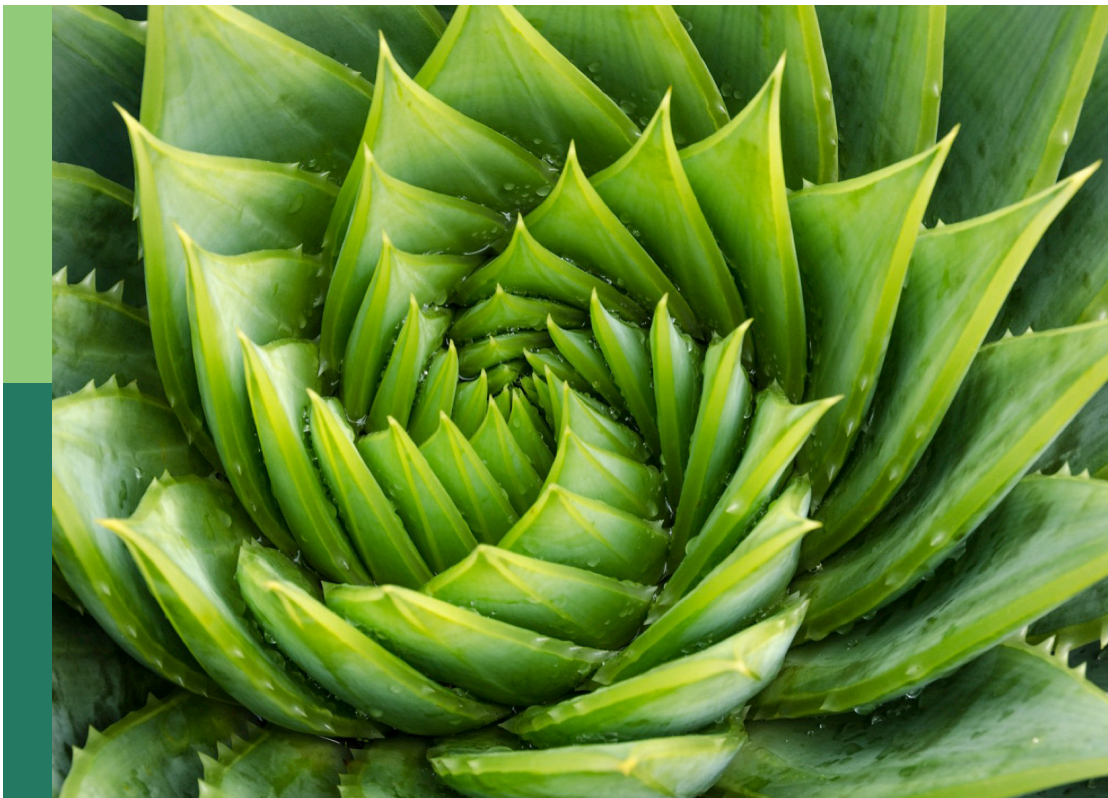
frontiers | Research Topics

Applications of novel gene editing tools and approaches in plants

Edited by
Changjun Huang, Cheng Yuan and Tong Zhang

Published in
Frontiers in Plant Science
Frontiers in Genome Editing



ISSN 1664-8714
ISBN 978-2-8325-4791-5
DOI 10.3389/978-2-8325-4791-5

About Frontiers

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

Frontiers journal series

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

Dedication to quality

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

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Applications of novel gene editing tools and approaches in plants

Topic editors

Changjun Huang — National Tobacco Genetic Engineering Research Center, Yunnan Academy of Tobacco Agricultural Sciences, China

Cheng Yuan — National Tobacco Genetic Engineering Research Center, Yunnan Academy of Tobacco Agricultural Sciences, China

Tong Zhang — South China Agricultural University, China

Citation

Huang, C., Yuan, C., Zhang, T., eds. (2024). *Applications of novel gene editing tools and approaches in plants*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4791-5

Table of contents

frontiers | Frontiers in Genome Editing

TYPE Editorial
PUBLISHED 08 April 2024
DOI 10.3389/fgeed.2024.1404959

OPEN ACCESS

EDITED AND REVIEWED BY
Qunxin She,
Shandong University, China

*CORRESPONDENCE
Cheng Yuan,
craig.cy@foxmail.com
Tong Zhang,
zhangtong@scau.edu.cn
Changjun Huang,
cjhuang@zju.edu.cn

RECEIVED 22 March 2024
ACCEPTED 28 March 2024
PUBLISHED 08 April 2024

CITATION
Yuan C, Zhang T and Huang C (2024), Editorial: Applications of novel gene editing tools and approaches in plants.
Front. Genome Ed. 6:1404959.
doi: 10.3389/fgeed.2024.1404959

Editorial: Applications of novel gene editing tools and approaches in plants

Cheng Yuan[1]*, Tong Zhang[2]* and Changjun Huang[1]*

[1]Yunnan Academy of Tobacco Agricultural Sciences, Key Laboratory of Tobacco Biotechnological Breeding, National Tobacco Genetic Engineering Research Center, Kunming, China, [2]Guangdong Province Key Laboratory of Microbial Signals and Disease Control, College of Plant Protection, South China Agricultural University, Guangzhou, China

KEYWORDS

genome editing tools, trait improvement, gene function, gene knockout, genetic transformation

Editorial on the Research Topic
Applications of novel gene editing tools and approaches in plants

The integration of gene editing technologies, particularly CRISPR/Cas systems, into plant biotechnology marks a groundbreaking advancement in the manipulation and understanding of plant genomes (Knott and Doudna, 2018; Manghwar et al., 2019). In this Research Topic, experts from nine groups have illustrated the rapid progress in the field, showcasing how these novel tools are revolutionizing the way we enhance crop resilience, yield, and quality traits through precise genetic modifications.

The discussion initiated by Ramesh et al. underscores the transformative potential of CRISPR/Cas9 in palm breeding and addresses the critical needs for sustainability and productivity in palm cultivation. Further expanding the scope of CRISPR/Cas9's impact, Yao et al.'s comprehensive review explores its applications in soybean molecular breeding. The authors detail how gene editing is being utilized to confer resistance to abiotic stresses and diseases, improve seed quality, and manipulate plant architecture for better yields. Vu et al.'s review delves into the utilization of CRISPR-Cas systems for enhancing plant tolerance to stress. Focusing on the unfolded protein response (UPR) as a mechanism to combat stress-induced damage (Liu et al., 2022), the review highlights how targeting specific UPR-related genes can lead to the development of crops with improved stress tolerance. The original research from Bhowmik et al. focuses on modifying the lipoxygenase (LOX) gene in yellow peas using CRISPR/Cas9, aiming to enhance sensory qualities by reducing off-flavors. This targeted genetic modification exemplifies the nuanced applications of gene editing in improving the nutritional and sensory attributes of food crops, offering new possibilities for food application without the need for post-harvest processing.

Optimizing Agrobacterium-mediated genetic transformation is crucial for CRISPR genome editing in plants, as it significantly enhances the efficiency and reliability of gene delivery, facilitating precise genetic alterations and advancing plant biotechnology (Chen et al., 2022). Ma et al. presents a groundbreaking *Agrobacterium rhizogenes*-mediated genetic transformation technology for citrus. This method streamlines the genetic modification process, demonstrating its efficacy across various citrus genotypes and enabling efficient study of genes previously challenging to analyze. While Yuan et al. develops a highly efficient CRISPR/Cas9 system delivered by Agrobacterium for genome

editing in wild tobacco (*Nicotiana alata*), overcoming the challenge of lacking an efficient genetic transformation and genome editing system in this species. This study not only advances genetic research in tobacco, but also sets a precedent for applying genome editing technologies to other species lacking efficient genetic transformation and editing systems.

