtain tomato homozygous lines for endogenous *SlPG* mutations without any transgenic element, "transgene-clean" plants were sought *via* a PCR strategy that used four sets of primer pairs spanning four distinct regions in the T-DNA of sgRNA/Cas9 vectors and one region in the vector backbone. In this assay, we found that 8, 2, and 2 out of 20 T_3 *slpg* mutants from *slpg*-T_2#2 (**Figure 4A**), *slpg*-T_2#3 (**Figure 4B**), and *slpg*-T_2#5 (**Figure 4C**) and their offspring plants were transgene-clean homozygous *slpg* mutants, respectively.

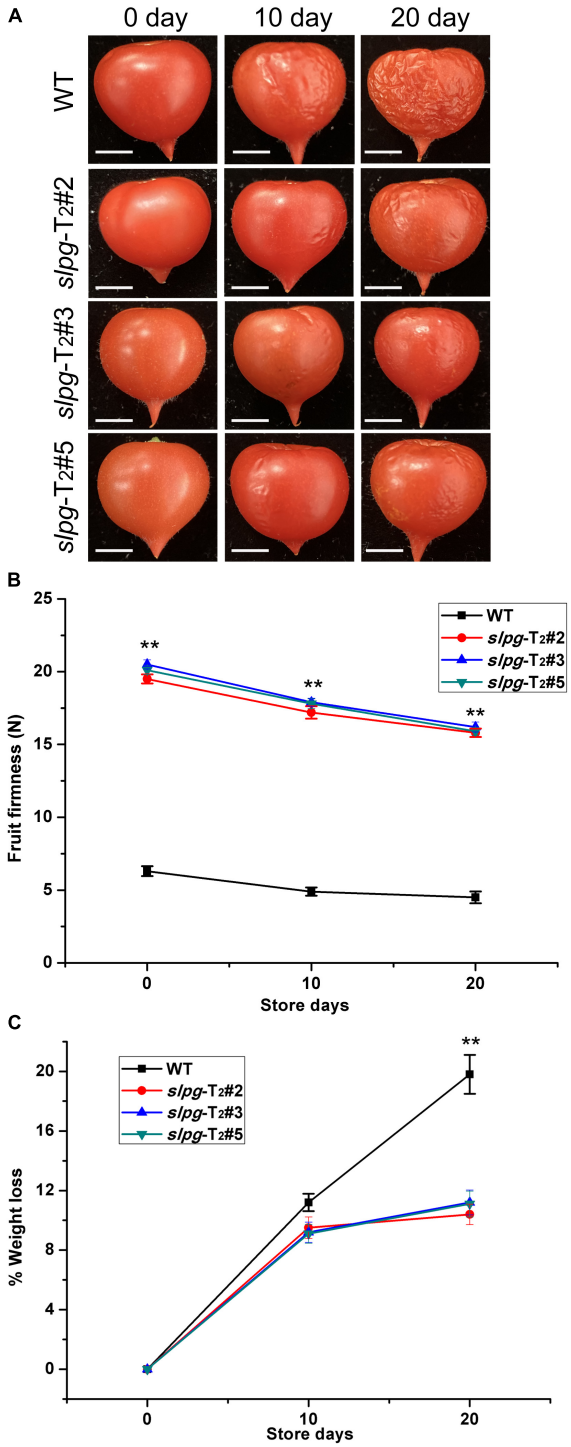


FIGURE 3 | Three *slpg* mutants (*slpg*-T_2#2, *slpg*-T_2#3, and *slpg*-T_2#5) show extended softening time. **(A)** RR fruit from three *slpg* mutants and WT plants after storage at room temperature for 0, 10, and 20 days. Scale bar, 0.5 cm. **(B)** Change of fruit firmness in three *slpg* mutants and WT plants. **(C)** Physiological loss of water (PLW) in three *slpg* mutants and WT plants. The fruit firmness per fruit and PLW were measured after storage of 0, 10, and 20 days. Average and SE (standard error) values were shown for each data point. ** Indicate significant differences between WT plants and three *slpg* mutants with $p < 0.01$, as determined by the *t*-test.

DISCUSSION

The CRISPR/Cas9 system has emerged as a robust technology for efficient genome editing and has been successfully applied

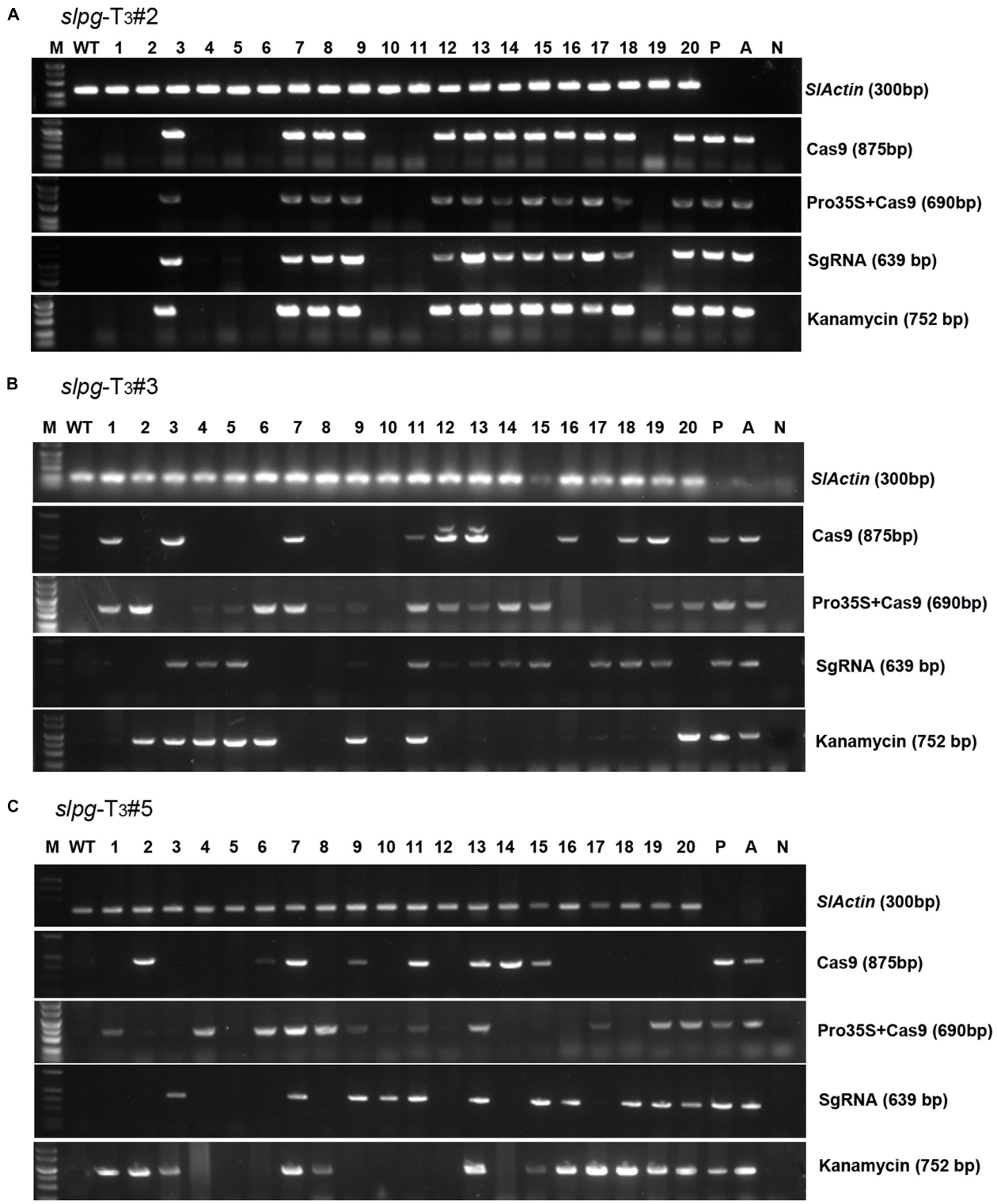


FIGURE 4 | Identifying transgene-free homozygous *slpg* mutants [**(A)** *slpg*-T_3#2, **(B)** *slpg*-T_3#3, and **(C)** *slpg*-T_3#5]. Gel image of PCR products obtained with primer sets for four T-DNA regions of sgRNA/Cas9 vector. *SlActin* (300 bp), part of the tomato Actin coding sequence, was used as a normalization control; Cas9 (875 bp), part of the Cas9 coding sequence; Pro35S + Cas9 (690 bp), region from the downstream of the 35S promoter to the upstream of the Cas9 coding sequence; SgRNA (639 bp), region from the AtU6 promoter to the downstream vector sequence spanning the sgRNA; Kanamycin (752 bp), part of the *nptII* coding sequence; M, DL5000 ladder DNA marker; WT, DNA of WT plant as a template; Lane 1-20, individual mutant lines; P, positive control (sgRNA/Cas9 vector plasmid as template); A, positive control (*Agrobacterium* containing sgRNA/Cas9 vector as template); N, negative control (water as template).

to many crops (Shan et al., 2013; Upadhyay et al., 2013; Michno et al., 2015; Svitashev et al., 2015). However, some genome editing events do not generate desired specific mutation because of unknown reasons. To overcome this problem, *A. rhizogenes*-mediated transient transformation is an attractive system for assessing the efficiency of sgRNAs in generating InDels at target sites. Different from *A. tumefaciens*, *A. rhizogenes* promote the rapid proliferation of adventitious roots that emerge at the wounding site, which are called hairy roots. The gene-editing efficacy of the sgRNAs in generating mutation at target sites can be evaluated in 15 days by sequencing PCR products amplified from the genomic DNA of pooled hairy roots. Before the whole plant's stable transform, this transient system can be time-saving, less labor-intensive, and more effective, and many researchers have already applied this system in their works (Pavli and Skaracis, 2010; Li et al., 2019). In this work, we designed five sgRNAs and evaluated the gene-editing efficacy of each sgRNA in the tomato *SlPG* gene. Our data demonstrated that the editing efficacy of sgRNA SP1 is relatively high (75%, in **Table 1**), and SP1 was chosen and applied in the next stable transformation.

Tomato genomes are *ca*. 900 Mb arranged in 12 chromosomes with 35,768 loci containing protein-coding transcripts[3]. It offers many possible binding sites for CRISPR/Cas9 to generate some additional unwanted mutations. Therefore, a certain risk of off-target activity always remains in the application of CRISPR/Cas9. Previously, several measures, such as choice of suitable nuclease (Fauser et al., 2014), target site choice with CRISPOR (Concordet and Haeussler, 2018), and nuclease delivery method (Hendel et al., 2015), should be considered to minimize the chances for an off-target cleavage to occur. A suitably engineered nucleases can increase the efficiency of targeted mutagenesis. Different types of codon-optimized Cas9 nucleases, including maize codon-optimized (Svitashev et al., 2015), were expressed in our transient assay and the results showed that maize codon-optimized nucleases possess the highest rate of targeted mutagenesis in monocot and dicot plants (paper unpublished). In this study, to avoid potential off-target mutations, a zCas9 nuclease (maize codon-optimized deactivated Cas9), a high specificity score (94) target site SP1 from CRISPOR, and Agrobacterium-mediated transformation were applied.

CRISPR/Cas9-induced mutations were determined from the T_0 to T_1 generation. All T_0 heterozygous biallelic and homozygous *slpg* edited lines could transmit the targeted mutations to the T_1 generation. We still obtained some novel targeted mutagenesis in T_1 generation from T_0 heterozygous monoallelic *slpg* lines. In these lines, CRISPR/Cas9-induced mutation exists only in one allele and the other allele without any mutation, and the targeted mutations of the *SlPG* allele induced by CRISPR/Cas9 were stably inherited and maintained consistent mutation types from T_0 to T_1 generation. At the same time, the other allele without any mutations can be induced by CRISPR/Cas9 and generate novel targeted mutagenesis in the T_1 generation. These results demonstrated that CRISPR/Cas9 can induce targeted mutagenesis in the next generation if monoallelic edits are in the previous generation. This phenomenon is consistent with the previous research on soybean (Bai et al., 2020).

Fruit softening is one of the most characteristic physiological processes that occur during the ripening of fleshy fruits (Garcia-Gago et al., 2009). A softening process results from the degradation of cell wall polymers by the action of the cell wall-associated hydrolytic enzymes. In the past few decades, many enzymes, such as pectate lyase (PL) (Uluisik et al., 2016; Yang et al., 2017), β-galactosidase (Smith et al., 2002), expansin (Brummell et al., 1999), and GA2-oxidase (Li et al., 2020), have been studied as candidates for delaying fruit softening, and some changes were observed in fruit softening. For example, the PL gene has already been chosen to manipulate fruit softening in tomatoes (Marín-Rodríguez et al., 2002; Yang et al., 2017), bananas (Payasi and Sanwal, 2003), and mangos (Chourasia et al., 2006). Among the cell hydrolytic enzymes involved in fruit softening, PG is the best and the earliest characterized (Brady et al., 1982). In 1986, the *PG* gene was first cloned and sequenced (Grierson et al., 1986). In 1994, The FLAVR SAVR^{TM} tomato is the first genetically engineered commodity to be sold in commerce (Kramer and Redenbaugh, 1994). By 2000, the members of the homologs of *SlPG* have reached 7 (Hong and Tucker, 2000). The last cloned *PG* gene, *TPG7*, is highly expressed in pistils and shares 39% sequence identity with the tomato fruit *PG* genes. The *PG* gene locus, solyc10g080210, was proved to be highly expressed in tomato fruit, especially in fruit PK and LR stage (**Figure 2A**). These results were also basically consistent with the data obtained from the TomExpress platform[4], thereby further implying a possible crucial role of *SlPG* during fruit ripening and softening. The FLAVR SAVR^{TM} tomato was developed by using antisense RNA to suppress the expression of PG in ripening tomato fruit. Subsequently, this antisense RNA strategy was applied to other fruits to produce firmer fruits (Garcia-Gago et al., 2009; Zhang S. et al., 2019; Huang et al., 2020). However, these commodities were developed through a transgenic approach, which may generate negative results, such as the disruption of plant endogenous genes or exogenous gene silencing (Napoli et al., 1990). Although no scientific evidence suggests that genetically modified organism (GMO) products are harmful to human health, debates about GMO safety continue. Previously, many transgene-free targeted mutagenesis plants, such as soybean (Cai et al., 2018), rice (Li et al., 2016), wheat (Zhang et al., 2016), and tomato (Soyk et al., 2017), have been generated by CRISPR/Cas9. In this study, CRISPR/Cas9 was applied to induce targeted *SlPG* mutagenesis, which could delay fruit softening without any exogenous gene fragment. Moreover, CRISPR/Cas9 was proven to enhance fruit firmness without compromising other fruit qualities (Li et al., 2020), indicating that the targeted mutagenesis of the *SlPG* gene in tomatoes could delay fruit softening without impacting the tomato flavor, aroma, taste, and nutritional value. This conclusion was supported by Kramer and Redenbaugh's research

[3]https://phytozome-next.jgi.doe.gov/info/Slycopersicum_ITAG3_2

[4]http://tomexpress.toulouse.inra.fr/

(Kramer and Redenbaugh, 1994). This is a key target characteristic in modern tomato breeding.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

HN and GX designed the project. HN and YS performed the experiment. HN and XG analyzed the data. HN wrote the manuscript. GX made suggestions for improving the procedure and proofread the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.729128/full#supplementary-material

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Molecular and Computational Strategies to Increase the Efficiency of CRISPR-Based Techniques

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The prokaryote-derived Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas mediated gene editing tools have revolutionized our ability to precisely manipulate specific genome sequences in plants and animals. The simplicity, precision, affordability, and robustness of this technology have allowed a myriad of genomes from a diverse group of plant species to be successfully edited. Even though CRISPR/Cas, base editing, and prime editing technologies have been rapidly adopted and implemented in plants, their editing efficiency rate and specificity varies greatly. In this review, we provide a critical overview of the recent advances in CRISPR/Cas9-derived technologies and their implications on enhancing editing efficiency. We highlight the major efforts of engineering Cas9, Cas12a, Cas12b, and Cas12f proteins aiming to improve their efficiencies. We also provide a perspective on the global future of agriculturally based products using DNA-free CRISPR/Cas techniques. The improvement of CRISPR-based technologies efficiency will enable the implementation of genome editing tools in a variety of crop plants, as well as accelerate progress in basic research and molecular breeding.

Keywords: CRISPR/Cas9, base editing, prime editing, genome editing efficiency, DNA-free CRISPR/Cas

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INTRODUCTION

The capability of creating genetic variation has always been the key for crop improvement (Griggs et al., 2013). While this is traditionally achieved through conventional plant breeding or random mutagenesis induced by ionizing radiation and chemical mutagens, nowadays, agricultural biotechnology has entered a new era of nucleotide-scale precision (Jung and Till, 2021).

Site-directed nucleases are used to produce single- or double-strand breaks at specific DNA target sites. These DNA breaks stimulate either the stochastic non-homologous end joining (NHEJ) or homology directed repair (HDR) pathway, resulting in diverse outcomes such as site-directed mutagenesis, gene replacement, and nucleotide insertions or deletions (Kumar and Jain, 2015). While hybrid enzymes, like meganucleases, Zinc Finger Nuclease (ZNF), and Transcription activator-like Effector Nucleases (TALEN), have been successfully used for genome editing in plants (Curtin et al., 2012; Osakabe and Osakabe, 2015) the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system is the

new gold standard, because it is more versatile, less expensive, simpler and more reliable (Belhaj et al., 2013).

CRISPR/Cas9 was first discovered as a bacterial and archaeal defense system against transmissible elements from virus or exogenous DNA (Mojica et al., 2000; Mojica and Rodriguez-Valera, 2016). The rapid adoption of CRISPR/Cas9 technique originated from *Streptococcus pyogenes*, enabled the initial application as a genome editing tool in plants and animals (Makarova et al., 2011a,b; Hsu et al., 2014).