Transformation in soybean is challenging due to its complex genetic structure and low efficiency in traditional methods, making protoplast transfection crucial for enabling direct gene editing within cells (Xu et al., 2022). The use of ribonucleoprotein (RNP) complexes in this process offers a significant advantage by enhancing precision, reducing off-target effects, and facilitating transient expression without integrating foreign DNA into the genome. Subburaj and Agapito-Tenfen develops a CRISPR/Cas9 RNP-based genome editing method for soybean protoplasts using electro-transfection, enhancing targeted mutagenesis in soybean by bypassing the need for PEG-mediated transfection methods. This innovation represents a significant leap in soybean genetic engineering, providing a more precise and efficient approach to improving crop traits and conducting functional genomics studies.

Optimizing and discovering promoters is pivotal for plant transformation and gene editing as it directly influences gene expression levels and specificity, thereby ensuring the effectiveness and precision of genetic modifications in plants. Ye et al. identifies and applies novel promoters from Chinese fir to enhance the efficiency of CRISPR/Cas-mediated genome editing in plant protoplasts. This strategy not only advances genetic engineering in forestry but also showcases the potential for these promoters to facilitate genetic improvements across a broader range of plant species.

Using CRISPR for pathogen detection is important because it offers a highly specific, sensitive, and rapid method to identify pathogens, significantly improving disease management and prevention strategies in agriculture. Wang et al.'s research introduces a novel CRISPR/Cas12a-based visual nucleic acid detection system for identifying plant viruses, such as *sorghum mosaic virus* (SrMV) and *rice stripe mosaic virus* (RSMV), in the field. This rapid and sensitive method offers a practical tool for early detection and management of viral infections, thereby mitigating potential impacts on crop production.

Collectively, these articles underscore the profound impact of CRISPR/Cas9 and related technologies on plant biotechnology. From enhancing crop resilience and yield to innovating disease diagnostics and genetic studies, gene editing tools are paving the way for the development of next-generation crops with improved performance and sustainability (Rodriguez-Leal et al., 2017; Gao, 2021). As these technologies continue to evolve, their application in addressing the complex challenges of modern agriculture holds great promise for advancing global food security and environmental sustainability.

Author contributions

CY: Funding acquisition, Writing–original draft, Writing–review and editing. TZ: Funding acquisition, Writing–original draft, Writing–review and editing. CH: Funding acquisition, Supervision, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by the NSF of China (Nos 31960530, 31860499 and 32222071).

Acknowledgments

We thank all authors and reviewers for their contributions to this Research Topic and appreciate the support of the editorial office.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

Chen, Z., Debernardi, J. M., Dubcovsky, J., and Gallavotti, A. (2022). Recent advances in crop transformation technologies. *Nat. Plants* 8 (12), 1343–1351. doi:10.1038/s41477-022-01295-8

Gao, C. (2021). Genome engineering for crop improvement and future agriculture. *Cell* 184 (6), 1621–1635. doi:10.1016/j.cell.2021.01.005

Knott, G. J., and Doudna, J. A. (2018). CRISPR-Cas guides the future of genetic engineering. *Science* 361 (6405), 866–869. doi:10.1126/science.aat5011

Liu, Y., Lv, Y., Wei, A., Guo, M., Li, Y., Wang, J., et al. (2022). Unfolded protein response in balancing plant growth and stress tolerance. *Front. Plant Sci.* 13, 1019414. doi:10.3389/fpls.2022.1019414

Manghwar, H., Lindsey, K., Zhang, X., and Jin, S. (2019). CRISPR/Cas system: recent advances and future prospects for genome editing. *Trends Plant Sci.* 24 (12), 1102–1125. doi:10.1016/j.tplants.2019.09.006

Rodriguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., and Lippman, Z. B. (2017). Engineering quantitative trait variation for crop improvement by genome editing. *Cell* 171 (2), 470–480. doi:10.1016/j.cell.2017.08.030

Xu, H., Guo, Y., Qiu, L., and Ran, Y. (2022). Progress in soybean genetic transformation over the last decade. *Front. Plant Sci.* 13, 900318. doi:10.3389/fpls.2022.900318

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 27 October 2022
DOI 10.3389/fpls.2022.1039094

OPEN ACCESS

EDITED BY
Basavaprabhu L Patil,
Indian Institute of Horticultural Research (ICAR), India

REVIEWED BY
Simon Sretenovic,
University of Maryland, College Park, United States
S. Kanakala,
North Carolina State University, United States

*CORRESPONDENCE
Shuo Duan
duansure@163.com
Xuepeng Sun
xs57@zafu.edu.cn
Jinhua Liu
jhliu11@mail.yst.com.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Plant Biotechnology, a section of the journal Frontiers in Plant Science

RECEIVED 07 September 2022
ACCEPTED 10 October 2022
PUBLISHED 27 October 2022

CITATION
Ma H, Meng X, Xu K, Li M, Gmitter FG, Liu N, Gai Y, Huang S, Wang M, Wang M, Wang N, Xu H, Liu J, Sun X and Duan S (2022) Highly efficient hairy root genetic transformation and applications in citrus. *Front. Plant Sci.* 13:1039094. doi: 10.3389/fpls.2022.1039094

Highly efficient hairy root genetic transformation and applications in citrus

Haijie Ma[1†], Xinyue Meng[1†], Kai Xu[1], Min Li[2], Fred G. Gmitter Jr[3], Ningge Liu[1], Yunpeng Gai[4], Suya Huang[1], Min Wang[1], Min Wang[2], Nian Wang[3], Hairen Xu[1], Jinhua Liu[5*], Xuepeng Sun[1*] and Shuo Duan[2*]

[1]Collaborative Innovation Center for Efficient and Green Production of Agriculture in Mountainous Areas of Zhejiang Province, College of Horticulture Science, Zhejiang A&F University, Hangzhou, Zhejiang, China, [2]China-USA Citrus Huanglongbing Joint Laboratory (A Joint Laboratory of The University of Florida's Institute of Food and Agricultural Sciences and Gannan Normal University), National Navel Orange Engineering Research Center, Gannan Normal University, Ganzhou, Jiangxi, China, [3]Citrus Research and Education Center, Horticultural Sciences Department, University of Florida, Lake Alfred, FL, United States, [4]School of Grassland Science, Beijing Forestry University, Beijing, China, [5]Natural Medicine Institute of Zhejiang YangShengTang Co., LTD, Hangzhou, Zhejiang, China

Highly efficient genetic transformation technology is greatly beneficial for crop gene function analysis and precision breeding. However, the most commonly used genetic transformation technology for woody plants, mediated by *Agrobacterium tumefaciens*, is time-consuming and inefficient, which limits its utility for gene function analysis. In this study, a simple, universal, and highly efficient genetic transformation technology mediated by *A. rhizogenes* K599 is described. This technology can be applied to multiple citrus genotypes, and only 2–8 weeks were required for the entire workflow. Genome-editing experiments were simultaneously conducted using 11 plasmids targeting different genomic positions and all corresponding transformants with the target knocked out were obtained, indicating that *A. rhizogenes*-mediated genome editing was highly efficient. In addition, the technology is advantageous for investigation of specific genes (such as *ACD2*) for obtaining "hard-to-get" transgenic root tissue. Furthermore, *A. rhizogenes* can be used for direct viral vector inoculation on citrus bypassing the requirement for virion enrichment in tobacco, which facilitates virus-induced gene silencing and virus-mediated gene expression. In summary, we established a highly efficient genetic transformation technology bypassing tissue culture in citrus that can be used for genome editing, gene overexpression, and virus-mediated gene function analysis. We anticipate that by reducing the cost, required workload, experimental period, and other technical obstacles, this genetic transformation technology will be a valuable tool for routine investigation of endogenous and exogenous genes in citrus.

KEYWORDS

citrus, genetic transformation, genome editing, *Agrobacterium rhizogenes*, virus

Introduction

Citrus is among the most important fruit crops worldwide and is grown in more than 114 countries (Talon and Gmitter, 2008). Global predicted citrus production exceeded 146 million tons (FAOSTAT; https://www.fao.org/faostat/en). The citrus industry currently requires new cultivars with desirable traits to improve yields, nutritional value, and adaptability to biotic and abiotic stresses. The application of genetic transformation to improve citrus has increased in recent years (Peña, 2000; Boscariol et al., 2006; Fagoaga et al., 2006; Febres et al., 2008; Gambino and Gribaudo, 2012). *Agrobacterium*-mediated transformation of epicotyl segments requires tissue culture, which is widely employed to produce disease-resistant materials in the laboratory, remains the quickest method for improvement of citrus cultivars. To date, many important traits have been successfully introduced into different citrus species and hybrids, such as lime, sweet orange, and grapefruit (Domínguez et al., 2002; Fu et al., 2011; Jia et al., 2017; Peng et al., 2017; Jia et al., 2021). However, transformation mediated by *Agrobacterium tumefaciens* has many disadvantages, including that the procedure is time consuming, laborious, expensive, and inefficient. The low frequency of rooting is an additional limitation of *A. tumefaciens*-mediated transformation of epicotyl segments of citrus (Gutiérrez-E. et al., 1997). As a result, micrografting is frequently used for maintenance of transgenic plants (Poles et al., 2020). Therefore, a rapid and highly efficient genetic transformation method bypassing the need for tissue culture is critical for gene function analysis and genetic improvement of citrus.

Agrobacterium species are widely used to generate transgenic plants as the agrobacteria can integrate transfer DNA (T-DNA) into a host plant's nuclear DNA genome. *Agrobacterium tumefaciens* transfers the tumor-inducing (Ti) plasmid into the host nucleus to incorporate exogenous DNA into a host chromosome and subsequently cause formation of a tumor at the plant wound site. This mechanism has been utilized for *A. tumefaciens*-mediated plant transformation of many plant species to improve crop traits and for research on gene function (Chetty et al., 2013; Kaur and Sah, 2014). In recent decades, *A. tumefaciens* has been widely applied in citrus breeding and gene functional research (Domínguez et al., 2002; Stover et al., 2013; Hongge et al., 2019). However, for most plants, especially woody species, when using *A. tumefaciens*, the generation of stable transformants requires plant regeneration from a few cells or even a single cell using exogenous phytohormones, and thus the process is time consuming and laborious. In addition, *Agrobacterium rhizogenes* has been successfully used in plant genetic transformation technologies (Estrada-Navarrete et al., 2007). *Agrobacterium rhizogenes* can infect plants to induce formation of hairy roots from wounded tissue owing to the expression of *rol* genes encoded in the Ri plasmid. The T-DNA cassette from the exogenous binary vector can be transferred and integrated into the host cell genome together with T-DNA from the Ri plasmid (White et al., 1985). Compared with *A. tumefaciens*, *A. rhizogenes*-mediated hairy root genetic transformation technology bypassing the requirement for tissue culture and antibiotic screening is highly efficient and has been widely used in many herbaceous plants for rhizosphere physiology research and recombinant protein production (Tsuro et al., 2005; Majumdar et al., 2011; Habibi et al., 2016; Beigmohamadi et al., 2019). However, *A. rhizogenes*-mediated genetic transformation in woody plants bypassing tissue culture remains at an early stage of application (Irigoyen et al., 2020).

In this study, we describe a rapid and highly efficient root genetic transformation and genome-editing protocol for citrus using *A. rhizogenes* strain K599. This technique requires only 2–8 weeks for completion, bypasses tissue culture, and has applicability for diverse citrus accessions. To date, no report is available on a highly efficient endogenous gene-editing technology for citrus that bypasses tissue culture. In addition, the proposed protocol can be used for viral vector inoculation bypassing tobacco-mediated virion enrichment, which can improve the efficiency of virus-mediated analysis of citrus gene function. We anticipate that the protocol will be a valuable tool for routine investigation of endogenous and exogenous genes in citrus.

Materials and methods

Bacterial strains, plant material, and growth conditions

The *Escherichia coli* (DH5α) competent cells (CAT#: DL1002), *A. rhizogenes* (K599) competent cells (CAT#: AC1080), and *A. tumefaciens* (EHA105) competent cells (CAT#: AC1012) were obtained from Shanghai Weidi Biotechnology Co., Ltd. All transformed bacterial strains were stored in 15% glycerol and preserved in a freezer at −80° C. *Escherichia coli* cells were cultured in lysogeny broth medium at 37°C. The K599 and EHA105 strains were recovered and cultured at 28°C in tryptone yeast medium with corresponding antibiotics.

Branches of *Citrus medica*, *C. limon*, *C. sinensis*, and citrange 'Carrizo' were obtained from the National Citrus Engineering Research Center, Chongqing, China. Plants with transgenic hairy root were grown in a greenhouse at 26°C with a 16 h/8 h (light/dark) photoperiod. All citrus plants were cultured in a net greenhouse under natural conditions.

Agrobacterium-infiltrated citrus hairy root transformation

Recombinant *A. rhizogenes* strains were cultured in fresh yeast extract peptone medium with appropriate antibiotics at 28° C. The resuspended *A. rhizogenes* K599 cells at the final concentration (OD_{600} = 0.6) were diluted into the MES solution (10 mM $MgCl_2$, 10 mM MES [pH 5.6], and 200 μM AS). Blade-removed citrus branches (approximately 2 months old) were collected from the greenhouse. We cut the stems into ~10 cm sections using sterilized shears by keeping the smooth surface of the cross-section.

The base of the stems sections was soaked in the *A. rhizogenes* K599 suspension and vacuum infiltrated for approximately 25 min using a standard vacuum. The stem sections were cultured in a dome tray filled with vermiculite-mixed soil in the greenhouse at 26°C with 90% relative humidity and a 16 h/8 h (light/dark) photoperiod. Hairy root development began after approximately 2–4 weeks (*C. medica*) or 4–8 weeks (*C. limon* and citrange 'Carrizo') after agroinfiltrated transformation. Potential transgenic roots were detected by the fluorescence signal with a portable excitation lamp (Luyor-3415RG, Shanghai, China). The fluorescence-positive hairy roots were incubated in liquid modified Hoagland's nutrient medium (Coolaber, Beijing, China) in sterile tubes for observation of symptoms. Transgenic roots were cultured at 26°C under a 16 h/8 h (light/dark) photoperiod. The symptoms were captured with a digital camera (Canon EOS 200D, Tokyo, Japan). Those shoots were further confirmed by PCR analysis. The hairy root transformation efficiency was calculated using the following formula: [(Number of GFP-containing roots)/(Total number of roots)] × 100. The GFP fluorescence in transgenic citrus roots was observed with a confocal microscope (LSM 780, Carl Zeiss, Jena, Germany) with 488 nm excitation and 505–530 nm emission wavelengths.