Even though CRISPR/Cas9 technology has been rapidly adopted and successfully implemented in plants, its gene editing efficiency rate varied greatly (Howells et al., 2018). Recently, several robust technical improvements have emerged based on the necessity to improve efficiency and specificity. This review focuses on highlighting the different computational and experimental base and prime editing strategies developed to increase the efficiency of CRISPR/Cas-based genome editing systems. We first describe recent advances in deep learning tools used during CRISPR/Cas constructs design. We then present a comprehensive overview of the recently developed Cas9 and prime editing (PE) tools and their implications on enhancing editing efficiency. We also provide a perspective on the global future of agricultural based products using DNA-free CRISPR/Cas techniques. The advances we discuss here point to several venues of experiencing that are paving the way to increase the efficiency in producing homozygous edits, the occurrence of off target editing, the broadening of possibilities of target sites in the genome and to enhance homologous recombination. Addressing these key issues will have a major impact on the way we can modify the genome of crops, leveraging the efforts to a sustainable agriculture and preparing crops to a changing environment.

THE CRISPR/Cas9 SYSTEM

Deep-Learning Strategies to Increase CRISPR Cleavage Efficiency

The rapid progress of experimental procedures implementing CRISPR/Cas technology in plants over the past decade has been accompanied by equally impressive advances in the computational methods for gRNA design and off target prediction (Wang et al., 2017b, 2020b; Lowder et al., 2018; Hajiahmadi et al., 2019). As genetic transformation methods and CRISPR implementation grew, the emerging algorithm developments for accurate target prediction revealed increasingly complex facets of the underlying biology, from sequencing composition to epigenetic regulation (Wang et al., 2019; Yan et al., 2020). At the same time, rapid growth has forced constant reevaluation of the underlying algorithms and statistical models used by these computational tools when implemented in CRISPR studies (Sledzinski et al., 2020).

The development of computational tools has become essential in the process of single guide RNA (sgRNAs) design. However, one of the major challenges of the CRISPR system is to precisely predict the sgRNA on-target knock-in or knock-out efficacy, while avoiding undesired cuts to similar DNA sequences in the target genome creating off-targets. In plants as well as in other organisms, a large difference in cleavage efficiency has been observed, which suggests that several factors regulate the binding and cutting efficacy of the sgRNA-Cas complex (Raitskin et al., 2019; Ren et al., 2019b; Banakar et al., 2020; Wolabu et al., 2020; Grützner et al., 2021). Those factors include sequence composition, nucleotide position, GC content, chromatin accessibility, gene expression profile, RNA secondary structure, melting temperature and free energy (Kim et al., 2019; Safari et al., 2019; Xiang et al., 2021). Thus, a careful design of the sgRNA is essential for optimal Cas9 activity. It is critical to search for conserved domains, motifs, or regions within the target sequence among different genotypes or related species to make sure that there are no point mutations at the gRNA binding site or at the PAM site. A second important aspect to take into consideration is the presence of favorable nucleotides at the seed sequence (10 nucleotides closest to the PAM site; Doench et al., 2014; Xu et al., 2015). To date, the methods developed for sgRNA efficacy prediction and on-target identification can be classified into three major groups:

(i) Alignment-based methods: sgRNAs candidates are designed based on an alignment from the target sequence and the respective genome purely by locating the PAM sequence.
(ii) Hypothesis-driven: sgRNAs efficiencies are scored empirically by compiling information related to the factors that impact the genome context.
(iii) Machine and Deep learning-based: sgRNAs are predicted from a training model by considering different features.

Over the last couple of years, solid evidence has shown that hypothesis-driven and learning-based strategies outperform alignment-based strategies (Wang et al., 2020c; Yan et al., 2020; Zhang et al., 2020). However, the popularity gained by artificial intelligence has positioned learning-based methods as the leading strategies to increase efficiency efforts. Building reliable models to design highly efficient sgRNA using learning-based strategies largely depends on the critical factors that the models are built on. For instance, it is required a considerably large amount of data of sgRNAs designed from different platforms and their respective experimental efficiencies to build reliable models. In addition, experimental data on real quantification of off-target site prediction based on all the possible nucleotide mismatch loci at the whole genome level are limited (Zischewski et al., 2017). And finally, other genomic and epigenetic features that affect sgRNA remain unclear and could improve its efficacy.

Several machine and deep learning-based methods have been developed to predict CRISPR on-target activity. However, most of them use data sets exclusively for animal models (Chuai et al., 2018; Kim et al., 2019; Yan et al., 2020; Xiang et al., 2021). Even though animals and plants share genetic and epigenetic features, models specifically for plants are needed. Based on their statistical methods (Feng et al., 2017; Lin and Wong, 2018; Chen et al., 2019b; Wang et al., 2019, 2020c; Liu et al., 2020; Padilha et al., 2020; Yan et al., 2020; Zhang et al., 2020; O'Brien et al., 2021), most of the models developed can be roughly classified into six categories: (i) CNN: Convolution Neural Network; (ii) L1-Reg: L1-Regression; (iii) SVM: Support

Vector Machine; (iv) RF: Random Forest; (v) GBRT: Gradient Boost Regression Tree; and (vi) SVM (C): using SVM to classify (+1 represents high activity, −1 represents low-activity).

Many of these statistical methods use algorithms to recognize targets based on the gRNA-DNA pairing. For instance, CRISPR-P, one of the most popular tools for sgRNA design in plants, supports sgRNA design for almost 50 plant species including gene annotation. In addition, it provides a sgRNA scoring system for on-target efficiency and off-target (Lei et al., 2014). Interestingly, it also supports various CRISPR-Cas systems including Cas12a (formerly Cpf1). Nevertheless, CRISPR-P allows uploading custom sequences and identifying sgRNAs if the genome of interest is not listed between the ones available. However, no off-target is available for that option (Liu et al., 2017a). Another interesting web-based tool, CRISPR-PLANT, calculates specificity of all gRNA spacer sequences based on both mismatches number and position in their alignments with other spacer sequences for any given sequence (Xie et al., 2014; Minkenberg et al., 2019). However, CRISPR-PLANT only allows searching for NGG-PAM sites. It would be interesting to have the possibility to upload genome sequences from other species, as implemented in CRISPR-P.

Learning Cas9 Machines

Rational engineering of Cas9 nuclease has required conformational and mechanistic understanding of the CRISPR-Cas system, which has contributed greatly to push the boundaries of this technology. Currently, a vast repertoire of engineered or evolved SpCas9 variants (e.g., evoCas9, xCas9, HypaCas9, SpCas9-HF1, eSpCas9, and HiFi Cas9) have been created by rational design or directed evolution, altering catalytic function, PAM requirements and reducing off-target activity (for a detailed review on these Cas9 variants see Meaker et al., 2020). As such, the different combinations of key amino acid changes in the functional domains of Cas9 have improved its specificity, genome coverage and consequently, its efficiency. Nonetheless, despite remarkable progress, the existence of a guide-dependent trade-off between specificity and cleavage activity has been described among the panel of these novel variants (with the exception of xCas9), still leaving researchers puzzled by which Cas9 variants to use at a given locus (Schmid-Burgk et al., 2020).

Different features associated with gRNA activity (e.g., differences of nucleotide preference, and position-dependent nucleotide composition) between wild-type SpCas9 and these highly specific Cas9 proteins, explain to some extent the high dependence of these variants on the target sequences. Yet, far from meeting an ideal Cas9 (i.e., with high specificity and activity regardless of the guide RNA used), the use of techniques of relatively low-throughput and the lack of extensive comparisons among variants, due to an exceedingly costly task and labor-intensive, have limited novel biological insights.

These unprecedented challenges regarding the analysis and interpretation of Cas9 variants along with the astronomical numbers of theoretical targetable sequences (4^{20} = ca. 10^{12} molecules) have recently directed researchers to look at other alternatives to handle these massive amounts of data. Recently, for instance, an elegant bacterial system (termed Self-targeting sgRNA Library Screen—SLS) was developed to characterize these tailored Cas9 proteins, exploiting more than a million sequence libraries, wherein over 60 physicochemical parameters were considered (Tálas et al., 2021). Interestingly, the study identified various sequence features that positively or adversely impact on SpCas9-HF1 cleavage. This variant prefers a slightly higher GC-content in the middle region compared to other ones of the spacer. However, the presence of motifs corresponding or overlapping to the GTNAC sequence, also in the middle region (positions 10–14), affects its ability to form cleavage-competent conformation. Besides, it is noteworthy to mention that the SLS approach presented a robust prediction tool for mammalian cleavage activities too, which might open the use of a dozen SpCas9 variants.

Another recent high-throughput methodology that has been gaining a growing interest for decoding the variant activities dependency on target sequences is deep learning. Briefly, deep learning is a set of machine learning techniques based on stacked artificial neural network layers that can learn rich data representations from raw inputs through affine transformations and non-linear activation function (Xu et al., 2020c). Recently, using a combination of lentiviral libraries and DL-based computational models, Kim et al. (2019) have assembled an extensive comparison of SpCas9 variants, providing a helpful and general guide (Kim et al., 2020b). This online tool called DeepSpCas9variants allows users to select the most appropriate and effective variant to use at a given target sequence according to 20 most important features for predicting their activities. However, the greatest challenge will be to obtain the best identified Cas9 variants and optimize them for wet lab experiments.

Interestingly, CRISPRon, a recently developed tool, provides more accurate gRNA efficiency prediction outperform the existing tools developed so far (Xiang et al., 2021). When compared with DeepSpCas9variants, Azimuth, and DeepSpCas9, CRISPRon exceeded the performance showing a higher Spearman's rho correlation (0.80; Xiang et al., 2021).

Given the Cas9 dependency of the protospacer DNA and the above-mentioned astronomical amount of possible permutations, it might seem unrealistic a library of Cas9 variants suitable for any possible composition of the target sequence. On the other hand, this raises the question of whether, in the near future, an ideal Cas9 suitable for all target DNA might be achievable. Fortunately, the field of deep learning for protein engineering is also moving rapidly and outperforming conventional methods. Besides, this approach has not yet been explored and used for the Cas9 protein structural diversity to identify good adaptive routes to higher fitness (Gao et al., 2020). Concomitant with that, the need of multiple mutated residues in Cas9 has been predicted to improve specificity and efficiency by changing the conformational dynamics and biophysical properties of the iCas9 at different stages of its activity (Palermo et al., 2017, 2018; Boyle et al., 2021; Wang et al., 2021). Mostly, these residues mediate the allosteric communication of Cas9 (i.e., flexibility of the three conformational stages) and the RNA/DNA heteroduplex to

ensure the proper positioning of the catalytic site and the proofreading step (Yang et al., 2018). However, to find multiple mutations at one time is beyond the capacity of most screenings. In addition, the extreme epistatic interactions, so-called sign epistasis (i.e., the result of a mutation depends on the preceding mutations), in Cas9 variants may be a constraint on the path to an optimum fitness peak (Poelwijk et al., 2007; Romero and Arnold, 2009). Thereby, the use of conservative computational approaches such as UniRep (Alley et al., 2019), low-N (Biswas et al., 2021), and 1D convolutional neural network (CNN) is gaining much ground nowadays to the study of large-scale conformational changes of Cas9 and to accelerate engineered proteins (Orellana, 2019; Gao et al., 2020).

INCREASING THE PAM SEQUENCE SCOPE

Streptococcus pyogenes Cas9 is the most widely used protein in the CRISPR/Cas system for genome editing. However, the number of sequences it can recognize is limited by its strict dependence on the PAM (5′-NGG-3′) motif (Zeng et al., 2020). In case non-canonical PAM motifs could be recognized, the number of sequences that can be edited would increase remarkably, for instance: 1.36 times in rice (Hu et al., 2016) and 1.62 in pepper (Li et al., 2020a). Therefore, several studies have focused on developing variants that improve PAM recognition sequence flexibility allowing broader screens on the target genome. Variants with different PAM preferences have been reported, such as VQR-Cas9 (NGA PAM), VRER-Cas9 (NGCG PAM), EQR-Cas9 (NGAG PAM; Kleinstiver et al., 2015), xCas9 (NG, GAA, and GTA PAM; Hu et al., 2018), SpCas9-NG (NG PAM; Nishimasu et al., 2018), SpG, and SpRY (Walton et al., 2020).

SpCas9-VQR, EQR, and VRER have been used to edit Arabidopsis (Yamamoto et al., 2019) and rice genomes (Hu et al., 2016, 2018), but their cleavage efficiency was lower in comparison with wild type SpCas9 (Kleinstiver et al., 2015; Hu et al., 2016, 2018; Yamamoto et al., 2019). On the other hand, SpCas9-NG allows a broader sequence recognition, identifying at least four types of atypical PAMs without showing a preference for the third nucleotide (NAC, NTG, NTT, and NCG; Hua et al., 2019; Ren et al., 2019a; Zhong et al., 2019) which increases the possibility of genetic editing in any given target of a plant genome. The most effective xCas9 variant is xCas9-3.7, showing high targeting fidelity, although a lower editing efficiency compared to SpCas9-NG (Hua et al., 2019; Zhong et al., 2019).

Interestingly, the development of variants partial or totally independent to the PAM sequence (SpG and SpRY) increases the possibility to edit a wider variety of genomic loci (Walton et al., 2020). Both variants, SpRY and SpCas9-NG, are so far the most efficient in the context of site-directed mutagenesis not only in plants (Ren et al., 2021; Xu et al., 2021) but also in single-celled organisms (Asano et al., 2021) Nevertheless, the relaxation that allows them to expand their compatibility with shorter PAM sequences can also lead to the recognition of a greater number of potential off-target sites (Endo et al., 2019). Comparison of the editing specificity of these variants with Cas9-WT demonstrated that SpCas9-NG has comparable editing activity (Hua et al., 2019; Zhong et al., 2019), while the broad flexibility of SpRY increases the editing of off-target sites in all plant genomes (Walton et al., 2020; Xu et al., 2021). Fortunately, it was shown that SpRY-HF1 can almost completely mitigate off-target site editing while increasing the fraction of total events edited at on-target sites in human cells (Walton et al., 2020).

Improved Templates for Homologous Recombination Using Cas9

In eukaryotic cells, the two repair mechanisms, NHEJ and HR, compete with each other, and NHEJ is by far the preferred choice (Miyaoka et al., 2016). In the presence of a repair template containing a region with homology to the region flanking the DSB, HR can take place, replacing part of the gene of interest (Begemann et al., 2017; Oz et al., 2021; Wei et al., 2021). The frequency of HR in plants is very low (Butt et al., 2017; Miki et al., 2018; Que et al., 2019; Ali et al., 2020) and the delivery of the DNA template to different cell types in the right amount to stimulate recombination is not an easy task (Schindele et al., 2018; Huang and Puchta, 2019). HR can also be directed by the addition of an RNA template molecule. Li et al. (2016) used a pair of gRNA targeted to adjacent introns of the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). By adding a donor DNA template containing a few mutations the rice endogenous gene was replaced at a 2% frequency, producing glyphosate-resistant plants. With the use of one sgRNA targeting one intron the gene replacement frequency was 2.2%. Furthermore, the site-specific gene replacements were transmitted to the next generation.

Several methods for template delivery aiming to increase HR efficiency have been developed and the majority lies on the type and/or quantity of the molecule used as template. Other studies aimed to increase efficiency by co-localizing the donor template and the RNA-guided nuclease. Aird et al. (2018) demonstrated in mammal cells that a 30-fold increase in HDR can be obtained by covalently tethering a single-stranded oligonucleotide (ssODN) to the Cas9 complex using a HUH endonuclease, creating a stable complex between the ribonucleoprotein (RNP) and the ssODN without the need for its chemical alteration, changes in the sgRNA or additional proteins. Butt et al. (2017) verified that the template can be delivered as an RNA molecule fused with the sgRNA, producing one single RNA molecule that will set the specificity of the nuclease and serve as the HR template. However, this technique has some limitations, such as low efficiency, low versatility and the need for long homology arms. Lu et al. (2020) improved dsDNA template stability and consequently the insertion efficiency (compared to unmodified dsDNA and to ssDNA) by adding at the 5′ and 3′ ends of both strands two phosphorothioate linkages. This methodology was also efficient to insert short (<70 bp) and longer (526 and 2,049 bp) modified dsDNA donors. These authors also developed the tandem repeat-HDR strategy (TR-HDR) in which the desired

insertion is flanked by a repeat sequence, increasing HR, providing a robust successful mean of base substitutions.

The integration efficiency can also be enhanced by the use of engineered DNA virus-based replicons to amplify the number of template copies available to stimulate HR. Several studies were successful, and insertion of desired DNA sequences were achieved in some species such as tomato (Čermák et al., 2015; Dahan-Meir et al., 2018), rice (Wang et al., 2017a), and hexaploid wheat (Gil-Humanes et al., 2017).

The microhomology-mediated end joining (MMEJ) pathway happens when the donor template has short homology arms (5–25 pb) to be used during the HR system (Nakade et al., 2014). Although this repair mechanism has low occurrence in G0/G1 phase compared to S and G2 phases of plant cell cycle (Truong et al., 2013) and is highly error-prone (García-Medel et al., 2019). Some studies suggest that MMEJ, especially in the hexaploid wheat, may be an effective HR strategy (Nakade et al., 2014; Wang et al., 2014; Arndell et al., 2019).