DNA and RNA extraction

Genomic DNA from roots was extracted using the cetyltrimethylammonium bromide method (Richards et al., 1994). Total RNA from the root and callus was extracted using the RNA Isolater Total RNA Extraction Reagent (R401-01, Vazyme, Nanjing, China). Gel electrophoresis and a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) were used to assess RNA quantity and quality. The cDNA was synthesized using the HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (R312-01, Vazyme). RT-qPCR analysis was conducted using the AceQ Universal SYBR qPCR Master Mix (Q511-02, Vazyme) and a real-time PCR system (Q2000A, LongGene, Hangzhou, China). The internal control gene used was actin and the corresponding primers were listed in Table S1.

Transcriptome analysis

The RNA-sequencing experiments were conducted using three biological replicates of each sample. Sequencing libraries were generated using the NEBNext Ultra II RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) and were sequenced on an Illumina NovaSeq 6000 Sequencing System in paired-end mode. The raw reads were processed with Trimmomatic v. 0.36 (Bolger et al., 2014) to remove adaptor sequences and low-quality reads. The cleaned reads were aligned to the Citrus *medica* genome using HISAT2 v. 2.2.1 (Kim et al., 2015). The number of reads mapped to each gene was counted with htseq-count v. 1.99.2. Differential expression between the C. *medica* wild type and C. *medica* agroinfiltrated explants was analyzed using the 'DESeq2' R package (Love et al., 2014). Genes with an adjusted *p*-value ($p_{adj} \leq 0.05$) and at least two-fold change in expression were assigned as DEGs. To explore the functions and pathways of the DEGs, GO terms and KEGG pathway enrichment analyses were performed using the 'ClusterProfiler' R package (Wu et al., 2021) and were visualized using the 'ggplot2' R package. The selected KEGG pathways associated with "plant hormone signal transduction", "amino acid biosynthesis", "plant–pathogen interaction", and "MAPK signaling pathway" were visualized using the 'Pathview' R package (Weijun and Cory).

Sequencing analysis

All transgenic roots and the wild-type plants were subjected to PCR (P111-01, Vazyme) using gene-specific primers (Table S1) to amplify DNA insertions or fragments including the target sites. The PCR amplicons were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) for Sanger sequencing. The sequence chromatograms were analyzed with SnapGene software.

Visualization of GFP fluorescent signal

Transgenic roots were first confirmed with a hand-held excitation lamp (Luyor-3415RG). For microscopic inspection, roots were rinsed with ddH_2O and photographed under a Leica-M205FA stereomicroscope (Leica Microsystems, Wetzlar, Germany). Two different autofluorescence emission wavelength bands were used for detection: green (505–550 nm) and red (>560 nm), defined by optical filters. Transverse sections of the transgenic roots were cut with a razor blade as thinly as possible. The sections were mounted on a glass microscope slide in ddH_2O. The GFP signal was observed under a Leica-SP8MP confocal fluorescence microscope (Leica Microsystems). The fluorescence was observed under excitation

at 488 nm and emission at 505–550 nm. Simultaneously, images (1920 × 1024 pixels) were captured with a suitable scale bar.

β-Glucuronidase expression

The GUS (*uidA*) gene expression was detected using a GUS gene quantitative detection kit (SL7161, Coolaber) following the manufacturer's instructions. Briefly, roots and hairy roots were incubated in 0.1 M sodium phosphate buffer with GUS substrate for 12 h at 37°C. The enzymatic reaction was stopped with 70% ethanol. Tissues were observed with a light stereomicroscope after GUS staining.

GRNA design

sgRNAs applied in this study were designed using the online tools CRISPRP v2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) based on their evaluation score (Rank from high to low), GC content (40%-60%) and putative off-target sites (Rank from low to high). target sequences with 20-bp were designed for each gene in the first exon. The purpose of this design will increase the possibility of affecting protein function and the likelihood that at least one site would be edited.

Virus inoculation

Prepare the *A. rhizogene* strain K599 harboring corresponding CLBV-based vector. Grow the corresponding *A. rhizogene* strain in YEP medium till OD_{600} = 0.8. Spin down the pellets and wash twice with infiltration medium (MES medium: 10 mM MES, 10 mM $MgCl_2$, 200 μm acetosyringone). Re-suspend the pellets using infiltration medium till OD_{600} = 0.5. Place the infiltration medium at 25°C for 3 hours in dark condition. Conduct vacuum agroinfiltration using citrus explants and maintain explants in greenhouse at 25°C. Observe the phenotypes after several months and confirm the transcription of target genes by RT-qPCR. All the experiments were conducted using at least three replicates.

Data availability statement

All datasets supporting the conclusions of this article are included in the article and supplementary files. The transcriptome project has been deposited at NCBI BioProject under the accession PRJNA800116 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA800116). All gene sequences, genome, gff3 file, and proteomes, gene ontology (GO), COG category, CAZy, KEGG, PFAMs, NR, eggNOG annotation of C. medica for bioinformatic analysis are available in the Zenodo repository at https://doi.org/10.5281/zenodo.5902607. The high-resolution figures are available on the figshare repository: https://doi.org/10.6084/m9.figshare.19060802.v1. The raw sequence data used for transcriptome analysis are available in NCBI under the Sequence Read Archive (SRA) with the SRA accession number for *C. medica* GFP agroinfiltrated explants: SRR17731820, SRR17731819, SRR17731818; *C. medica* wild-type: SRR17731817, SRR17731816, SRR17731815.

Results

Explants survival of different citrus genotypes in vermiculite

Agrobacterium rhizogenes integrates T-DNA from the Ri plasmid into the host plant genome when sensing signal substances, such as acetosyringone (AS), so as to induce formation of hairy roots and to synthesize substances needed for growth of the bacteria (Figure S1). To achieve genetic transformation bypassing tissue culture, the percentage explant survival of 22 citrus genotypes in vermiculite was first analyzed, ranging from 0% to 95% at 45 days post-incubation (Table S2). The four citrus genotypes with the highest percentage survival (>80%) were *Citrus medica*, *C. limon*, *C. grandis* 'Shatianyou', and *C. hystrix*. (Figure 1A). Most branches of these four genotypes eventually produced roots for more than 10 genotypes, the percentage survival was less than 40% within 45 days and most of the branches failed to produce roots (Figure 1B).