Recently, Schubert et al. (2021), from Integrated DNA Technologies (United States) evaluated a series of parameters affecting homologous recombination efficiency using single-stranded oligodeoxynucleotide (ssODN) as donor templates, probing 254 loci in Jurkat cells and another 239 loci in HAP1 cells. Among several parameters tested, it is worth noting the selection of the targeting strand is highly dependent on the loci and also on the cell type and that the position of the DSB site has a critical impact. Moreover, the introduction of blocking mutations, inhibiting the re-cleavage of the recombined product by Cas9, also had a positive effect, namely when targeting the PAM sequence. The company has a website[1] that uses all their findings to create optimized sequences for homologous recombination. The evaluation of these rules to plant genome editing awaits further experimentation.

Cas9-coupled deaminases and prime editing techniques were developed as an alternative to overcome those problems and precisely and efficiently produce specific edits without both DSB and a repair template, as discussed below.

Introducing Base Edition With Nuclease Fused Deaminases

Another variation of the CRISPR/Cas9 system is the base edition technique (**Figure 1**). Mutations are created due to the capacity of cytidine deaminases to convert C/G to A/T and adenine deaminases to convert A/T to C/G (Nishida et al., 2016; Schindele et al., 2018; Chen et al., 2019a). These editor proteins are fused to Cas9, Cas13, or Cas12a, that guide them to the desired locations in the genome. This approach allows the modification of only one nucleotide in a specific sequence. Using mammalian cells, it was shown that two mutations (Asp10Ala and His840Ala Cas9) can be introduced in the Cas9 protein, generating catalytic dead protein (dCas9) unable to produce a double-strand break, but still capable of being guided to a specific DNA target by a gRNA (Komor et al., 2016; Rees and Liu, 2018). The dCas9 protein is then fused with a deaminase allowing the substitution of the desired base. Amino acid substitution or stop codon generated by a single nucleotide mutation may lead to losing or changing the protein function. Komor et al. (2016) found that a Cas9 containing only the Asp10Ala mutation (Cas9 nickase, or nCas9) was able to introduce nicks at the target strand and performed better than dCas9. nCas9 also performed better than dCas9 in rice (Shimatani et al., 2017) and has been widely used in other plant species (Li et al., 2020c; Qin et al., 2020a).

More recently, several groups have reported the development of new deaminases able to perform transversion base changes, known as C-to-G base editors (CGBEs), by fusing rAPOBEC1 to either uracil DNA glycosylase (UNG; Kurt et al., 2021; Zhao et al., 2021) or XRCC1, a base excision repair protein (Chen et al., 2021). Besides their ability to perform new editions, a R33A mutation in the rAPOBEC1 deaminase and the use of UNG or rXRCC1, reduced off-targets of these new deaminases in the human genome (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). Interestingly, these authors also found that in the target sequences, these CGBE have a stronger bias towards the cytosine positioned at the sixth position. Sretenovic et al. (2021) produced versions of these three CGBE with codon optimization for plants, and their performance showed a dependence on the species. For example, in rice, monoallelic editing efficiency with a rXRCC1-based CGBE produced efficiencies up to 38%, while in poplar the best performance (6.25%) was obtained with the UNG-rAPOBEC1 (R33A)-based CGBE. Assays using rice and tomato protoplasts also showed C to T editions and indels as major byproducts. By contrast, in transgenic rice plants, no indels were observed in most CGBE constructs, indicating that these byproducts might be dependent on cell cycle and DNA repair mechanisms. It is worth noting that G to C edition had lower efficiency when compared to C to T conversion by the regular CBE system.

The use of nucleases that recognize different PAM can add versatility to genome editing. This is the case of Cas12a, which recognizes TTTV (V = A/G/C) as the PAM. However, Cas12a-based base editors (BEs) have lower editing efficiencies compared to SpCas9-based BE systems, probably because Cas12a has a looser binding to DNA targets when compared with SpCas9 (Bin Moon et al., 2018; Chen et al., 2020). The efficiency of base editing was also influenced by the context of the PAM sequence, indicating that the distinct gene context of the edition site should be carefully evaluated when using different combinations of nuclease:deaminase.

The addition of a protein from *Bacillus subtilis* bacteriophage PBS1, presenting uracil DNA glycosylase inhibitor (UGI), allowed a 3-fold enhancement in base edition in human cells (Komor et al., 2016). This is because this protein inhibits uracil DNA glycosylase (UDG), an enzyme involved in base-excision repair, reverting the edited U:G pair to the original C:G pair (Kunz et al., 2009). More recently, Qin et al. (2020b) produced an enhanced BE3 (eBE3), by adding three copies of UGI to the 3′ end of the BE3 sequence, which increased efficiency up to 2.8 times. Moreover, eBE3-edited plants had no indels which were observed in up to 25% of the edited plants using BE3 with a single UGI. The rates of undesired C>A and C>G conversion, and "clean edits" (only C>T substitutions) were

[1]https://www.idtdna.com/HDR

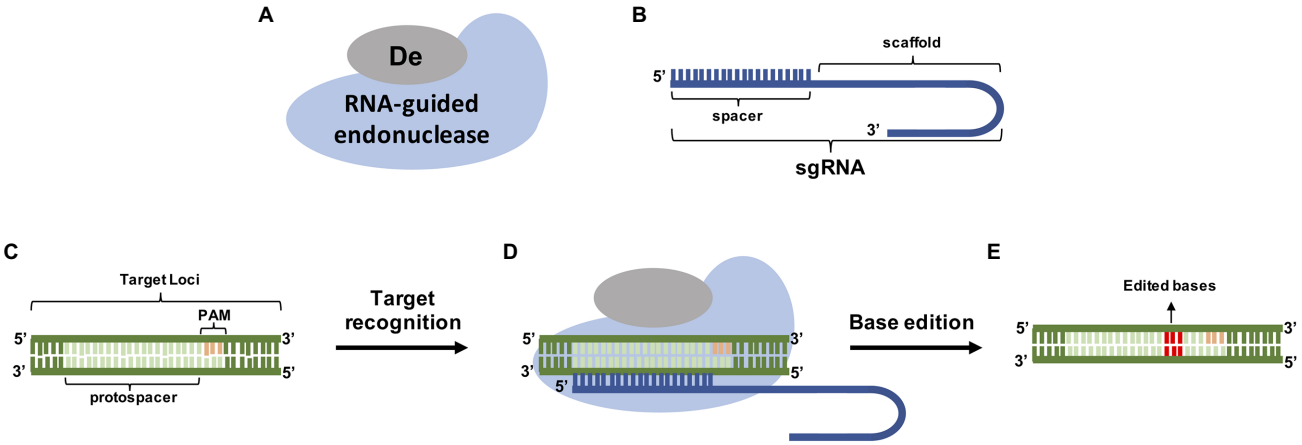


FIGURE 1 | Genome editing using base editors **(A)** RNA-guided endonucleases and specific base Deaminases (De) are guided by a sgRNA **(B)** to its PAM containing target **(C)**. After target recognition **(D)** the deaminase precisely edited the bases inside the spacer site **(E)**.

1.14–3.81 higher with eBE3 compared to BE3 in several gene targets. Interestingly, Nishida et al. (2016) also found evidence that UGI also helped to decrease indels at the edited site. It is worth noting that in their seminal work, Gaudelli et al. (2017) found that adenine BEs have lower rates of indels when compared to editions using Cas9. In plants, Jin et al. (2019) also found that adenine BEs have a low frequency of changes in off target sites, when compared to cytosine BEs.

The ability of the protein::RNA complex to be transported to the nucleus is a key step in genome editing. Koblan et al. (2018) found that a bipartite nuclear localization signal (bpNLS) positioned at the N and C terminus of the coding region of an APOBEC deaminase::Cas9::UGI fusion enhanced 1.3 fold the editing process in human cells. The same authors also found that the algorithms used by different authors and companies to optimize the coding regions also influenced the efficiency of the cytidine base editor, ranging from 20% to up to 60% of total sequencing reads with target C:G converted to T:A in human cells (Koblan et al., 2018). In the case of an adenine base editor, these authors found increases in editing efficiency from 1.3- to 7.9-fold when the optimization algorithms from two private companies were compared. Anyway, these enhancements are correlated with an improved nuclear localization of the editing protein fusion, as observed by Zafra et al. (2018), using the BE3 cytidine base editor in mice cells.

The influence of different NLS as well as the effect of multiple copies of NLS in base editing in rice was reported by Wang et al. (2020a). These authors found that in some genome sites, the efficiency of the different configurations of the base editor was similar. However, the efficiency was 3-fold higher in the case of the *OsSLR1* gene edited with a construct containing two different NLS compared to a construct containing one single copy. Li et al. (2018a), using a transient assay with a mutated version of the GFP gene in rice protoplasts, found no differences when constructs containing one or two NLS were used in vectors expressing an adenosine deaminase (ecTadA-ecTadA*) with nCas9 (D10A), while the insertion of a third copy of a NLS increased the efficiency by 25%. However, the increase in efficiency was less pronounced, around 10%, when several genes in the rice genome were assayed.

Changes in the sequence of the sgRNA can enhance A to G edition by more than two-fold in 13 target sites in both rice and wheat (Li et al., 2018a). Qin et al. (2020b) also observed editing frequencies of the enhanced gRNA (esgRNA) 1.9–2.1 fold higher than the usual gRNA in five genes from rice. These changes seem to increase not only the assembly of gRNA with the dCas9 protein, the gRNA stability, but also eliminate a putative Pol-III terminator (Chen et al., 2013). However, Wu et al. (2019) produced another esgRNA, introducing mutations in the native gRNA scaffold slightly different from those made by Qin et al. (2020), and observed only modest increases in BE efficiency. Although these studies tested the distinct esgRNA in different target genes, the data suggest that changes in the native gRNA need to be carefully tested.

PRIME EDITING

Prime editing was first described at the end of 2019 in human and yeast cells (Anzalone et al., 2019) and since then it has been used broadly in several plant species (Butt et al., 2020; Hua et al., 2020; Jiang et al., 2020; Li et al., 2020b; Lin et al., 2020; Xu et al., 2020a,b; Lu et al., 2021; Perroud et al., 2022). PE is based on a "search and replace" approach in which a nickase Cas9 (nCas9) is fused to a reverse transcriptase (RT), coupled to a prime editing guide RNA (pegRNA) that specifies both the target site and provides the template for the edition (**Figure 2**). The mechanism of PE action is described in **Figure 3**.

PE is divided into three main strategies: PE1 strategy 1 (PE1) uses a wild type Moloney murine leukemia virus (M-MLV) RT fused to a nCas9 and a pegRNA. PE strategy 2 (PE2) uses an engineered RT to increase the edition efficiency (mutations that affect thermostability, processivity, DNA–RNA substrate affinity and RNaseH activity) and therefore, exhibited

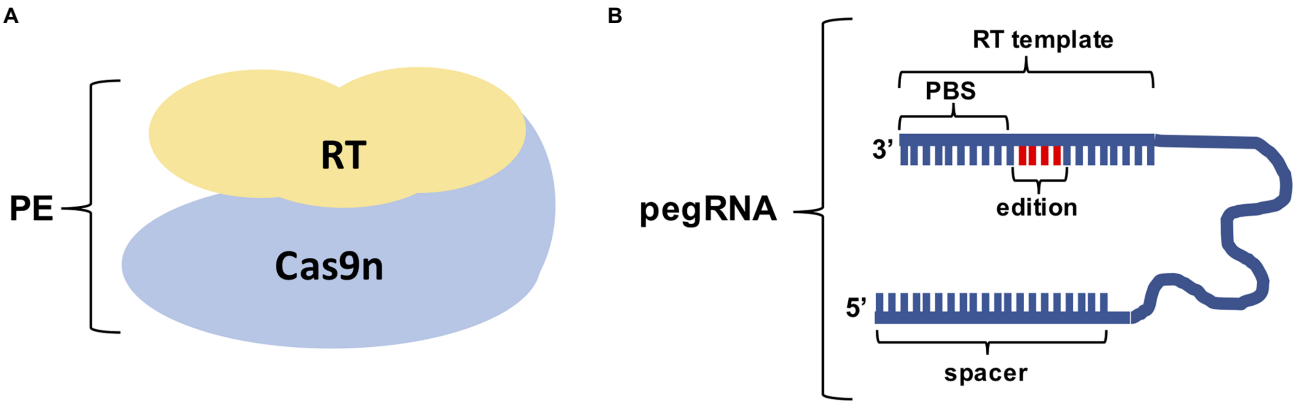


FIGURE 2 | Components of the Prime editing technique. **(A)** Cas9 nickase (Cas9n) is fused to a Reverse Transcriptase (RT) to form the Prime editing protein complex. **(B)** The pegRNA contains the Prime Binding Site (PBS) which is used to prime the reverse transcriptase reaction, the template containing the desired edit and the spacer that will guide the Cas9n to the target.

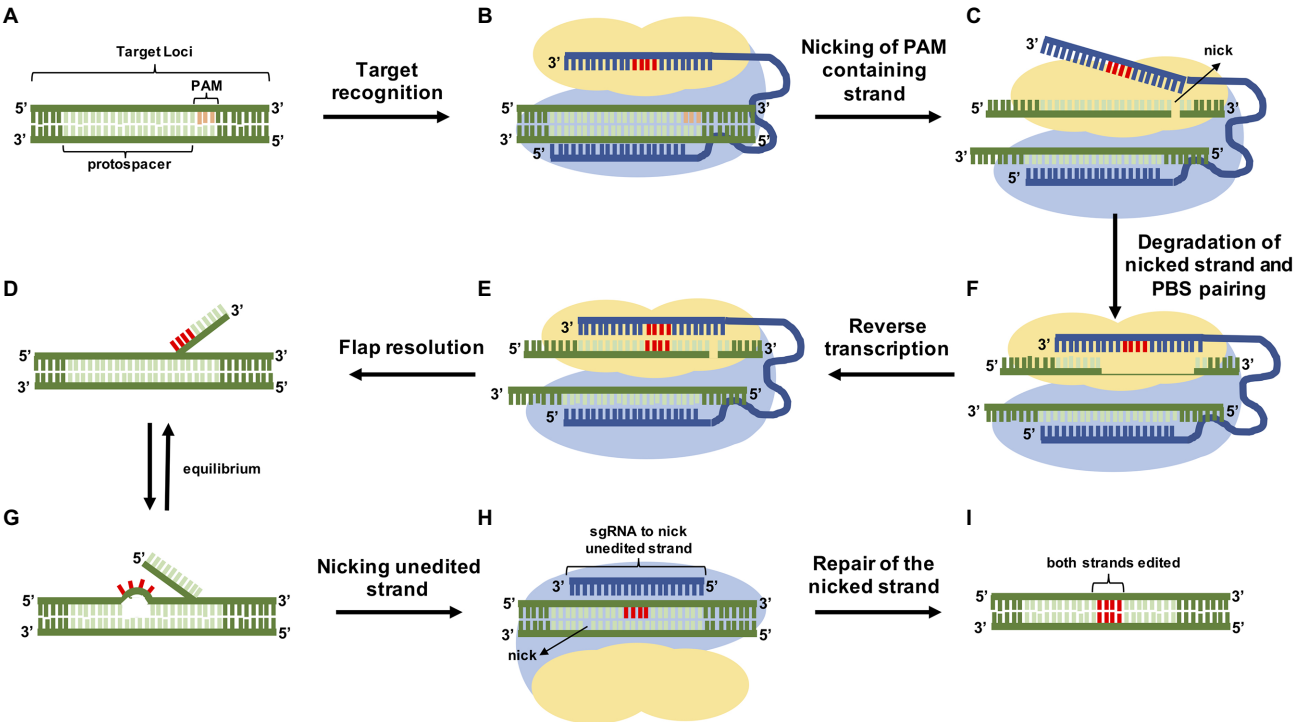


FIGURE 3 | Overview of precise genome modification using prime editing. At the genomic site **(A)**, the nCas9 (H840A) is led to the target by the pegRNA **(B)** and breaks just the strand containing the PAM of the DNA duplex **(C)** exposing a 3′-hydroxyl group (3′ flap), that, together with the extension of the pegRNA PBS **(D)** is used to prime the RT reaction, inserting the edit **(E)**. The editing area contains two single-stranded flaps in equilibrium: the 3′ flap containing the edition **(F)** and the 5′ unedited flap **(G)**. The endogenous cellular endonucleases prefer 5′ flaps as a substrate digesting it and leaving the 3′ flap to be ligated. At the end of the process the nicked DNA strand is replaced by the newly synthesized strand that had the information copied from the pegRNA generating a heteroduplex. In this case, the repair mechanism is going to resolve the mismatch using one of the strands, so there is a 50% chance of the edition being repaired by the cell. To overcome this setback, the induction of a second nick on the unedited strand using a sgRNA **(H)** stimulates the repair by copying the information present on the edited strand **(I)**.

a 1.6–5.1-fold improvement in introducing point mutations. PE strategy 3 (PE3) uses a second sgRNA to perform a nick on the non-edited strand to induce its replacement and increase the edition efficiency. PE strategy 3b (PE3b) uses a sgRNA that contains mismatches in order to be complementary to the edited strand, but not the original one, therefore, being active after the flap resolution. PE3b has 13-fold less indels compared to the PE3 in human cells (Anzalone et al., 2019).

The technique was successful to induced insertions (up to 44 nt), deletions (up to 80 nt), all types of transitions (C to

T, G to A, A to G, and T to C), and transversions (C to A, C to G, G to C, G to T, A to C, A to T, T to A, and T to G) without inducing DBS. However, the authors describe that each prime editing experiment must be optimized because several factors can affect the efficiency, such as the source of RT enzyme, thermostability and binding capacity of the RT enzyme, length of the RT template, length of the PBS, position of the nicking sgRNA in the unmodified strand, secondary structure of the pegRNA, G/C content of the PBS, the target gene and location of the mutation relative to the PAM site (Anzalone et al., 2019).

In plants, these strategies have been tested in rice and the evidences show that PE3 does not increase the efficiency in relation to PE2 (Butt et al., 2020; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a). PE3b also does not increase the efficiency in relation to PE3 (Jiang et al., 2020; Lin et al., 2020; Xu et al., 2020a). For PE3 and PE3b a second nick is performed on the non-edit DNA strand to stimulate the repair using the new reverse transcribed strand as a template. Besides the different PE strategies, PBS, template length, nicking positions also have to be optimized for each target gene (Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a,b; Lu et al., 2021). However, Lu et al. (2021) concluded that closer second nicks and simpler base changes affect the efficiency positively.

Lin et al. (2020) did an extensive evaluation of all types of base editions, deletions and insertion on several rice targets. They concluded that, although successful, each of them presented different efficiencies in different targets and that editing efficiencies decreased as the size of the intended deletions or insertions increased. Hua et al. (2020) showed in rice that only 9.1% of the regenerated plants were edited for a simple S627N change in the *OsALS* gene, while other targets (APO1, SLR1, OsSPL14, and APO2) aiming C42F change, 3-bp, 24-bp, and 24-bp insertion respectively, had no mutated lines. These data reinforce that the efficiency of even simple edits varies at different sites and longer insertions are harder to achieve. Xu et al. (2020a) using the *OsPDS* as a model analyzed different types of editions (insertion of 1, 2 or 3 nt; 28 bp deletion; A to T or A to C modification) and also observed a wide amplitude of edition frequencies (from 0 to 31.3%), with transversion being the most efficient edit.

Optimizations of nCas9 and RT

The system can be modified by exchanging the nCas9-RT fusion and its expression. Hua et al. (2020) tested SaCas9, a multiple-turnover enzyme that releases DNA faster than SpCas9 (Yourik et al., 2019). SaCas9 was used to target the mutated version of the EGFP gene expressed in rice calli, but only 5.12% expressed GFP, much less than the 52.5–55.5% successfully edited using SpCas9. SaCas9 may require a different sequence and structure of sgRNA, and optimization of the pegRNA is required for this type of protein.

Xu et al. (2020b) fused a hygromycin phosphotransferase to the c-terminus of the nSpCas9-M-MLV region with a self-cleaving 2A peptide. Overall, most of the edits had their efficiency significantly increased (when compared to the nSPCas9-M-MLV alone) and it was especially efficient to induce multiple bases mutations.

Codon optimization and the use of alternative promoters are also strategies tested in order to increase PE efficiency. Tang et al. (2020) optimized both Cas9 and M-MLV codons for plants and added a nuclear localization signal and even though the efficiencies in rice protoplast were very low (0.1–1.55%), the optimization increased slightly the number of edited reads. Lu et al. (2021) also showed that codon optimization of the M-MLV RT increased by three times the editing efficiency in tomato, while the RPS5A promoter was also three times more effective than the usual 35S promoter.

Lin et al. (2020) evaluated the replacement of the engineered M-MLV RT by the cauliflower mosaic virus RT (RT-CaMV; Plant et al., 1985) or a retron-derived (RT-retron) from *Escherichia coli* BL21 (Lim and Maas, 1989) in an assay to convert the blue fluorescent protein (BFP) to GFP (Zong et al., 2017). Rice protoplasts were analyzed by flow cytometry and the M-MLV RT presented a 4.4% efficiency, RT-CaMV presented 3.7% and RT-retron 2.4%. M-MLV RT and RT-CaMV were also tested in an endogenous gene and showed significantly higher efficiency.

pegRNA Optimizations

Some pegRNA design tools have been reported, such as Multicrispr (Bhagwat et al., 2020), PrimeDesign (Hsu et al., 2021), and PlantPegDesigner (Lin et al., 2021), but both of them are suitable for human/mouse genomes only. Despite that, pegRNAs can be optimized by changing their promoters, and therefore increasing their expression, changing the PBS or the template lengths. One study reported a high-throughput evaluation of PE2 activities in human cells using 54,836 pairs of pegRNAs to develop computational models to predict pegRNA efficiency and an accuracy by Spearman's correlations was between 0.47 and 0.81. The author's recommendations were: (1) use a 13-nt PBS and a 12-nt RT template; (2) use a high GC count in the PBS region if possible; (3) use a G at the last templated nucleotide when the RT template length is $\leq$12 nt; and (4) include PAM editing for human cells (Kim et al., 2021).

For plants, Lin et al. (2020) tested a variety of pegRNAs with differential PBS and RT template lengths and also nicking positions. The authors concluded that these factors strongly affect the editing frequencies in rice, and each target site presents optimal parameters. Jiang et al. (2020) hypothesized that the efficiency could be improved by enhancing the expression of pegRNAs. However, they verified that doubling the pegRNA expression cassettes in rice did not increase PE frequency.

The common conclusion of all studies that tested different PSB and RT length in plants, such as tomato (Lu et al., 2021), rice (Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a,b), is that each target there has its optimal combination of PBS and template length, and for every study several tests must be performed to find the best strategy. Lin et al. (2021) described the in rice the optimal performance was achieved when the PBS had a melting temperature of 30°C and using two pegRNAs in trans coding for the same edits (called paired pegRNA) increased the edition efficiency from 2.9-fold to 17.4 fold and also launched a web application (PlantPegDesigner) for optimal pegRNA and paired pegRNA design.

A recent study (Liu et al., 2021) described the enhanced prime editing system (ePE) in which to avoid the circularization of the pegRNA due to complementarity between the PBS 3′ the spacer 5′ the 20-nt Csy4 recognition site was added to the 3′ end of the pegRNA. This site forms a hairpin therefore, avoiding pegRNA circularization and increased editing efficiency although the addition of Csy4 slightly increased indels frequency. This technique needs further optimization and there are no reports of its use in plants yet.

Reducing Off-Targets in Prime Editing

Prime editing has a lower incidence of off-targets when compared to conventional CRISPR/Cas9 approach (Anzalone et al., 2020). The higher efficiency may be due to the three-step hybridization necessary for the editing. The first one is between the target DNA and the spacer present in the pegRNA, the second one between the PBS and the target DNA to start the RT priming and finally, the hybridization of the DNA flap. In the conventional CRISPR/Cas9 approach, only one hybridization is necessary, between the target DNA and the spacer in the sgRNA. The off-target rate in each experiment can also be influenced by the target DNA configuration in the chromosome, as heterochromatin or euchromatin (Kallimasioti-Pazi et al., 2018; Verkuijl and Rots, 2019). However, only one of all the studies in plants reported the evaluation of off-targets (Li et al., 2020b). Authors used the CRISPR-GE software[2] and posterior PCR amplification and sequencing of 10 putative off targets of four genes targeted by prime editing, and they found no mutations, indicating a high specificity.

Kim et al. (2020a) developed a strategy named Nickase-based Digenome-seq (nDigenome-seq), based on next generation sequencing to recognize all off-targets produced by experiments using PE2 *in vitro* in human cells. They demonstrated that undesired DNA modifications were detected in 0.1–1.9% of the cases, which can be considered as very low. Despite the importance of this type of evaluation, only Li et al. (2020b) described that no off-targets were detected; however, several by-products such as chimeras and unexpected insertions were detected in rice plants. Kim et al. (2021) also found that PE specificity in human cells could be further improved by incorporating mutations from engineered Cas9 variants, particularly eSpCas9 and Sniper Cas9, into PE, and these strategies can also be used for plants.

One of the main pitfalls of PE technique is the relative high frequency of by-products such as incorporation of pegRNA scaffolds, incomplete editions due mistakes of the repair system and unexpected deletions and insertions. Reports indicate up to 37.2% frequency of by-products in maize (Jiang et al., 2020) and 2.23% in tomato (Xu et al., 2020b). In rice, by-products are also reported (Lin et al., 2020; Tang et al., 2020; Xu et al., 2020a; Li et al., 2020b). Lin et al. (2020), studying rice and wheat, tested several targets and pegRNAs for each one of them and concluded that different PBS length did not affect the frequency of by-products. Meanwhile, template length not only dramatically affected the proportion of by-products, but were also variable depending on the target locus. Interestingly, OsCDC48-T3 and OsEPSPS-T2 target sites were less edited, despite the high indel frequency generated by nCas9, indicating that prime editing activity is independent from Cas9 activity at some targets. Xu et al. (2020a) described that PE3 with second nick closer to the first original nicking site was more efficient in producing edited plants, but also produced a higher amount of plants with by-products.

Some studies were capable of regenerating plants of rice (Butt et al., 2020; Hua et al., 2020; Li et al., 2020b; Lin et al., 2020; Xu et al., 2020b), tomato (Lu et al., 2021), and maize (Jiang et al., 2020). However, despite all the efforts, these works have found that the production homozygous plants in the first generation has a very low efficiency. Most works reported heterozygous or chimeras which leads to the conclusion that this technique needs more improvement before being widely applied, namely in polyploid species.

CRISPR/Cas12a SYSTEM

The Cas12a endonuclease belongs to type V of class 2 effector proteins (Koonin et al., 2017) and has a size between ~1,200 and ~1,500 aa as a single subunit protein (Zetsche et al., 2015). Guided by a single mature crRNA of 42–44 nt length, Cas12a binds upstream of a typically thymidine-rich PAM TTTV (V = A, C, and G) and cleaves DNA 18–23 nt distal of the PAM *via* 5 bp staggered double-stranded breaks (Zetsche et al., 2015). Different from the Cas9 endonuclease which requires a tracrRNA and RNase III, Cas12a maturates the pre-crRNA by its intrinsic ribonuclease activities (Fonfara et al., 2016). The action mode of Cas12a is depicted at **Figure 4**.

The CRISPR/Cas12a system has been used mostly for mutagenesis through NHEJ repair in plants, but also HDR (Begemann et al., 2017; Li et al., 2019) and base editing have been demonstrated (Li et al., 2018b; Kleinstiver et al., 2020). With its great potential as a genome editing tool in plants, the CRISPR/Cas12a system has been improved to achieve higher editing efficiencies, more relaxed PAM requirements (Gao et al., 2017) and better efficiencies in multiplexed systems (Zhang et al., 2021).

Assessment of "New" Cas12a Orthologs and Variants

In plants, mainly three Cas12a orthologs—FnCas12a (*Francisella novicida* Cas12a), LbCas12a (*Lachnospiraceae bacterium ND2006* Cas12a), and AsCas12a (*Acidaminococcus* sp. BV3L6)—have been used for genome editing (Zhong et al., 2018; Bandyopadhyay et al., 2020). Because of Cas12a's restrictive PAM site requirement, TTTV, one major goal of researchers is to increase the PAM flexibility of Cas12a (Bandyopadhyay et al., 2020). While FnCas12a was initially shown to recognize TTV PAMs *in vitro* (Zetsche et al., 2015) and *in vivo* (Endo et al., 2016), Zhong et al. (2018) showed that in rice protoplasts FnCas12a mediated a high editing efficiency at all eight tested TTTV PAM sites (10–35%), while the efficiency at TTV PAM sites was relatively low (0–10%). A systematical comparison of all possible VTTV and VTTTV PAM sites while using the same protospacer sequences showed that ATTG/ATTTG, CTTC/CTTTC, and

[2]http://skl.scau.edu.cn/

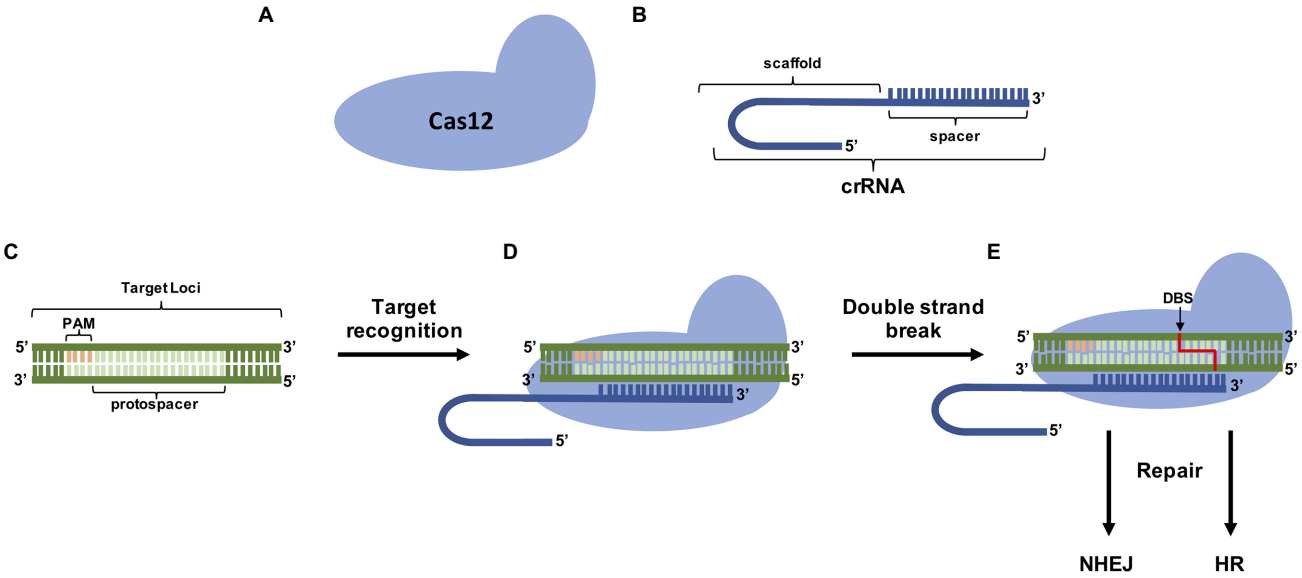


FIGURE 4 | CRISPR/Cas12a genome editing components. **(A)** The Cas12a protein capable of provoking double-strand breaks (DSBs) in specific target sites with the help of **(B)** the CRISPR RNA (crRNA) that contains the sequence (spacer; **C**) complementary to the target 3′ from the Protospacer Adjacent Motif (PAM) site. **(D)** The ribonucleoprotein complex leads to specific DSB located generally cleaves DNA 18–23 nt distal of the PAM *via* 5 bp staggered double-stranded breaks. **(E)** Repair mechanisms (NHEJ and HR) fix DSBs.

CTTG/CTTTG were equally edited by FnCas12a, while FnCas12a failed to edit only at GTTA or GTTC PAM sites.

The use of RR and RVR variants of AsCas12a and LbCas12a *in vitro* and in human cells were shown to overcome the strict requirement for TTTV PAM sites (Gao et al., 2017). In a similar fashion, LbCas12a-RR/FnCas12a-RR and LbCas12a-RVR/FnCas12a-RVR were tested in rice protoplasts at CCCC/TYCV PAMs (Y is C or T) and TATV PAMs, respectively (Zhong et al., 2018). The editing activity of LbCas12a-RR was tested at all tested CCCC and 10/11 TYCV PAM sites between 2 and 40% (11/13 higher than 10%) and outperformed FnCas12a-RR. The RVR variants were not able to mediate editing at TATC PAM sites and while both variants showed activity at two out of three TATG PAM sites, LbCas12a-RVR outperformed FnCas12a-RVR. In rice T0 plants LbCas12a-RR showed the highest and most robust editing frequency at two tested TTCC PAM sites (93.3 and 100%), while no or low editing activity of FnCas12a-RR at all tested PAM sites was observed.

Zhang et al. (2021) tested eight Cas12a orthologs in plants, which previously showed cleavage activity preferentially at TTV PAM sequences *in vitro* (Zetsche et al., 2020). From these, four orthologs [ErCas12a (MAD7, Inscripta, Inc.), Lb5Cas12a, BsCas12a, and Mb2Cas12a] showed high editing efficiencies at two different target sites in rice protoplasts (*OsDEP1-TTTC* and *OsEPFL9-TTTG*), which were comparable to those of LbCas12a (>30%). Also in transgenic rice lines, most of the tested Cas12a orthologs showed medium to high editing efficiencies at both target sites compared to LbCas12a.

Interestingly, Mb2Cas12a induced genome editing at 13 out of 18 tested VTTV PAM sequences with ~15% or higher efficiency. The editing efficiency of Mb2Cas12a at two more relaxed target sites (*OsROC5-GTTG* and *OsDEP1-GTTC*) was ~10% and Mb2Cas12a outperformed all other tested Cas12a orthologs. Introducing a RVR mutation into Mb2Cas12a (Mb2Cas12a-RVR) resulted in editing efficiencies from 15 to 35% at all TATV PAM sites which were higher than those from all other tested RVR variants (Zhang et al., 2021). The RVRR variant (Mb2Cas12a-RVRR) was active at TTTV, VTTV, TATV, TYCV, CCCV, and CTCV PAM sites and the genome-wide PAM analysis revealed that all targetable sites of Mb2Cas12a-RVRR cover 22.2% of the total rice genome (SpCas9 10.5%; Zhang et al., 2021). Recently, an engineered variant of LbCas12a (impLbCas12a) was shown to cleave TNTN PAMs with higher activity (Tóth et al., 2020) and could be a promising tool in plants.

Overcoming Temperature Sensitivity of Cas12a

The above-mentioned temperature sensitivity of Cas12a nucleases is another drawback for their application in plants, because most plant transformation and cultivation processes are performed at lower temperatures (Bernabé-Orts et al., 2019; Lee et al., 2019; Malzahn et al., 2019). Malzahn et al. (2019) showed that AsCas12a, FnCas12a, and LbCas12a performed significantly better at temperatures ≥28°C compared to 22°C. Enhanced AsCas12a (enAsCas12a) showed two times higher activity at lower temperatures compared to AsCas12a in human cells (Kleinstiver et al., 2020), but was still outperformed by LbCas12a *in vitro* (Schindele and Puchta, 2020). Schindele and Puchta (2020) engineered, based on the findings from Kleinstiver et al. (2020), two LbCas12a variants (enLbCas12a and ttLbCas12a) and found that editing efficiencies of ttLbCas12a were 2- to 7-fold higher than those

of LbCas12a at 22°C and were even higher than those of the other variants at 28°C.