K599-mediated genetic transformation of citrus bypassing tissue culture

Based on the percentage survival, we selected *C. medica*, *C. limon*, and citrange 'Carrizo' for assessment of the efficiency of genetic transformation of citrus branches mediated by *A. rhizogenes* K599 harboring a binary plasmid (1380-Cas9-HA) overexpressing *GFP* (Figure S2; Table S3). Briefly, the genetic transformation protocol comprised three steps: K599 and explant preparation, vacuum infiltration, and explant incubation in vermiculite (Figure 2A; Table S4). The fluorescent transgenic hairy roots began to develop 2 weeks (*C. medica*) or 4 weeks (*C. limon* and citrange 'Carrizo') post-incubation in vermiculite (Figure S3). The length and number of fluorescent hairy roots increased significantly after 4 months (Figure 2B). Non-transgenic hairy roots lacking GFP signal also emerged, but these roots had no impact on subsequent research as they were easily distinguishable based on GFP fluorescence and were readily removed. Confocal microscopic examination confirmed that GFP fluorescence was universally distributed in transgenic hairy roots but was not observed in non-transgenic

FIGURE 1
Comparative analysis of percentage explant survival of citrus accessions in vermiculite. **(A)** Citrus explants were incubated in vermiculite [28°C, 16 h/8 h (light/dark), and 90% relative humidity] after vacuum infiltration. Statistical analysis was performed on the survival data for explants at 15, 30 and 45 days post-infiltration (dpi), respectively. **(B)** Statistical analysis of explant survival.

hairy roots (Figure 2C). Subsequently, genetic transformation in citrange 'Carrizo' was performed using a different binary plasmid carrying a gene encoding protein β-glucuronidase (GUS) (Table S3). Staining of GUS revealed that dark blue-stained transgenic hairy roots were induced by K599 harboring the corresponding plasmid, whereas no GUS staining was detected in the control (Figure 2D). The transformation rate of corresponding plasmids or citrus species was listed in Table S5. Compared with *A. tumefaciens*-mediated citrus genetic transformation, which usually takes 3–6 months with a low success rate, the K599-mediated genetic transformation method was time-saving and cost-effective.

K599-mediated genetic transformation and genome editing is highly efficient

To verify the efficiency of K599-mediated genetic transformation and genome editing, transformation was performed using 18 transformed K599 strains, each containing a different plasmid. All 18 plasmids carried the *GFP* and *Neo* encoding genes, and 11 contained genome-editing elements targeting different loci in the citrus genome (Figure 3A; Figure S4; Tables S3 and S6). Branches of *C. medica* were vacuum infiltrated using these transformed K599 strains. After one month, transgenic hairy roots corresponding to these 18 plasmids were obtained. Owing to the presence of the *GFP* selection marker, non-transgenic hairy roots were easily detected and removed, and thus *C. medica* with only transgenic hairy roots was obtained (Figure 3B). The *GFP* and *Neo* genes were both amplified from genomic DNA isolated from the transgenic hairy roots (Figure S5). Sequencing revealed that the gRNA target in the corresponding transgenic hairy roots had been successfully edited (Figure 3C). The missing nucleotides in edited transformants were mainly located at the 5' end of the PAM site, which is consistent with the properties of Cas9-mediated DNA cleavage. To detect possible chimeras in the transgenic hairy root tissue, primers (Table S1) were designed to amplify the edited loci based on the sequencing results. No fragments were amplified by PCR from the edited hairy roots, and specific fragments of the expected length were amplified from wild-type explants (Figure 3D), confirming the absence of chimeras in the edited hairy roots. Furthermore, PCR amplification and sequencing were performed on one randomly selected edited hairy root (gRNA10) using primers that flanked the gRNA-targeted locus (Table S1). A total of 20 random colonies were sequenced, which all contained changed sequences at the gRNA targeted locus, further confirming that the tested transformant was not a chimera (Figure S6).

Agrobacterium rhizogenes significantly affects expression of genes involved in hormone signal transduction

Compared with the wild-type explant lacking fluorescence, several GFP fluorescence spots were observed in callus formed at the wound surface of *C. medica* explants at 10 days post-agroinfiltration (Figure 4A), indicating that K599 successfully transferred the binary plasmid into numerous cells.

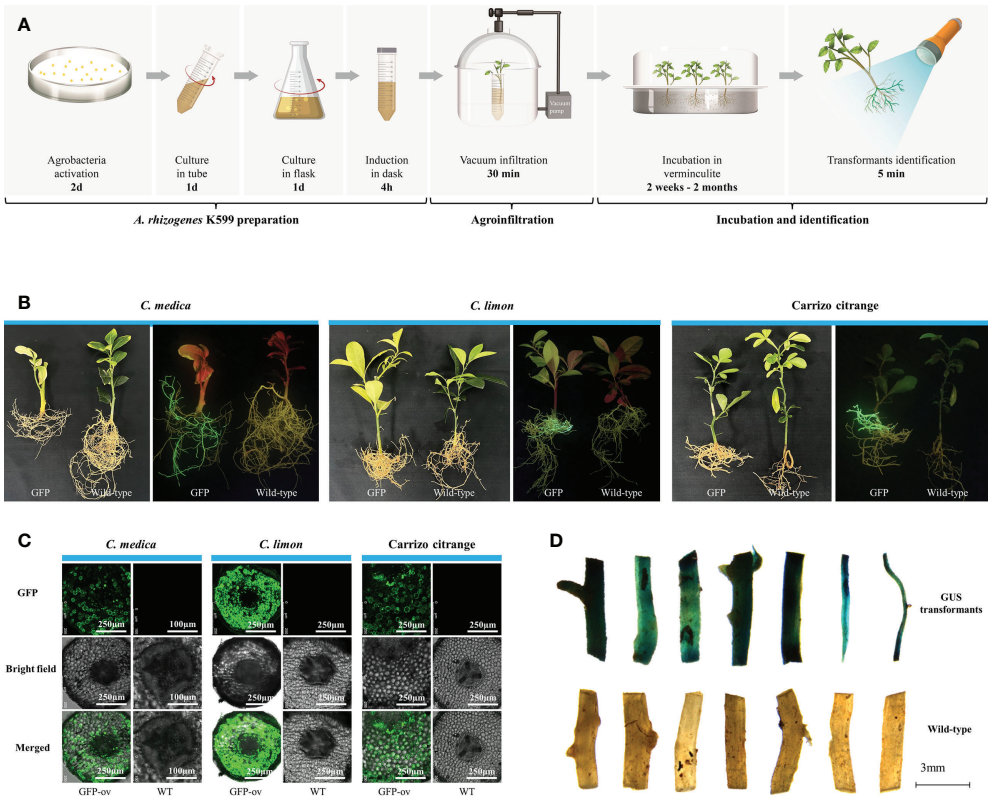


FIGURE 2
Agrobacterium rhizogenes K599 mediated highly efficient genetic transformation bypassing tissue culture. **(A)** Schematic diagram of the protocol for conducting A. *rhizogenes*-mediated genetic transformation bypassing tissue culture. **(B)** Images of *Citrus medica*, *C. limon*, and citrange 'Carrizo' at 4 months post-agroinfiltration. GFP, citrus explants infiltrated with K599 harboring a binary plasmid containing *GFP*; Wild-type, untreated citrus explants. The left images were taken under white light, and the right images were taken under excitation light. **(C)** Green fluorescent protein (GFP) signal visualized by laser scanning confocal microscopy in transgenic tissues of citrus roots. GFP, green fluorescent protein expression signal observed with a Leica-SP8MP confocal fluorescence microscope (excitation 488 nm, emission 505–550 nm); Bright, brightfield; Merged, merged GFP and brightfield images. **(D)** GUS staining of roots.