Interestingly, Mb2Cas12a was less sensitive to lower temperatures (22°C) compared to other Cas12 orthologs and the activity could be further improved by introducing mutations equivalent to enAsCas12a (Kleinstiver et al., 2020; Zhang et al., 2021).

Improving cRNA Design

Cas12a presents both endoribonuclease and endonuclease activity (Fonfara et al., 2016; Creutzburg et al., 2020). Consequently, it is not sufficient to predict Cas12a's editing efficiency only based on the spacer-protospacer complementarity, but also on the efficiency of pre-crRNA maturation (Creutzburg et al., 2020). The maturation of the pre-crRNA is catalyzed by the ribonuclease domain of Cas12a after recognition of the hairpin structure formed by the repeat sequence at the 5′ end of the pre-crRNA (Fonfara et al., 2016; Creutzburg et al., 2020). The presence of surrounding nucleotides that base pair with nucleotides in the repeat sequence can result in alternative secondary structures which are not recognized by Cas12a (Creutzburg et al., 2020). While Creutzburg et al. (2020) provide valuable recommendations for crRNA design, it would be required to develop tools that predict efficient crRNAs also based on secondary structures.

While typically a crRNA of FnCas12a has a 24-nt guide sequence, Negishi et al. (2020) showed that the length of this guide sequence (18–30 nt) can positively influence the editing efficiency, while off-targets are not affected. It seems that there is an optimal length for each target sequence that must be tested individually. Tang et al. (2017) investigated the target specificity of LbCas12a by introducing double mismatch mutations into the protospacer and found that mismatches in the first 18 nt of the 23 nt of the protospacer abolished cleavage activity and mismatches at position 21–22 reduced cleavage activity by ~50%. These findings are in line with data from Zhang et al. (2021) that also confirm the high targeting specificity of several Cas12a orthologs.

Increasing Multiplexing Efficiency in Cas12a Systems

Multiplex genome editing allows the introduction of two or more precise gene mutations in one plant generation and has the potential to significantly accelerate breeding processes (Abdelrahman et al., 2021). Several multiplex Cas12a systems in plants have been developed, but the efficiency of high biallelic editing frequencies in all targeted genes were very low (Wang et al., 2017b, 2018; Tang et al., 2019). An extensive comparison of different multiplexed systems indicated that the editing efficiency of all tested Pol II promoter-based systems were superior to Pol III promoter-based systems (Zhang et al., 2021). The most efficient combination was LbCas12a with a pZmUbi-HH-crRNA-HDV system, which resulted in 26/36 T_0 lines carrying biallelic mutation in all four targeted genes. The same system, in combination with Mb2Cas12a, resulted in 8/11 T_0 lines with biallelic mutations at all four sites. Mb2Cas12a-RVRR was able to edit seven targets with canonical and altered PAM sequences with editing efficiencies ranging from 5.1 to 31.1% in rice protoplasts. The power of this system was further demonstrated by simultaneously targeting 16 genomic sites across nine chromosomes in rice plants. While 20/21 T_0 lines contained at least 13 edited sites of which at least seven were biallelic edits, 11/21 T_0 lines had 15 edits and 1/21 T_0 line showed editing in all 16 targeted genes. The proposed system allows editing of 14 sites simultaneously with biallelic mutations.

Increasing HDR Efficiencies in Cas12a Systems

Cas12a is particularly suited for HDR as it induces a double-strand break 18 nt distal of the PAM and does not destroy the target sequence (Moreno-Mateos et al., 2017). Begemann et al. (2017) first demonstrated the capability of FnCas12a for an efficient targeted insertion in rice plants (8%), which performed better than LbCas12a under comparable conditions (up to 3%).

One strategy to improve the low HDR efficiency is to optimize the template delivery and Vu et al. (2020) utilized a geminiviral DNA replicon to express LbCas12a in tomato. The HDR efficiency mediated by LbCas12a expressed from the construct containing a geminiviral replicon (~4.5%) was significantly higher than the control and similar to the CRISPR/SpCas9-based construct (~3.6%). Further results showed that at higher temperatures (31°C) the efficiency can be significantly improved (9.8%). The use of a T-DNA system containing multiple replicons further increased the levels of donor template after the transformation step and resulted in higher HDR efficiencies up to 12.79%. In Arabidopsis, the variant ttLbCas12a outperformed LbCas12a at 22°C/28°C and ttLbCas12a showed a higher HDR efficiency at 22°C compared to LbCas12 at 28°C (Merker et al., 2020).

CRISPR/Cas12b SYSTEM

Cas12b (formerly C2c1) is a class 2 type V-B endonuclease and represents the third major CRISPR system that has been used for plant genome editing (Shmakov et al., 2015; Ming et al., 2020; Wang et al., 2020d; Wu et al., 2020). Like Cas12a, Cas12b recognizes T-rich PAM sequences, TTN, and generates staggered DSBs (7-nt 5′-overhangs) 23 bp upstream of the PAM on the non-target strand (Shmakov et al., 2015; Liu et al., 2017b). Unlike Cas12a, Cas12b is a dual-RNA-guided endonuclease which utilizes a cRNA and tracrRNA (or combined engineered sgRNA) for DNA recognition (Shmakov et al., 2015). The highest cleavage activity of Cas12b was achieved in the presence of Mn^{2+} and at temperatures between 37°C and 60°C (Shmakov et al., 2015; Yang et al., 2016; Liu et al., 2017b).

The first identified Cas12b orthologs, *Alicyclobacillus acidoterrestris* Cas12b (AacCas12b) and *Bacillus thermoamylovorans* Cas12b (BthCas12b) required high temperatures (48–50°C) for optimal activity *in vitro* (Shmakov et al., 2015). Wang et al. (2020d) used an AacCas12b-based system to edit the *GhCLA* gene in cotton, which as a thermophilic plant is able to survive higher temperatures. From three different temperatures (42°C, 45°C, and 48°C) and eight incubation times (ranging from 6 h to 15 days), the highest editing efficiency was achieved at 45°C after 4 days (Wang et al., 2020d).

The discovery of *Alicyclobacillus acidiphilus* Cas12b (AaCas12b; Teng et al., 2018) and later *Bacillus hisashii* Cas12b (BhCas12b; Strecker et al., 2019) which showed activity at lower temperatures, enabled the broader use of Cas12b for plant genome editing. In a comparative study, Ming et al. (2020) showed that AaCas12b has an editing efficiency of ~10% in rice protoplasts, while AacCas12b (~5%) and BthCas12b (very low) were less efficient. All three tested endonucleases generated 4–14 bp deletions located 12–24 bp distal to the PAM sites. Testing different PAM requirements for AaCas12b and AacCas12b revealed that both are capable of editing VTTV PAM sites with a preference for ATTV and GTTG sites. AaCas12b was further shown to be very sensitive to 2-pb mismatches in the protospacer and a protospacer length of ≤18 bp abolished the editing efficiency. 54.2% of the tested T_0 lines were edited by AaCas12b and of these 46.2% of the T_0 lines carried biallelic mutations.

Cas12b was further used in a multiplexed system with dual Pol II promoters and a hammerhead–sgRNA–hepatitis delta virus array to target three genes simultaneously (Ming et al., 2020). Compared to AacCas12b, AaCas12b performed better at two out of three target sites, but showed no activity at one site. At the two edited sites, 29.2 and 45.8% of the tested T_0 line carried biallelic mutations.

CRISPR/Cas12f SYSTEM

Recently, two Class 2 miniature type V-F Cas nucleases, SpCas12f1 (497 aa) and AsCas12f1 (422 aa), have been used for genome editing in maize (Bigelyte et al., 2021). With their compact size compared to Cas9 and Cas12 endonucleases, Cas12f nucleases could be a good choice when using viral-based delivery systems. Furthermore, their dependence on higher temperatures for optimal activity (45–55°C) could help to better control off-target effects in some plant species (Bigelyte et al., 2021).

FINAL REMARKS

There is an urgent need to improve crop sustainability and the use of gene editing technologies will not only to increase our ability to modify traits already present in the crop, but also introduce new traits that so far have only been achieved with regular transgenesis, such as tolerance to herbicides (Dong et al., 2021) and insects (Tyagi et al., 2020). The use of deep learning technologies certainly will help our ability to engineer new proteins, with not only higher specificity, but also higher efficiency. In this sense, there is a plethora of organisms that have CRISPR-like system, including extremophyles, that remain to be explored (Salwan et al., 2020). In **Table 1**, we have compiled components that can be manipulated in order to increase the editing efficiency of CRISPR-based methods. It is worth mentioning that high throughput methods to screen new versions of all the editing components will improve our ability to discover new improvements in gRNA and protein structures, new editing features, among others.

Genome editing technologies are a new addition to the toolbox of genetic engineering that so far has relied extensively in the addition of DNA sequences from other organisms. In spite of all the scientific evidence indicating genetically modified organisms (GMOs) are safe for human consumption, skepticism still remains among consumers, with 46.7% having a negative view on GM food in China (Cui and Shoemaker, 2018), 57% in the United States (Pew Research Center, 2015), and 34.7% in Canada (Charlebois et al., 2019). Although increased knowledge from qualified sources are known to move this trend towards a positive view on GMOs (Wunderlich and Gatto, 2015; Hunt and Wald, 2021), there is evidence that precision genome editing, without the incorporation of recombinant DNA in plant genomes will have a higher acceptance than GMOs because no exogenous DNA would be introduced (Muringai et al., 2020).

Additionally, after all components of the gene-editing system have been integrated into the host genome, chances of off-target indels increase with time (Lawrenson et al., 2015). The capability of producing transgenic plants without the integration of foreign DNA would alleviate the regulatory concerns in order to commercialize new varieties (Jones, 2015).

The delivery of reagents for genome editing without incorporating DNA into the genome results in mutations identical to naturally occurring events. This approach, also called DNA-free genome editing, has been reported in protoplast from several plant species with a mutagenesis frequency of up to 46% in Arabidopsis, tobacco, rice, and lettuce plants (Woo et al., 2015; Kim et al., 2017). However, regeneration of plants from transformed protoplasts, especially monocots, can be challenging. To overcome this, Zhang et al. (2016) used biolistics to introduce Cas9 mRNA and gRNA into immature wheat embryos that were allowed to regenerate plants without selection. This strategy resulted in genome editing at a ratio of 1.1 plants per 100 bombarded embryos. In addition, transformed plants were transgene-free, since no DNA was used in the process.

The use of DNA-free CRISPR/Cas techniques in crop plants is particularly interesting due to the regulatory issues involving the release of commercial products from genetically modified organisms. Recently, the USDA-APHIS confirmed that plants with phenotypes developed through genome editing without foreign DNA inserted in the genome will not require regulatory approval for commercial production in the United States (Waltz, 2016). Products to be commercialized will be evaluated on a case-by-case basis, but certainly will result in a remarkable reduction of regulatory costs for cultivar development. In Brazil, genome editing technologies will not be regulated as GMOs, on a case-by-case analysis (CTNBio, 2018). On the contrary, in Europe, the decision on regulating precision breeding technologies as GMOs has created concerns on the continent's economy (Hjort et al., 2021). On a global scale, local authorities are still discussing regulatory frameworks for genome-edited plants in other countries. In the meantime, delivery methods have been developed that introduce targeted mutations without any transgenic footprint of the genome editing tools (Liang et al., 2017). This transformative technology will allow a paradigm

TABLE 1 | Overview of tools and techniques to increase genome editing efficiency.

Component	Strategies	Impact	Future developments
Guide RNA/ pegRNA	- New computational algorithms allow the design of gRNA sequences with high affinity and specificity to the target sites, as well as higher affinity with the nuclease - Larger gRNAs (pegRNA) provide an HR template when combined with nuclease/ reverse transcriptases	- Reduced binding to non-target sites - Enhanced binding to target sites - Higher nuclease activity	- Improved algorithms to decipher the best gRNA sequence - Improve prediction for best optimal combination of PBS and template length
Nucleases	- Protein engineering to produce evolved nucleases - Association with proteins or domains with new features - Discovery of new nucleases	- Higher DNA cutting activity - Increased affinity to gRNA - Broader target selection, allowing to edit the entire genome (PAM-less nucleases) - New functions by fusion with other proteins, such as deaminases and reverse transcriptase - Improved nuclear localization - Higher activity at temperatures used in plant cell tissue culture	- Computational tools to create new protein structures - Identification of proteins from a broader range of organisms, with new abilities - Increase the editing power in order to decrease chimera or heterologous plants
Deaminase	- Protein engineering - Fusions with new proteins or protein domains	- New editing capacities, such as transversions - Higher predictability of the edited base	- Discovery of new deaminases or other protein modules that can improve the editing process
HR templates	- Increased template availability by using synthetic DNA more resistant to nucleases, higher copy number using virus replicons, and co-localization of the HR template to the RNP complex by fusions with the gRNA	- Higher probability of HR, therefore, increased desired editing rate	- Better understanding of key cellular components involved in HR enhancement and also in HR inhibition - New strategies to increase HR stability and proximity to the editing site
Computational tools	- Identification of new nuclease variants with increased specificity and efficiency - Increased accuracy of both target and off-target predictions		- New experiments designed to gather data to feed the algorithms - New algorithms and publicly available servers - Decipher the role of epigenomics and chromatin structure in the gRNA binding and nuclease activity
Plant tissue culture	- Protocols that allow the selection of edited cells without transgenes, due to mutations in genes encoding herbicide tolerance	- GMO-free edited plants	- Multiplex editions, targeting the herbicide tolerance gene and other genes related to other traits
Proteins components in general	- Codon optimization - Promoters and terminators with higher activity	- Enhanced levels of the proteins involved in gene editing	- Identification of new promoters and terminators with higher activity in the cell types and initial stages of the transformation

shift in improvement and streamline regulatory approval of genetically modified crops.

AUTHOR CONTRIBUTIONS

LM, MR, MS, RT, JS, KB, and MM: wrote and reviewed the paper. All authors contributed to the article and approved the submitted version.

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Increased sulfur-containing amino acid content and altered conformational characteristics of soybean proteins by rebalancing 11S and 7S compositions

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Soybean proteins are limited by their low contents of methionine and cysteine. Herein, 7S globulin accumulation was reduced using RNA interference to silence *CG-β-1* expression, and the content of the A2B1a subunit was largely increased under the soybean seed-specific oleosin8 promoter. The results showed that the sulfur-containing amino acid content in soybean seeds drastically improved, reaching 79.194 nmol/mg, and the 11S/7S ratio had a 1.89-fold increase compared to the wild-type acceptor. The secondary structures of 11S globulin were also altered, and the β-sheet content increased with decreasing β-turn content, which was confirmed by Fourier transform infrared spectroscopy, Raman spectroscopy and circular dichroism analysis. Our findings suggested that raising the accumulation of 11S glycinin at the expense of reducing the content of 7S globulin is an attractive and precise engineering strategy to increase the amount of sulfur-containing amino acids, and soybean proteins with A2B1a subunits of 11S isolates improved, and β-subunits of 7S fractions reduced simultaneously might be an effective new material for food production.

KEYWORDS

sulfur-containing amino acids, conformational characteristics, soybean proteins, RNA interference, 11S glycinin, 7S globulin

Introduction

Soybean [*Glycine max* (L.) Merr.] is a remarkable crop that serves as an excellent protein source for both humans and livestock (Krishnan and Jez, 2018). In contrast to the high protein content in soybeans, the sulfur-containing amino acids methionine and cysteine are deficient in monogastric diets and rations. Methionine cannot be synthesized in humans and monogastric animals and is considered an "essential" amino acid, and cysteine is a "conditionally" essential amino acid for animals that can convert methionine into cysteine

in vivo (Brosnan and Brosnan, 2006). There is a negative influence from an inadequate intake of sulfur-containing amino acids on the growth and development of animals. Therefore, it is therefore crucial to enhance the concentrations of these two sulfur-containing amino acids in soybeans (Imsande, 2001). Considerable efforts have been directed toward improving the content of sulfur-containing amino acids in soybean seeds, and multiple approaches, including traditional breeding and biotechnological methods, have been proposed to realize high concentrations of methionine and cysteine in proteins (Zhang et al., 2014). In the early stages of research, breeding methods that included the use of spontaneous or induced soybean mutants were used to increase the content of sulfur-containing amino acids (Paek et al., 1997, 2000). Imsande (2001) selected several methionine-overproducing genetic lines whose sulfur-containing amino acid concentrations were approximately 20% higher than those of the parent lines.

Biotechnological approaches have also been undertaken to increase the sulfur-containing amino acid content of soybean seeds. Several methionine-rich heterologous proteins have been overexpressed in transgenic soybean. For example, sulfur-rich zeins from maize have been expressed in transgenic soybean plants (Dinkins et al., 2001; Li et al., 2005; Kim and Krishnan, 2019), and even the *de novo* synthetic protein MB-16 has been introduced into soybean to improve protein quality (Zhang et al., 2014). Recent experiments have been developed to increase the sulfur-containing amino acid content of seeds by enhancing the expression of enzymes involved in the sulfur assimilatory and cysteine/methionine biosynthesis pathways. Overexpression of ATP sulfurylase increased the protein-bound cysteine and methionine contents in transgenic soybean seeds by 37–52% and 15–19%, respectively (Kim et al., 2020). A cytosolic isoform of O-acetylserine sulfhydrylase (OASS) has been introduced into soybean and could increase protein-bound cysteine levels by 58–74% compared with those of nontransformed wild-type seeds, and a 22–32% increase in free cysteine levels was also observed. Furthermore, the accumulation of a cysteine-rich protein, a Bowman-Birk protease inhibitor, markedly increased in transgenic soybean plants (Kim et al., 2012). The feedback-insensitive cystathionine γ-synthase obtained from *Arabidopsis* was also introduced into soybean, and the accumulation of methionine increased by 4.8-fold and 2.3-fold in seeds of transgenic Zigongdongdou and Jilinxiaoli 1 lines, respectively (Yang et al., 2018).

The major proteins stored in soybean seeds are β-conglycinin (a 7S globulin of 180 kDa) and glycinin (an 11S globulin of approximately 360 kDa), which account for 30–40% of the total weight (Nishinari et al., 2014). 7S β-conglycinin consists of three subunits, α, α' and β, with molecular weights of 67 kDa, 71 kDa and 50 kDa, respectively (Wu et al., 2016). The mature β-subunit of combined soybean seed storage proteins is composed of approximately 416 amino acid residues and lacks both methionine and cysteine (Paek et al., 1997). 11S glycinin is composed of six nonrandomly paired acidic and basic peptides. The acidic A polypeptide (37–42 kDa) and basic B polypeptide (17–20 kDa) are linked together by a disulfide bond (Wu et al., 2017). Soybean 11S globulin contains five major subunits, A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3, and has three to four times more sulfur-containing amino acids than 7S β-conglycinin does. A2B1a (encoded by *gy2*) is the most abundant Met+Cys and is present in all commercial soy protein isolates (Sui et al., 2021).

The specific units of protein secondary structure, including α-helix, β-sheet, β-turn, and random coil, are vital for the rigidity and flexibility of protein (Su et al., 2015). Native soybean glycinin and β-conglycinin are a combination of different isoforms with high heterogeneity, and at the tertiary level, the conformational stability/flexibility of their subunits or polypeptides is difficult to evaluate; in fact, each isoform possesses a quaternary structure. By comparison, the secondary structure in soy glycinin and β-conglycinin is highly ordered and preserved due to the strong restrictions from the presence of their quaternary structure (Tang, 2017). Globulins from soybean typically possess low levels of α-helices and very high β-sheet secondary structures, and they are often considered to be β-type proteins (Marcone, 1999). The conformational characteristics of soybean proteins are complicated by complex mixtures of different constituents with different structural and physicochemical properties. The properties and structural characteristics of soy proteins are highly dependent on the composition and proportion of 7S and 11S globulins (Tezuka et al., 2004; Tang, 2017). The β-sheet structure was most important for its significant role in denaturation of 7S globulin and subsequent formed aggregates and even in denaturation of 11S globulin, and biological structural-functional relationships are usually associated with protein applications (Wang et al., 2014).

In this study, the strong seed-specific promoters of soybean oleosin and α subunits were introduced to enhance the expression of *gy2* and to silence the *CG-β-1* gene by RNA interference, which sharply increased the content of sulfur-containing amino acids and the 11S/7S ratio in soybean seeds. Moreover, the soybean proteins with the A2B1a subunit of 11S isolates improved, and the β-subunit of 7S fractions reduced simultaneously, which would also alter conformational characteristics and supply a new formula for food products.

Materials and methods

Construction of vectors

pCambia1300-OsEPSPS-GmOle8pro was gifted by Dr. Zhu (Zhou et al., 2006). For β subunit of β-conglycinin silencing, 302-bp coding *CG-β-1* sequences were selected and amplified by high-fidelity *Taq* enzymes and then ligated into a pHANNIBAL vector in opposite orientations on either side of a PDK intron (Wesley et al., 2001). To more accurately interfere with the β subunit gene and reduce its influence on agronomic traits, the CaMV35S promoter was substituted with a seed-specific promoter from the α subunit of β-conglycinin of soybean. This *CG-β-1*

RNAi construct was then cloned into the pCambia1300-OsEPSPS-GmOle8pro plant expression vector to produce pCambia1300-OsEPSPS-RNAi-GmOle8pro. For overexpression of 11S glycinin containing high-sulfur-containing amino acids, the coding sequence of the A2B1a subunit obtained from soybean cultivar 'Tianlong No. 1' was cloned into the vector pCambia1300-OsEPSPS-RNAi-GmOle8pro to form pCambia1300-OsEPSPS-RNAi-GmOle8pro-A2B1a (Figure 1). The newly constructed plant expression vector was subsequently inserted into *Agrobacterium* strain EHA105 for transformation of soybean cotyledons.

Soybean cotyledon transformation

Transgenic soybean events were performed using a modified cotyledon transformation protocol (Du et al., 2016). Briefly, *Agrobacterium tumefaciens* strain EHA105 was chosen for the soybean cultivar 'Tianlong No. 1', cultured to an OD_{600} of 0.8–1.0, centrifuged and then suspended in cocultivation media (CCM liquid: 1/10 B5 salts, 30 g/l sucrose, 20 mmol/l MES, 1.67 mg/l BAP, 0.25 mg/l GA3 and 200 μmol/l acetosyringone) to an OD_{600} of 0.4–0.5. The soybean seeds were surface sterilized for 16 h using chlorine gas and germinated overnight on sterile filter paper. The cotyledonary nodes were cut into 5–7 slices vertical to the axis using a blade, dipped into the coculture suspension for 30 min at 28°C and then transferred onto solid cocultivation media (solid: CCM liquid plus 5 g/l agarose, 0.4 g/l L-cysteine, 0.154 g/l DTT, and 0.158 g/l nathiosulfate) for 5 days at 25°C under dark conditions. After cocultivation, the infected explants were washed in washing media (B5 salts, 30 g/l sucrose, 3 mmol/l MES, 1.67 mg/l BAP, 500 μg/ml carbenicillin and 50 μg/ml cefotaxime) and subsequently cultured on SI media (washing media plus 3.5 g/l phytagel) for 2 weeks at 25°C under a 16 h light and 8 h dark photoperiod. The explant tissues were then transferred to fresh SI media supplemented with 8 mg/l glyphosate for an additional 14 days. The differentiating explants were cut and transferred to SE media (MS salts, 30 g/l sucrose, 3.5 g/l Phytagel, 3 mmol/l MES, 5 mg/l asparagine, 10 mg/l pyroglutic acid, 0.1 mg/l IAA, 0.5 mg/l GA3, 1 mg/l zeatin riboside, 500 μg/ml carbenicillin and 50 μg/ml cefotaxime) to initiate shoot elongation with 8 mg/l glyphosate every 2 weeks. To initiate rooting, each elongated shoot (>3–4 cm) was cut and then transferred to rooting media (MS salts, 20 g/l sucrose, 3.5 g/l phytagel, 3 mmol/l MES and 0.5 mg/l IBA). The rooted plantlets were subsequently transplanted into soil and grown in a greenhouse. T_0 plants were identified *via* PCR and sprayed with 10 ml/l glyphosate. Each generation of positive plants was grown and selected by screening 10 ml/l glyphosate again, and homozygous T_5 transgenic plants were further analyzed.

Real-time RT–PCR analysis

Total mRNA was isolated from soybean seeds at the R6 stage using TRIzol reagent (Sangon Biotech, Cat # B610409) and a Spin Column Plant Total RNA Purification Kit (Sangon Biotech, Cat # B518661) and then treated with RNase-free DNase enzyme (Sangon Biotech, Cat. # B300066). For real-time RT–PCR analysis, 1 μg of total purified mRNA was used for cDNA synthesis (Sangon Biotech, AMV Reverse Transcriptase Kit, B600020). Amplifications were performed in a total volume of 10 μl consisting of SGExcel Ultra SYBR Mixture (Sangon Biotech, B110032), primer pairs (each at 0.2 μmol/l), and cDNA (10 ng of starting mRNA), and the soybean *actin* gene (GenBank: accession: V00450.1) was used as the control. The samples were amplified for 40 cycles, starting with an initial activation step of 3 min at 95°C, followed by denaturation for 10 s at 95°C, annealing for 10 s at 55°C and extension for 15 s at 72°C. At the end of PCR amplification, the temperature was increased from 50°C to 99°C at a rate of 0.2°C s^{-1}, and melting curves were constructed based on the fluorescence intensity. The primers used for qPCR are listed in Supplementary Table S1. The relative expression levels of the genes were calculated using the $2^{-\Delta Cq}$ method (Schmittgen and Livak, 2008).

ELISA assay

Soy protein isolates (SPIs) were obtained from defatted soybean flakes according to Thanh and Shibasaki's protocols

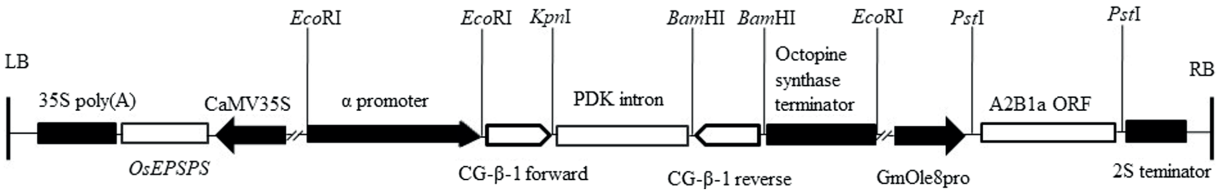


FIGURE 1
Schematic diagram of pCambia1300-OsEPSPS-RNAi-GmOle8pro-A2B1a. The soybean transformation construct contains the A2B1a subunit coding region (A2B1a ORF) under the control of the soybean oleosin8 promoter (GmOle8pro) and the 3′-end noncoding region of 2S albumin (2S terminator). The RNAi cassette under the control of the soybean α-promoter of β-conglycinin and the octopine synthase terminator, together with 302 bp fragments obtained from sequences of *CG-β-1* gene forward and reverse inserted side of PDK intron, respectively. The *OsEPSPS* gene under the control of the cauliflower mosaic virus 35S promoter (CaMV35S) and 35S poly(A) terminator acted as a selective marker. *Eco*RI, *Kpn*I, *Bam*HI and *Pst*I restriction sites are shown on the vector. LB, left border; RB, right border.

(Thanh and Shibasaki, 1976). The extracted total protein was resolved in sample buffer containing 62.5 mmol/l Tris–HCl, pH 6.8, 8 M urea, 15% SDS, 20% glycerol and 5% 2-mercaptoethanol, sonicated for 5 min, boiled for 5 min, and then centrifuged at 10,000×*g* for 10 min (Moriyama et al., 2005). The obtained clear supernatant was subsequently quantified using ELISA kits (AMEKO, Cat# AE98502G for glycinin, AE98605G for β-conglycinin) according to the manufacturer's protocol. Afterward, 100 µl of peroxidase substrate TMB solution was added to each well, and the reaction was terminated by the addition of 4 M H_2SO_4 solution (50 µl/well). The color change was measured spectrophotometrically at a wavelength of 450 nm. Calibration curves were then generated by plotting the optical density (OD) values against different gradient concentrations of standard samples.

Gel permeation chromatography

Gel permeation chromatography was performed using an EcoSEC HLC-8320 GPC system (Japan). Soybean protein isolate (consisting of 0.2% protein, w/v) was dissolved in DI water and filtered through a Millipore membrane (0.45 µm). The following conditions were used: injection volume, 2.0 µl; flow rate, 0.5 ml/min; elution solvent 1, 35 mm phosphoric potassium buffer (pH 7.6) including 0.1 mol/l sodium chloride; and elution solvent 2, 35 mm phosphoric potassium buffer (pH 7.6) including 0.1 mol/l NaCl and 6.0 M urea. The total elution time was 20 min, and the elution absorbance was measured at a wavelength of 280 nm. All the data were recorded and analyzed by Empower™ 3 software.

Amino acid analysis

The amino acid composition of soybean seeds was determined by an amino acid autoanalyzer (Model L-8900, Hitachi, Japan). In brief, 1.0 g samples were hydrolyzed with 10 ml of 6 M HCl for 24 h in Pyrex tubes for all amino acids, and then the hydrolysates were eluted through a concentration gradient starting at 0.2 M (pH 3.25) to 0.35 M (pH 5.25) at a rate of 0.4 ml/min. Amino acids were identified by an automatic amino acid analyzer equipped with an ion-exchange chromatography column. The total analysis time was 3.5 h per sample. The amino acid composition was reported in nmol/mg of protein powder on a dry weight basis. Each analysis was carried out for three replications.

Preparation of 11S and 7S proteins

The 11S and 7S proteins were isolated according to the method of Yang et al. (2020). The 11S resultant solution was subsequently dialyzed against deionized (DI) water for 48 h in a dialysis tube (MW of 300,000 Da) and freeze dried to obtain the 11S fraction. Similarly, the 7S resultant solution was subsequently dialyzed against DI water for 48 h in a dialysis tube (MW 100,000 Da) and then freeze-dried to obtain the 7S proteins.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Thanh and Shibasaki (1976) with 4% stacking gels and 12% running gels. The protein samples were diluted to a final concentration of 0.1 mg/ml and solubilized in 0.125 M Tris–HCl buffer (pH 6.8) consisting of 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol (2-ME), 5% (v/v) glycerol and 0.025% (w/v) bromophenol blue and heated in a water bath (100°C) for 5 min before electrophoresis. For each sample, 10 µg was added to each lane in the stacking gel and electrophoresed. The gel was fixed in 50% trichloroacetic acid and then dyed with 0.25% Coomassie blue (R-250; Neuhoff et al., 1988), and the band intensities were analyzed with Image Lab software (Bio-Rad, Hercules, CA, United States).

Fourier transform infrared spectroscopy

The 11S and 7S proteins were freeze-dried and characterized *via* a 100 FT-IR spectrometer (PerkinElmer, Akron, OH, United States). In brief, a mixture of 5 mg of sample and 500 mg of KBr powder was ground, pressed into a pellet and measured within the range of 400–4,000 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans.

Raman spectra

The Raman spectra of the 11S and 7S fractions were determined according to the modified method of Zhu et al. (2018). The 11S and 7S isolates were freeze-dried and spread on glass slides, followed by Raman experiments with an inVia Qontor Raman microscope (Renishaw, England). The samples were scanned in the measurement range of 400 cm^{-1} to 3,300 cm^{-1} under the following conditions: 3 scans, 3 s exposure time, 20× lens, and the use of a diaphragm with a 50 µm slit. The spectra were smoothed, and baselines were corrected and normalized against the phenylalanine band at 1003 cm^{-1}.

Circular dichroism analysis

Circular dichroism (CD) spectra of the 11S and 7S proteins were recorded using a circular dichroism analyzer (J-815, JASCO, Japan). The protein concentration was 0.2 mg/ml, and the path length was set to 0.1 mm. The spectra were measured in the

far-UV region of 190–260 nm under the following conditions: a response time of 1 s and a bandwidth of 2 nm. The secondary structure contents of the 11S and 7S fractions were analyzed *via* the online Circular Dichroism Website.[1]

Peak fitting and statistical analysis

The spectra were analyzed using OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA). PeakFit v4.12 software was used to calculate the nonlinear fitting of the peaks in the spectral data. Baseline corrections were performed using a second derivative method for finding anchor points and detecting the baseline. Statistical analysis was performed by Student's t tests at the level $p = 0.01$ and $p = 0.05$.

Results

Generation of transgenic soybean plants

The cotyledonary node method was used to transform pCambia1300-OsEPSPS-RNAi-GmOle8pro-A2B1a constructs into soybean, and 102 T_0 independent transgenic events were confirmed by both PCR analysis using *OsEPSPS* gene-specific primers and glyphosate (10 ml/l, v/v) screening. The next generation of transformants was validated *via* PCR and herbicide screening again and were grown in a greenhouse with 300 μmol m^{-2} s^{-1} illumination intensity and a 14/10 h photoperiod. Two independent transgenic events, T1000 and T1006, which showed high levels of *gy2* transcripts and a sharp reduction in *CG-β-1* expression according to PCR identification and displayed normal agronomic traits based on observations, were further advanced another four generations to obtain T_5 plants (Figures 2A,B). Homozygous transgenic T_5 plants were used to identify the expression levels of the *CG-β-1* and *gy2* genes by quantitative real-time PCR analysis with SYBR Green used as a reporter dye, and the actin gene served as an internal reference. The normalized expression levels of *CG-β-1* transcripts were downregulated sharply in the T1000 and T1006 independent transgenic events, with only 37 and 22% intensity in the R6 stage seeds compared with those of the nontransgenic control plants ('Tianlong No. 1'), respectively. In contrast, the expression levels of *gy2* in the T1000 and T1006 lines harvested from R6 period seeds increased significantly (by 1.63- and 1.81-fold, respectively) compared with their original transcript levels (Figure 2C). Taken together, these results showed that the construct was integrated into the T1000 and T1006 genomes and displayed stable inheritance. Moreover, *CG-β-1* expression levels decreased dramatically, and the amount of *gy2* transcripts largely increased in both T1000 and T1006 plants.

1 http://dichroweb.cryst.bbk.ac.uk

Accumulation of 7S globulins and 11S glycinin

ELISAs were used to quantify the 7S and 11S fractions in the transgenic soybean seeds. The 11S protein contents of the T1000 and T1006 plants ranged from 182.9 to 189.8 mg/g dry seed flour, respectively, which were significantly higher than the values for the nontransformed controls (140.5 mg/g). The amount of the 7S globulins in the T1000 and T1006 lines was reduced substantially in transgenic seeds, with total seed weights ranging from 125.9 to 122.6 mg/g, respectively (Figures 3A–C). Further statistical analysis *via* t tests ($p = 0.01$) showed that there was a significant difference in the 7S and 11S globulin contents in seeds between the T1000 and T1006 lines and nontransgenic plants. The 11S/7S ratio increased significantly compared with its original value, from 0.82 to 1.45–1.55, and increased by 1.8 and 1.9 times in the T1000 and T1006 lines, respectively, compared to those of the transformation acceptor materials. We used Osborne fractions to extract the total protein (Osborne, 1924) and found that the 11S/7S ratio was also markedly improved in transgenic lines, although the value of 11S/7S was slightly different from the results obtained from Thanh and Shibasaki's method (Thanh and Shibasaki, 1976; Supplementary Figures S1A–C). The contents of total seed protein harvested from continuous generations were also measured, and there was no significant difference in protein accumulation obtained from the seeds of T1000, T1006 and nontransgenic plants (Supplementary Table S2).