Transcriptome sequencing (PRJNA800116) of callus from wild-type and agroinfiltrated explants was conducted. A total of 4,233 differentially expressed genes (DEGs), comprising 2,744 upregulated and 1,489 downregulated genes, were identified in agroinfiltrated tissues compared with the non-treated control (Figure 4B; Table S7). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that many DEGs in agroinfiltrated tissue were enriched in the "transcription factors" (87), "plant–pathogen interaction (55)", "amino sugar and nucleotide sugar metabolism" (41), "plant hormone signal transduction" (56), "transporters" (136), "starch and sucrose metabolism" (37), "glycosyltransferases" (50), "cytochrome P450" (32), and "enzymes with EC numbers" (66) (Figure 4C; Table S8). Gene ontology (GO) enrichment analysis revealed that many DEGs in agroinfiltrated tissue were enriched in "DNA-binding transcription factor activity" (274), "transcription regulator activity" (290), "response to external stimulus" (326), "response to oxygen-containing compound" (347), "response to other organism" (243), "response to external biotic stimulus" (243), "response to biotic stimulus (243), and "defense response" (212) (Figure 4D; Table S9). Further analysis revealed that many DEGs enriched in the pathways "Plant hormone signal transduction" (Figure S7), "Phenylalanine, tyrosine and tryptophan biosynthesis" (Figure S8), "Plant–pathogen interaction" (Figure S9), and "MAPK signaling pathway" (Figure S10) were upregulated.

Agrobacterium rhizogenes-mediated genetic transformation complements "hard-to-get" transgenic materials and facilitates gene function analysis

Huanglongbing is among the most destructive citrus diseases worldwide and is caused by the phloem-limited bacterium *Candidatus* Liberibacter asiaticus (CLas). Citrus

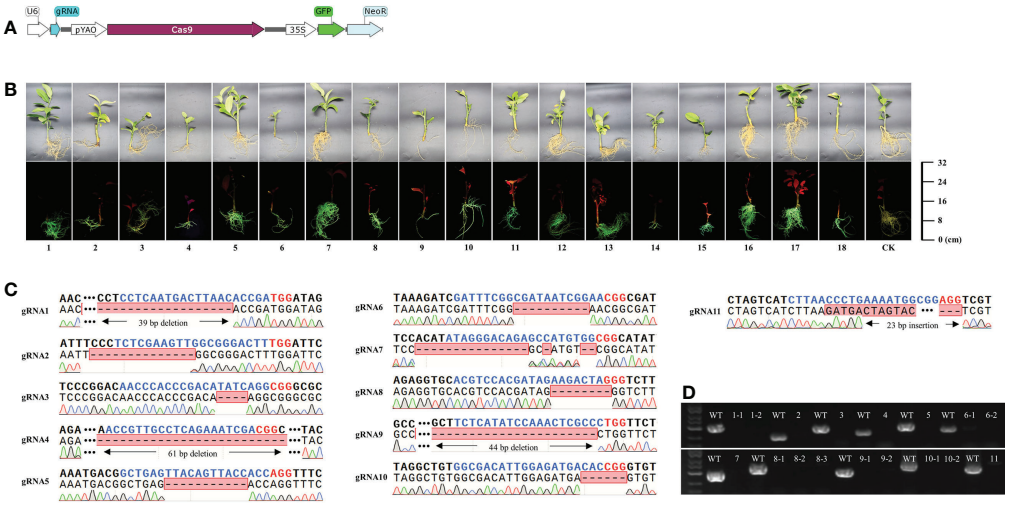


FIGURE 3
Agrobacterium rhizogenes K599-mediated genetic transformation and genome editing is highly efficient. **(A)** Schematic diagrams of genome-editing elements used in the study. **(B)** Green fluorescence signal visualized by laser scanning confocal microscopy in transgenic tissues of citrus roots. **(C)** Alignment of the genome-edited sequence of corresponding transgenic hairy roots with the gRNA-targeted loci. Blue nucleotides indicate gRNA and red nucleotides indicate PAM. **(D)** PCR assays conducted at gRNA-targeted sites of transgenic and wild-type roots.

roots harbor the pathogens during CLas infection and are difficult to treat with bactericides. Our previous studies revealed that overexpression of *ACD2* in citrus promotes CLas multiplication (Pang et al., 2020). However, *ACD2*-edited or *ACD2*-silenced citrus roots have never been obtained using *A. tumefaciens*-mediated genetic transformation because *ACD2* family genes are involved in the regulation of cell death and all corresponding transgenic shoots ultimately die. In the present study, we successfully obtained *ACD2*-edited citrus roots using K599-mediated genetic transformation (Figures 5A–C), indicating that this technology is advantageous for investigation of specific genes to obtain "hard-to-get" transgenic root tissue. In addition, *A. rhizogenes*-mediated citrus genetic transformation facilitates evaluation of tissue-specific promoters. When identifying exogenous genes that enhance resistance against a phloem-limited pathogen, a phloem-specific promoter is critical to reduce the impact of these genes on citrus biological traits (Dai et al., 2004; Tzean et al., 2020). In *Arabidopsis*, the companion cell-specific *AtSUC2* promoter has been widely used in gene function analysis of phloem-related genes (Paultre et al., 2016). In the present study, the homologous sequence of the *AtSUC2* promoter was identified in citrus and designated CsSUC2pro. Subsequently, we constructed three binary plasmids containing the *GFP* and *GUS* genes, of which CsSUC2pro, 35S, or no promoter was inserted at the 5′ end of GUS (Figure 5D). Using K599-mediated genetic transformation, we obtained transgenic citrus hairy roots harboring these three plasmids (Figure 5E). The results of GUS staining showed that all cells of 35S-GUS transgenic roots were stained blue, whereas only cells located in the phloem of CsSUC2-GUS transgenic roots were stained blue, and no cells of GUS (no promoter) transgenic roots were stained (Figure 5F).

Agrobacterium rhizogenes-mediated virus inoculation and its applications

Virus-induced gene silencing (VIGS), virus-mediated genome editing, or foreign gene overexpression technology have been widely used for gene function analysis in plants in recent years (Velázquez et al., 2016; Ellison et al., 2020). However, a viral vector cannot be transiently expressed in citrus leaves *via Agrobacterium* infiltration. Application of this technology to citrus is usually reliant on the use of tobacco (*Nicotiana* spp.) for virion enrichment. Given that K599-mediated genetic transformation of citrus is highly efficient, the feasibility of K599-mediated viral inoculation in citrus was examined. First, the *Citrus leaf blotch virus* (CLBV)-ChlI vector containing a partial sequence of *ChlI* (Magnesium chelatase subunit I) was constructed for *ChlI* silencing in citrus (Figure 6A). *Citrus medica* explants were infiltrated with K599 harboring the CLBV-ChlI viral vector. Agroinfiltrated explants displayed photobleaching phenotypes in new leaves after 3 months (Figure 6B). RT-qPCR results showed that the expression level of *ChlI* in CLBV-ChlI-transfected leaves was significantly downregulated, indicating that successful K599-mediated viral inoculation could be used for VIGS (Figure 6C). Subsequently, the CLBV-FT vector (Figure 6D)

containing the full-length sequence of the Arabidopsis thaliana *FLOWERING LOCUS* (*FT*) gene was constructed for *FT* overexpression in citrus based on a previously published method (Velázquez et al., 2016). The CLBV-FT vector was agroinfiltrated using 2-month-old *C. medica* seedlings, and the citrus infected with CLBV-FT flowered 9 months later (Figure 6E). The RT-qPCR results showed that the expression level of *FT* in CLBV-FT-transfected *C. medica* was significantly upregulated (Figure 6F).