SDS–PAGE showed that the electrophoretic band of the β subunit of 7S globulins obtained from the T1000 and T1006 lines was weaker than that of nontransgenic soybean seeds (Figure 3D). Under the same conditions, the A2B1a subunit in the protein profiles of 11S glycinin electrophoresis, which was separated into two fractions of approximately 38 and 19 kD, displayed stronger intensity in the transgenic T1000 and T1006 seeds than in the wild-type seeds (Figure 3D) and showed a similar electropherogram of the protein acquired from the Osborne fractions method (Supplementary Figure S1D). These results are consistent with the conclusions obtained from the ELISA data. Gel permeation chromatography (GPC) was performed to determine the protein size distribution and the 11S/7S ratio. As shown in Figures 4A–C, in the transgenic lines, the 11S soluble aggregates with slightly increased retention time had two peaks of 6.023 and 6.149 min for the T1000 lines and 5.943 and 6.257 min for the T1006 lines, respectively. Under the same elution conditions, there were 5.844 and 5.862 min peaks for 11S globulins in nontransgenic soybean plants. For 7S globulin, the profiles of soluble aggregates exhibited slight variability; there was a 5.419 min peak for the T1000 lines, the retention time was slightly longer than that of the transformation receptor (5.416 min), and the retention time (5.394 min) of the T1006 lines was slightly less than that of the control. The products collected from 5.0–6.5 elution time showed that the range of molecular weight contained α, α', β and 11S acidic subunits resolved by SDS–PAGE (Figure 4D). The 11S/7S ratio was also calculated according to the area of the corresponding

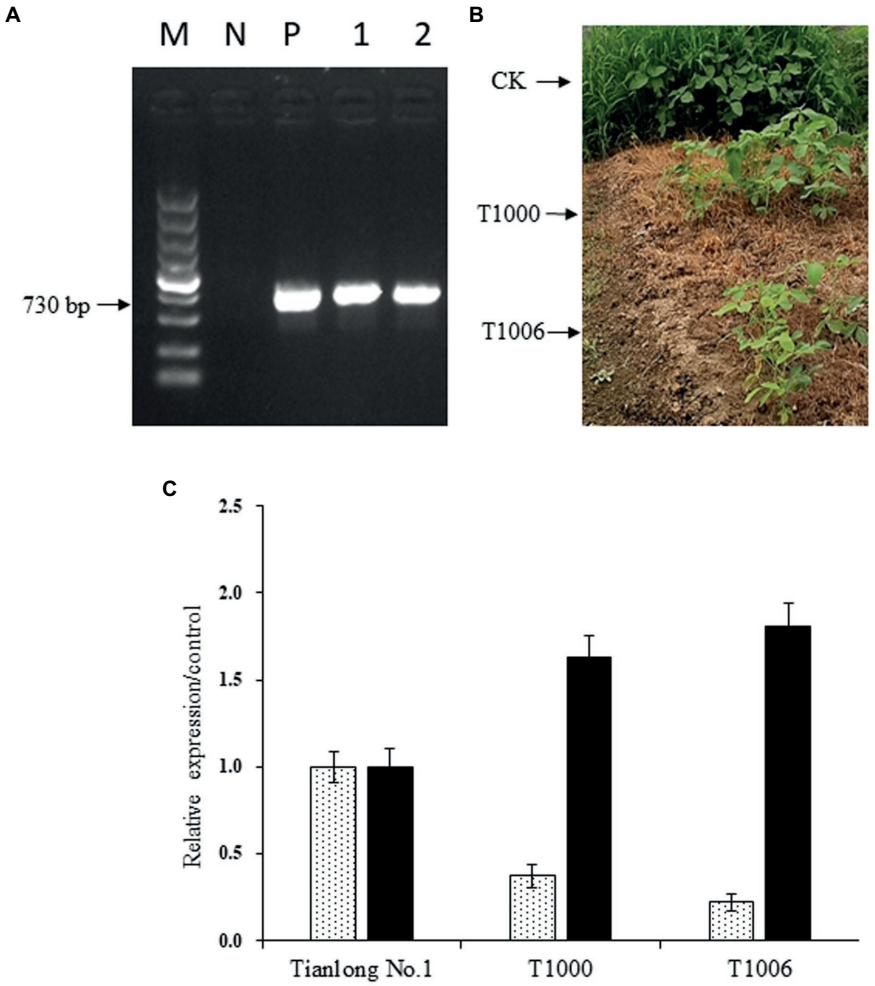
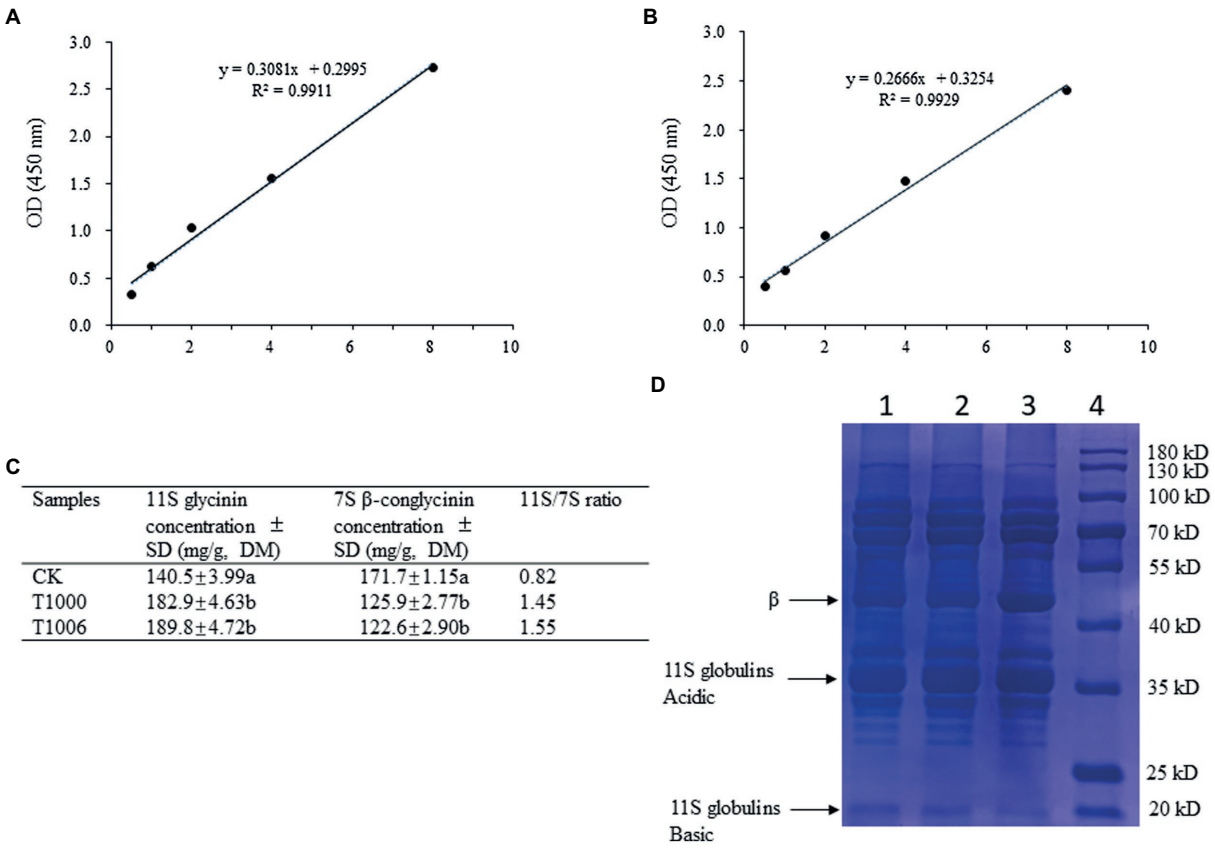


FIGURE 2
Expression of *OsEPSPS, CG-β-1* and *gy2* in transgenic soybean plants. **(A)** Detection of *OsEPSPS* transcripts by PCR amplification. M, DNA marker 5,000; N, the negative control 'Tianlong No. 1'; P, the positive plasmid; Lane 1, T1000 transgenic lines; Lane 2, T1006 transgenic lines. **(B)** Glyphosate screening for transgenic lines. CK, 'Tianlong No. 1' without spraying glyphosate; T1000, T1000 transgenic lines sprayed with glyphosate (10ml/l); T1006, T1006 transgenic lines sprayed with glyphosate (10 ml/l). **(C)** Detection of *CG-β-1* and *gy2 expression* using quantitative real-time PCR. CK, T1000, and T1006 represent 'Tianlong No. 1', T1000, and T1006 transgenic plants, respectively, and the levels of *CG-β-1* and *gy2* measured in 'Tianlong No. 1' were regarded as the control.

peak and was 2.43, 1.64, and 0.86 in T1006, T1000 and 'Tianlong No. 1′ plants, respectively (Figure 4E). The 11S/7S ratio acquired from the peak area of the gel filtration chromatography was higher than the results from the ELISA analysis, which might be affected by other proteins with molecular weights similar to those of 7S and 11S globulins. However, the trend of the 11S/7S ratio in the T1006, T1000 and 'Tianlong No. 1′ plants agreed with previous ELISA findings.

The sulfur-amino acid content of transgenic soybean seeds significantly increases

The amino acid profile analysis of dry soybean seeds showed that there was a dramatic increase in cysteine and methionine content in T1000 and T1006 transgenic lines compared to wild-type plants, 'Tianlong No. 1' (Table 1). In T1000 and T1006 seeds, the cysteine content improved on average by 11.9 and 48.0%, respectively, while the methionine content was enhanced by 32.1 and 53.8%, respectively, when compared to nontransformed seeds. The total content of sulfur-containing amino acids was also markedly improved, amounting to 79.194 nmol/mg in dry soybean seeds from T1006 transgenic plants, 1.51 times higher compared to nontransformed controls (52.316 nmol/mg). Transgenic soybean by silencing *CG-β-1* expression *via* RNA interference and specifically enhancing the *gy2* transcripts in seeds displayed a significant accumulation of cysteine and methionine content, which was also consistent with the increase in the 11S/7S ratio in protein isolates obtained from T1000 and T1006 transgenic lines. In addition, the nonsulfur-containing amino acid composition of transgenic seed samples



Samples	11S glycinin concentration ± SD (mg/g, DM)	7S β-conglycinin concentration ± SD (mg/g, DM)	11S/7S ratio
CK	140.5±3.99a	171.7±1.15a	0.82
T1000	182.9±4.63b	125.9±2.77b	1.45
T1006	189.8±4.72b	122.6±2.90b	1.55

FIGURE 3
Analysis of the subunit composition of 7S β-conglycinin and 11S glycinin in soybean seeds. **(A,B)** are standard calibration curves of competitive ELISA for 7S and 11S globulins using polyclonal antibodies, respectively. **(C)** The content of 7S β-conglycinin and 11S glycinin in dry soybean seeds from different plants determined by ELISA results (3 replicates). The letters indicate the level of significance at the level p=0.01. **(D)** Typical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12%) profiles of total proteins obtained from dry soybean seeds. The bands of the β subunit and the acidic and basic components of 11S globulins were noted. Lane 1, Lane 2, Lane 3, and Lane 4 represent T1000 and T1006 transgenic plants, 'Tianlong No. 1' and protein markers, respectively.

was only slightly different from that of nontransformed plants.

Secondary structures of the 11S and 7S isolates

Raman spectroscopy is a complement to infrared spectroscopy and is based on the inelastic scattering of light. The characteristic band of disulfide bonds is in the range of 500 to 550 cm^{-1}. The Raman shifts of disulfide bonds are different in different vibration modes: 500–510 cm^{-1} is the gauche-gauche-gauche (g-g-g) mode; 525–535 cm^{-1} is the gauche-gauche-trans (g-g-t) mode; and 535–545 cm^{-1} is the trans-gauche-trans (t-g-t) mode. As shown in Figure 5G, the disulfide bonds in the 7S fractions from the nontransgenic lines are mainly associated with the two vibration modes of g-g-g and t-g-t, and their contents are 42.51 and 43.11%, respectively. In the 7S fractions obtained from the T1000 and T1006 lines, the modes of g-g-g are decreased and those of g-g-t are increased, whereas in the 11S isolates originating from T1000 and T1006 plants, the modes of g-g-g decreased sharply, and the modes of both g-g-t and t-g-t were obviously augmented.

The secondary structure was defined by far-ultraviolet circular dichroism, Raman spectroscopy and Fourier transform infrared spectroscopy (FTIR). The orientation and energy transition of peptide bonds in the secondary structure of proteins or peptides can be reflected by the CD spectrum in the far ultraviolet region. The characteristic CD peaks of the 7S and 11S isolates obtained from T1006, T1000 and 'Tianlong No. 1' are shown in Figures 5A,B. With respect to water-soluble proteins, the optically active substance exhibits anisotropy in light absorption in far-UV CD spectroscopy, and the secondary structure was identified based on the following: α-helices have absorption peaks at 208, 198 and 222 nm; β-sheets have absorption peaks at 195 and 216 nm; β-turns have absorption peaks at 205, 180–190 and 220–230 nm; and irregular curls have absorption peaks at 200 and 212 nm (Yang et al., 1986). There was a negative absorption peak at approximately 208 nm for the 7S isolates and a positive absorption peak for the 11S fractions.

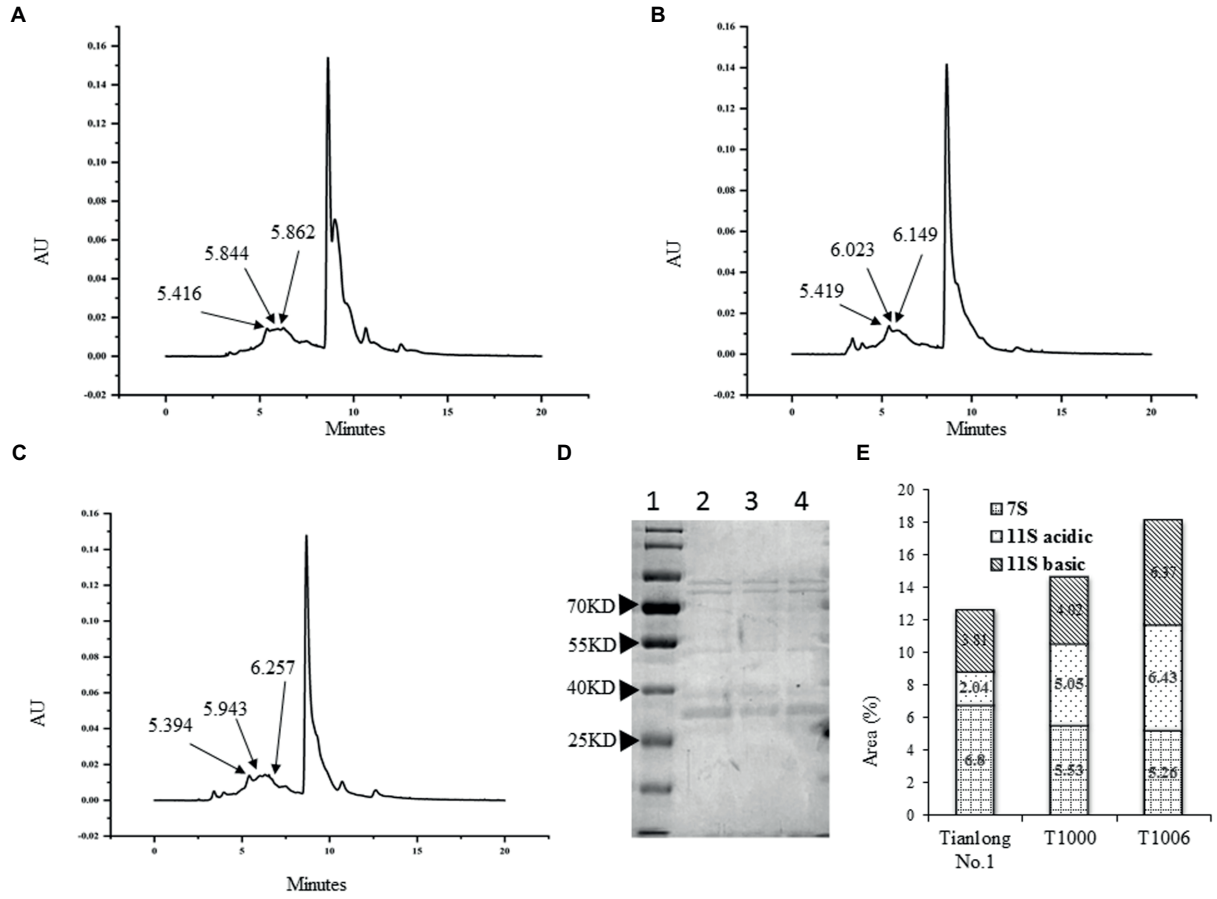


FIGURE 4
Gel permeation chromatography of SPI obtained from different plants. **(A–C)** are molecular weight (Mw) distribution profiles for the 'Tianlong No. 1', T1000, T1006 lines, respectively. **(D)** The proteins collected from elution times of 5.0–6.5 min were separated by SDS–PAGE. Lane 1, Lane 2, Lane 3, and Lane 4 represent the protein marker, T1000 lines, 'Tianlong No. 1', and T1006 transgenic plants, respectively. **(E)** The area distribution of 7S, 11S acidic, and 11S basic fractions from 'Tianlong No. 1', T1006 and T1000 plants, respectively.

The FTIR spectra of 11S and 7S in T1006, T1000 and 'Tianlong No. 1' plants are displayed in Figures 5C,D. The secondary structure of proteins can be quantitatively analyzed by means of the amide I band from Fourier transform infrared spectroscopy. According to the method of Qi et al. (2017), the corresponding secondary structures of proteins were calculated according to peak location attribution and peak area integral as follows: the α-helical structure ranged from 1,650 to 1,660 cm^{-1}, and the parallel β-folded structures ranged from 1,618 cm^{-1} to 1,640 cm^{-1}. The antiparallel β-folded structures ranged from 1,670 to 1,690 cm^{-1}, the β-angle structure ranged from 1,660 cm^{-1} to 1,670 cm^{-1}, and the random coiled structure ranged from 1,643 to 1,647 cm^{-1}. The secondary structure of the proteins was also deduced from the amide I band of Raman spectroscopy. According to the relevant references of Raman spectroscopy (Yu et al., 2021), the characteristic vibration frequency of the α-helix structure in the protein amide I band ranges from 1,645–1,660 cm^{-1}, the vibration frequency of the β-sheet ranges from 1,665–1,680 cm^{-1}, and the vibration frequency of the β-turn structure ranges from 1,680–1,690 cm^{-1} (Figures 5E,F). The secondary structure distribution of the 11S and 7S isolates in the T1006, T1000 and 'Tianlong No. 1' plants was determined according to the FTIR, Raman and CD spectroscopy results (Figure 5H). The contents of α-helices, β-sheets, β-turns and random coils isolated from 'Tianlong No. 1' materials were 15.39, 37.55, 12.06 and 43.49% for 7S isolates and 12.24, 26.65, 23.06, 41.79% for 11S isolates, respectively. For 7S isolates, the content of α-helices displayed slight variability in the T1000, T1006 and 'Tianlong No. 1' plants, ranging from 11.78–16.21%; however, the content of β-sheets in 11S isolates increased significantly, from 26.25 to 45.48%, and β-turns displayed the opposite trend, decreasing from 23.06 to 9.21%. The proportion of unordered coils ranged from 33.22 to 47.57%.

Discussion

Efforts have been going to improve the levels of the essential sulfur-containing amino acids methionine and cysteine in

TABLE 1 The content of amino acids (nmol/mg) in dry soybean seeds from different lines.

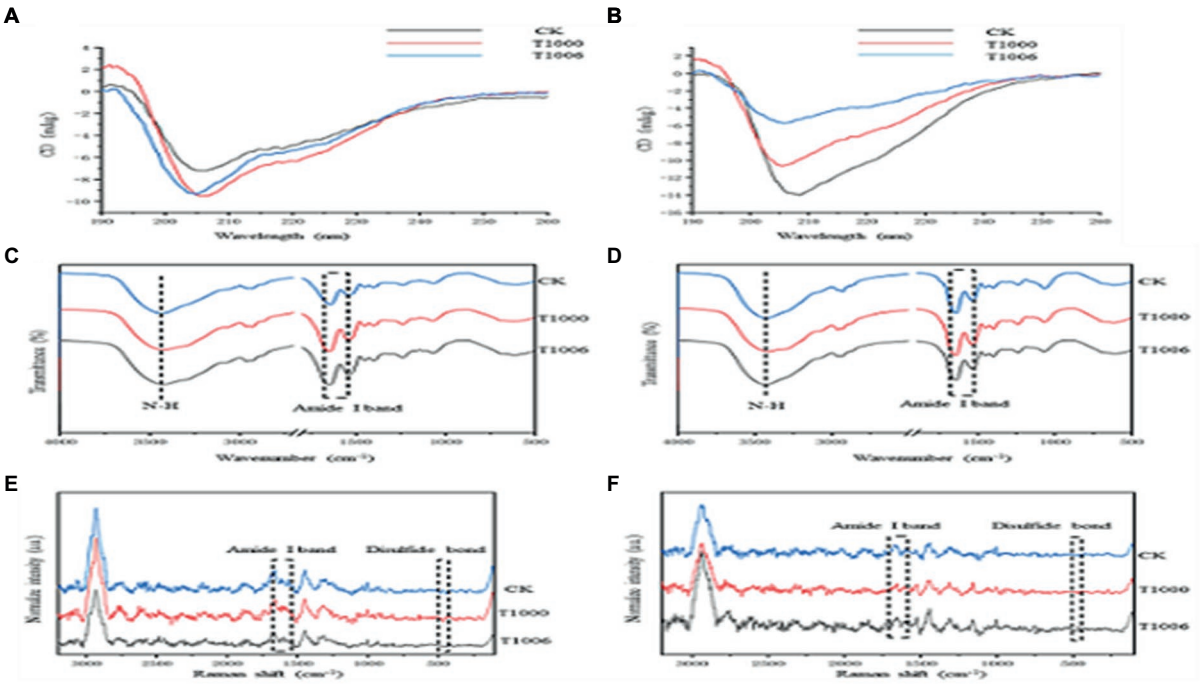
Amino acid	Tianlong No. 1	T1000	T1006
Asp	453.691 ± 15.79a	432.28 ± 18.42a	475.247 ± 14.42a
Thr	169.241 ± 8.49a	172.302 ± 6.79a	179.234 ± 11.43a
Ser	249.984 ± 19.67a	234.098 ± 13.41a	263.007 ± 9.41a
Glu	657.252 ± 25.32a	588.476 ± 18.80b	673.612 ± 17.83a
Pro	227.855 ± 13.45a	211.335 ± 14.03a	239.334 ± 11.21a
Gly	277.724 ± 19.94a	275.355 ± 17.73a	287.952 ± 5.06a
Ala	236.177 ± 13.17a	230.895 ± 13.31a	245.875 ± 12.59a
Cys	**21.494 ± 0.27a**	**25.656 ± 0.54b**	**31.803 ± 0.34c**
Val	206.826 ± 7.08a	201.005 ± 16.61a	214.037 ± 1.58a
Met	**30.822 ± 0.42a**	**40.71 ± 0.36b**	**47.391 ± 0.27c**
Ile	182.711 ± 8.42a	167.186 ± 8.30a	186.300 ± 9.47a
Leu	298.154 ± 10.24a	276.46 ± 9.23a	304.812 ± 16.60a
Tyr	104.871 ± 3.98a	102.998 ± 5.39a	108.779 ± 4.93a
Phe	161.642 ± 5.95a	143.296 ± 6.29b	161.491 ± 4.02a
Lys	221.734 ± 6.94a	205.647 ± 7.52b	232.490 ± 4.69a
His	88.576 ± 6.21a	86.003 ± 8.29a	117.088 ± 7.99b
Arg	223.767 ± 13.98a	213.22 ± 14.11a	251.747 ± 10.01b

Data is expressed as the mean value with 3 replicates ± standard error; Values of the same line followed by the same letter are not significantly different at the level $p = 0.05$. Bold fonts indicate sulfur-containing amino acids.

soybean seeds, including traditional plant breeding, cultivation techniques, and transgenic engineering. Variations in the concentration of sulfur-containing amino acids in soybean protein can be influenced by the nitrogen source, the availability of reduced forms of sulfur, and environmental factors. A survey of commercial soybean varieties and resources in North America revealed unexpectedly high sulfur-containing amino acids and indicated that soybean amino acid composition can be affected by both geographic and environmental factors (Pfarr et al., 2018). To date, new cultivars with high SAA contents have not been reported, although several QTLs and candidate alleles associated with amino acid contents have been found (Panthee et al., 2006; Ramamurthy et al., 2014). S ($MgSO_4$) application increased the proportion of Met + Cys in soybean seeds (Rushovich and WeilSulfur, 2021), and this required the correct application of S, which might be complex. Moreover, materials with high sulfur-containing amino acids have been influenced by environmental factors, nitrogen sources, and the availability of reduced forms of sulfur, which limits their use in field production. In this study, sulfur-containing amino acid content was significantly improved using *CG-β-1* RNA interference and the enhancement of A2B1a accumulation in seeds, and the level of Met + Cys maintained a stable ratio in various environmental factors.

Proteins rich in sulfur-containing amino acids, including synthetic methionine-rich proteins, MB-16, maize γ-zein proteins and delta-zein, have been successfully overexpressed in soybean to improve protein quality (Kim and Krishnan, 2004; Li et al., 2005; Zhang et al., 2014). However, the overall content of sulfur-containing amino acids in the transgenic soybean seeds increased only modestly, and it was not sufficient to meet the nutritional requirements of poultry or livestock. The insufficient accumulation of heterologous proteins in soybean typically accounts for approximately 1% of the total seed weight. In most cases, heterologous expression of sulfur-rich proteins in legumes is associated with a reduction in the accumulation of endogenous sulfur-rich seed proteins (Kim et al., 2014; Krishnan and Jez, 2018). Metabolic engineering of the sulfur assimilatory and methionine biosynthesis pathways has been applied to improve the cysteine and methionine contents of soybean. To date, cysteine biosynthesis OASSs, *Arabidopsis* cystathionine γ-synthase and ATP sulfurylase have been overexpressed in soybean (Song et al., 2013; Krishnan et al., 2018; Kim et al., 2020), and some encouraging results have been reported. Herein, metabolic engineering was adopted to improve the content of sulfur-containing amino acids, and no heterologous proteins were introduced into soybean by extremely increasing the 11S/7S ratio in seeds, which provides an attractive and precise engineering strategy to alter the functional and nutritional properties of soy protein.

Glycinin (11S) and β-conglycinin (7S) are the two main classes of multiple subunits of soybean seed storage proteins. Unfortunately, β-conglycinin is a major allergen for humans and is very deficient in sulfur-containing amino acids, especially the β subunit of 7S conglycinin (Herman, 2014). A2B1a subunit, which has the greatest abundance of sulfur-containing amino acids of all 11S glycinin fractions, cysteine and methionine account for 3 to 4.5% of the amino acid residues, similar to the levels of other high-quality dietary proteins (Paek et al., 1997). Hence, the glycinin fraction may be more desirable than β-conglycinin globulin in the production of foods with a well-balanced amino acid profile, and our study offered a very fine effective method to enhance the level of sulfur-containing amino acids in soybean seeds by raising the amount of 11S glycinin at the expense of reducing 7S globulin accumulation. There is a typical inverse relationship between 7S and 11S concentrations, and the glycinin content can be increased at the expense of β-conglycinin (Sui et al., 2021). Null mutations for each of the subunits of β-conglycinin were found to grow and reproduce normally, and the nitrogen content of seeds was similar to that of wild-type cultivars. Seeds of the induced mutant line appeared to compensate for the reduced nitrogen content by accumulating free amino acids (Takahashi et al., 2003) but had only a slight influence on the content of sulfur-containing amino acids. The total amino acid composition revealed a mild increase in transgenic soybean lines in which only β-conglycinin was knocked down (Kim et al., 2014), and in this study, the seed-specific promoter was introduced to enhance the accumulation of the A1B2a subunit in cooperation with reducing the total amount of β-subunits of 7S conglycinin, which increased the content of sulfur-rich amino acids to 79.194 nmol/mg and 51.38% higher than that of the wild-type control. This finding reflects a new promising approach to improve the level of sulfur-containing amino acids in dry soybean seeds. Moreover, the main agronomic



G

Samples	α- helix (%)	β- sheet (%)	β- turn (%)	Random coil (%)
CK-7S	15.39 ± 1.44a	37.55 ± 4.49a	12.06 ± 0.17b	43.49 ± 2.04b
T1000-7S	15.47 ± 1.88a	29.01 ± 5.14a	27.45 ± 10.28c	33.22 ± 2.66a
T1006-7S	12.10 ± 2.00a	37.45 ± 3.83a	10.30 ± 1.00a	47.57 ± 3.21b
CK-11S	12.24 ± 3.97a	26.65 ± 4.80a	23.06 ± 6.74b	41.79 ± 4.23a
T1000-11S	16.21 ± 3.00a	45.48 ± 3.10b	8.16 ± 0.03a	38.24 ± 2.41a
T1006-11S	11.78 ± 4.37a	42.17 ± 3.38b	9.21 ± 0.95a	39.92 ± 1.91a

H

Samples	g-g-g/%	g-g-t/%	t-g-t/%
CK-7S	42.51 ± 0.21c	14.38 ± 0.93a	43.11 ± 0.87b
T1000-7S	24.09 ± 0.51a	33.37 ± 0.14b	42.54 ± 0.34b
T1006-7S	27.28 ± 0.70b	39.75 ± 0.75c	32.97 ± 0.54a
CK-11S	39.08 ± 0.67c	33.23 ± 0.81a	27.69 ± 0.52a
T1000-11S	10.32 ± 0.29a	58.80 ± 0.25c	30.87 ± 0.46b
T1006-11S	14.62 ± 0.78b	43.82 ± 0.31b	41.56 ± 0.91c

FIGURE 5
Circular dichroism, FTIR and Raman spectra of 7S and 11S isolates from different lines. **(A,B)** CD spectra; **(C,D)** FTIR spectra; **(E,F)** Raman spectra; CK, T1000, T1006 represent the 'Tianlong No. 1', T1000, T1006 lines, respectively. **(G)** Disulfide bond configuration of 7S and 11S fractions from different samples. Different letters indicate the level of significance at the level p=0.05. **(H)** Secondary structure content of 7S and 11S fractions from different samples determined by CD, FTIR and Raman spectra. Different letters (a–c) indicate significant differences (p=0.05).

traits, including the yield and the content of proteins, were not altered.

When the alpha helix content in protein molecules is low, the beta folding and random coil contents are relatively high, and the surface hydrophobicity increases (Chen et al., 2011). It was found that the alpha helix content in the secondary structure decreased and the beta folding and random coil content increased in 7S globulins from red bean (Tang and Sun, 2011). The content of sulfur-containing amino acids may reflect the ratio of 11S and 7S subunits (Zhang et al., 2018). Hydrophobic interactions, disulfide bonds and nondisulfide covalent bonds maintained the structure of the protein molecular network, and more sulfur-containing amino acids and β-sheets and increasing 11S/7S ratios were found to improve the textural qualities of Chiba tofu. Moreover, the correlation between the content of disulfide bonds and sulfur-containing amino acids (Met+Cys) was significantly positive (Zheng et al., 2021). The more β-sheets that are ordered, the denser the network structure of the protein gel and the greater the gel hardness (Zhao et al., 2008). In the present study, the amount of the A1B2a subunit was sharply improved compared with its normal value in common 11S isolates, which was a new formula for 11S glycinin. Similarly, reducing the ratio of the β-subunit of 7S conglycinin would generate other kinds

of 7S globulins, which is different from ordinary 7S isolates. Our results showed that the content of sulfur-containing amino acids in the T1000 and T1006 lines was markedly improved, and the ratio of 11S/7S and β-sheets was also increased. As such, we provided a new soybean material for use in the production of foods with a well-balanced amino acid profile.

In summary, the present study demonstrated that sulfur-containing amino acid content was significantly improved using *CG-β-1* RNA interference and the enhancement of A2B1a accumulation in soybean seeds. The new formulae of the 7S and 11S fractions underwent conformational transitions accompanied by a decrease in the α-helix content and a corresponding increase in the β-sheet content.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

BW performed spectra and data analysis. DT conducted experiments of PCR identification and FTIR. CY performed amino acid analysis. LY conducted SDS-PAGE and ELISA experiments. XM constructed the vector. TW carried out soybean transformation and screening in the field. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.828153/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Analysis of subunit composition of 7S β-conglycinin and 11S glycinin in soybean seeds determined by Osborne fractions method. **(A,B)** are standard calibration curves of competitive ELISA for 7S and 11S globulins using polyclonal antibody respectively. **(C)** The content of 7S β-conglycinin and 11S glycinin in dry soybean seeds from different plants determined by ELISA results (3 replicates); The letters indicate the level of significance at the level $P=0.01$. **(D)** Typical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) profiles of total proteins obtained from dry soybean seeds. The band of β subunit, the acidic and basic components of 11S globulins were noted respectively; Lane 1, lane 2, lane 3, lane 4 represented 'Tianlong No. 1', T1000, T1006 transgenic plants and protein marker respectively.

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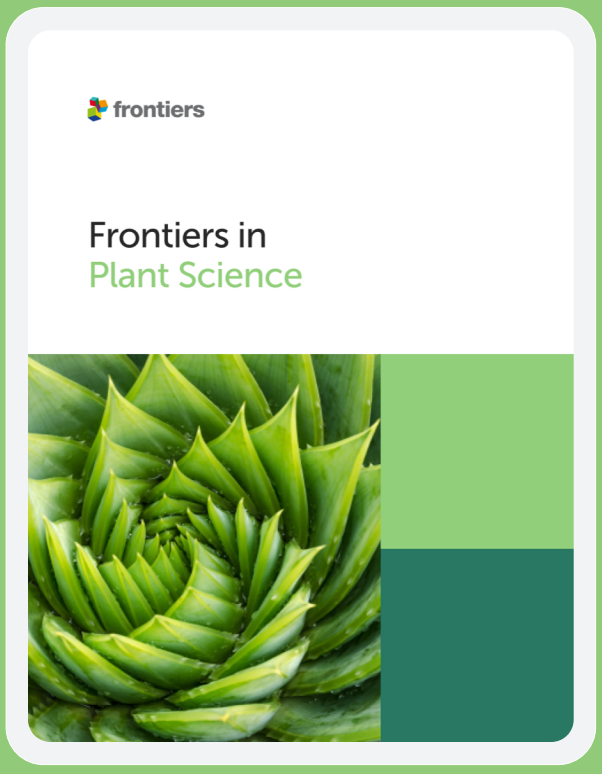
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