Discussion

Currently, *A. tumefaciens*, electroporation, particle bombardment, and RNA interference are used for citrus genetic transformation, but these methods are laborious, expensive, time-consuming, and inefficient. The limitation of these methods is largely due to their reliance on tissue culture, which requires an aseptic environment. Using *A. tumefaciens*-mediated genetic transformation as an example, the acquisition of transgenic citrus requires 4- to 5-week-old seedlings germinated on Murashige and Skoog medium, co-incubation of explants with *A. tumefaciens*, and shoot regeneration, and each step involves tissue culture under aseptic conditions (Orbović and Grosser, 2015). However, only few researchers have studied the genetic transformation of citrus bypassing tissue culture. With regard to *A. tumefaciens*, Chun-zhen Cheng developed an *in planta* genetic transformation approach for pomelo (*Citrus maxima*) and obtained transgenic plants using this method 3 months post-transformation (Zhang et al., 2017). On the other hand, virus-mediated genome editing in tobacco, Arabidopsis, and wheat has been reported (Ellison et al., 2020; Ghoshal et al., 2020; Ma et al., 2020; Luo et al., 2021). However, the aforementioned *in planta* genetic transformation

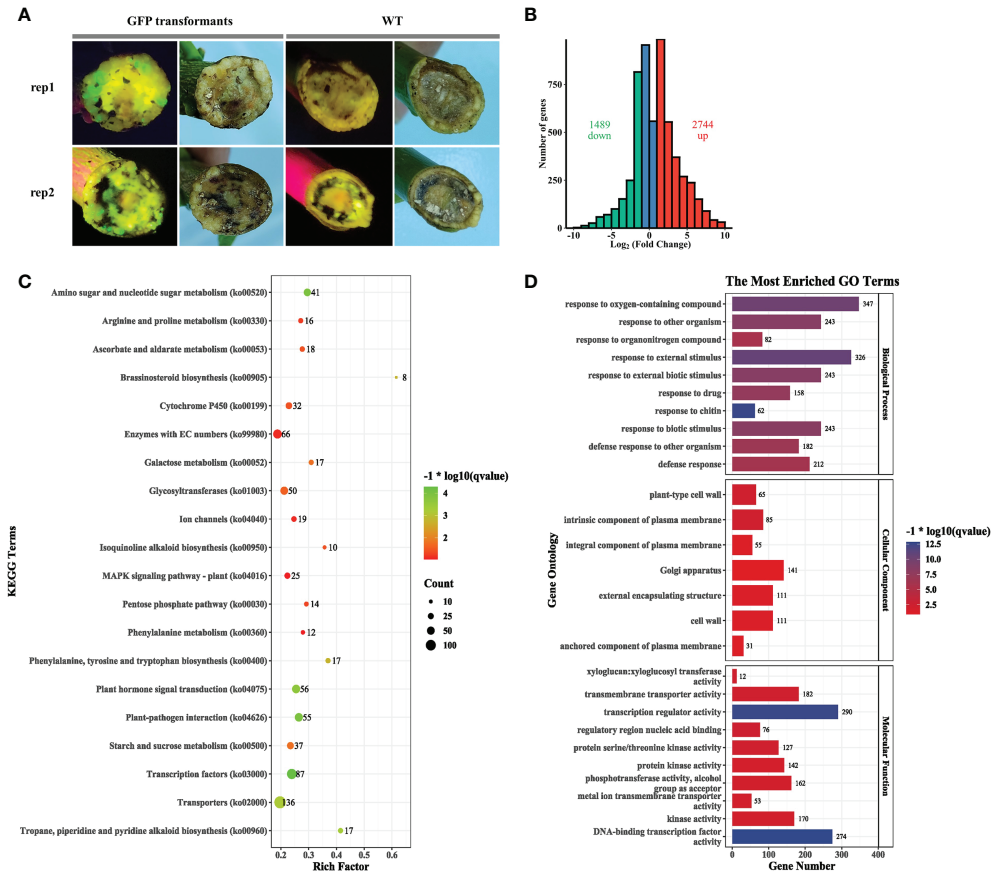


FIGURE 4
Agrobacterium rhizogenes K599 significantly affects expression of genes involved in hormone signal transduction. **(A)** Phenotype of callus generated on the wound surface of explants 2 weeks post-agroinfiltration. **(B)** Volcano plot (left) and histogram (right) of callus 2 weeks post-agroinfiltration compared with the wild type. **(C)** KEGG pathway analysis of differentially expressed genes (DEGs). The count represents the numbers of DEGs annotated for the pathway term. The *q* value is the adjusted *p* value. **(D)** Gene ontology (GO) enrichment analysis of DEGs. The results are summarized for the three GO categories (cellular component, molecular function, and biological process). The *x*-axis represents the number of DEGs in a category; the *y*-axis represents the GO term.

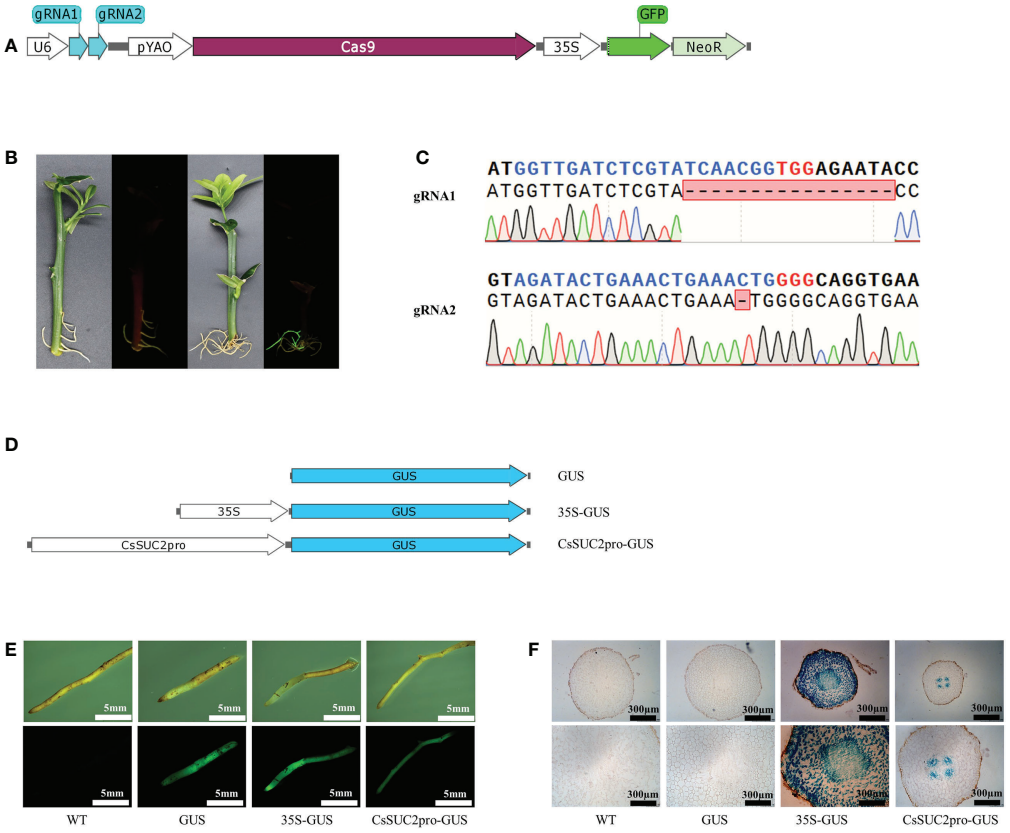


FIGURE 5
Agrobacterium rhizogenes-mediated genetic transformation complements "hard-to-get" transgenic materials and facilitates gene function analysis. **(A)** Schematic diagrams of *ACD2* gene-editing elements used in the study. gRNA1 and gRNA2 represent two gRNA scaffolds targeting *ACD2*. **(B)** Green fluorescence signal visualized by laser scanning confocal microscopy in transgenic tissues of citrus roots. **(C)** Alignment of the genome-edited sequence of *ACD2*-edited hairy roots with the gRNA-targeted loci. **(D)** Schematic diagrams of vectors containing different types of promoters. **(E)** Transgenic roots with GFP fluorescence were obtained after agroinfiltration using vectors containing different types of promoters. **(F)** GUS staining of transgenic roots.

technology requires 3–5 months to obtain transformants. In addition, virus-mediated genome-editing technology for woody plants has not been reported to date.

In recent years, the technology for obtaining transgenic roots using *A. rhizogenes* has been well developed in many herbaceous plants, such as soybean (Kereszt et al., 2007; Cao et al., 2009; Cheng et al., 2021), grain amaranth (Castellanos-Arévalo et al., 2020), rice (Raineri et al., 1990), and maize (Ishida et al., 2007). However, no report of *A. rhizogenes*-mediated endogenous gene editing bypassing tissue culture in citrus is available. In the present study, the genetic transformation procedure for citrus using *A. rhizogenes* bypassing tissue culture required only 2–4 weeks (*C. medica*) or 1–2 months (*C. limon* and citrange 'Carrizo'). The explants used in this study are branches, which are highly convenient to obtain. Subsequently, we established a highly efficient, convenient, and cost-effective genome-editing technology system in citrus using this technology. In addition, the procedure can be used for efficient inoculation with viral vectors. Furthermore, hairy roots induced by *A. rhizogenes* often develop from single cells, which leads to a lower incidence of chimerism in transgenic roots (Roychowdhury et al., 2017). This phenomenon was confirmed using the technology in the present study. In our study, the transcriptome analysis of citrus callus induced by *A. rhizogenes* revealed that hormone (IAA) pathway was significantly triggered, which provide evidence to explain the highly efficient transformation rate in citrus. To improve the efficiency of verification of transgenic hairy roots, the binary vector (1380-Cas9-HA) used contains the GFP reporter gene. Thus, the hairy roots can be identified by hand-held excitation light detection of GFP, which is time- and labor-saving. The establishment of a highly efficient genetic transformation technology mediated by *A. rhizogenes* for multiple citrus species is of great importance for gene functional analysis in citrus.

In summary, we established a highly efficient genetic transformation technology bypassing tissue culture for citrus, which can be used for genome editing, gene overexpression, and virus-mediated gene function analysis. The advantages of this

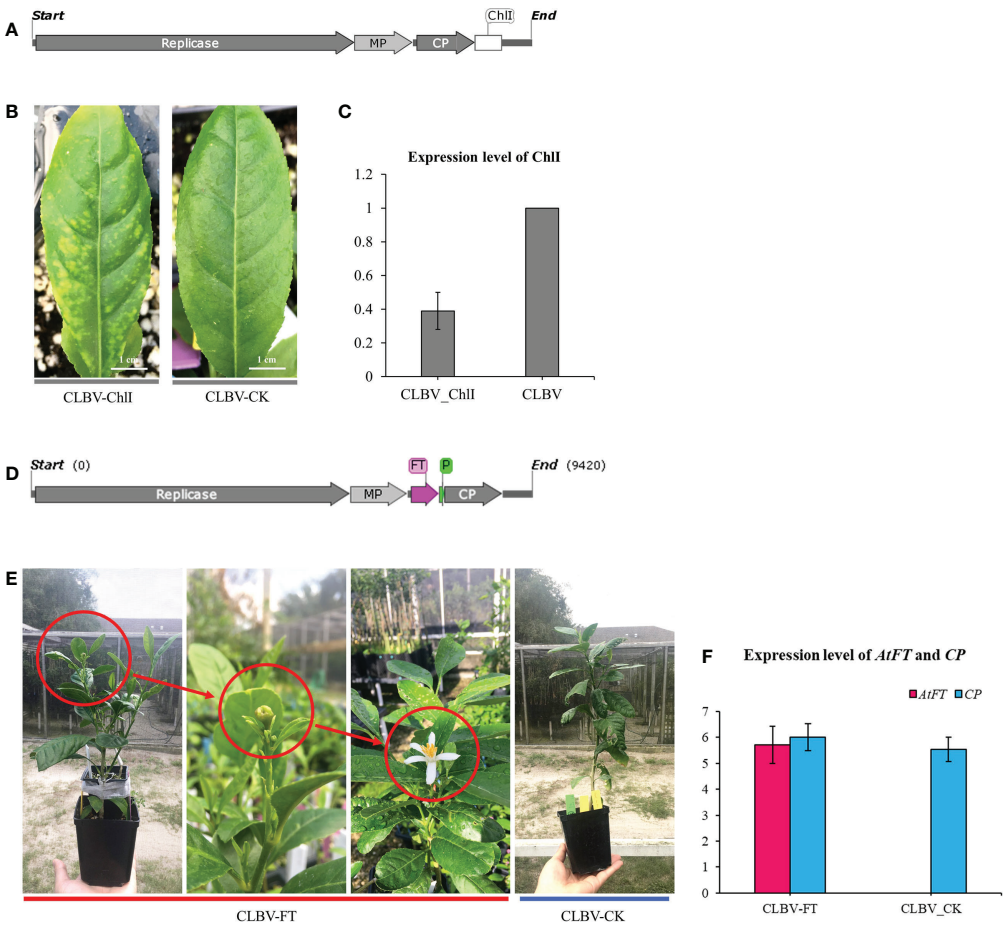


FIGURE 6
Agrobacterium rhizogenes-mediated citrus genetic transformation promotes the virus-induced application on citrus. **(A)** Schematic diagrams of the CLBV-ChlI vector. **(B)** Silencing phenotype of *Citrus medica* inoculated with wild-type CLBV (CLBV-CK) and CLBV-ChlI. **(C)** Relative accumulation of *ChlI* mRNA in *C. medica* inoculated with CLBV_CK or with CLBV_ChlI. **(D)** Schematic diagrams of the CLBV-FT vector generated by cloning the *FT* gene from *Arabidopsis thaliana*. **(E)** Flowering of a juvenile *C. medica* inoculated with the CLBV-FT. The juvenile *C. medica* inoculated with CLBV-CK did not flower. **(F)** Expression of *AtFT*.

technology are as follows: (1) the explant used for transformation are citrus branches, which is convenient to obtain; (2) the transformation process does not involve tissue culture and thus is convenient to implement; (3) the process is time-saving (2–8 weeks); (4) the procedure is less labor demanding (as few branches are required); (5) a high frequency of positive transformants is obtained (~57%, *C. medica*); (6) gene transformation or genome editing are achieved with high efficiency (Table S10). The problems that may be encountered during the experiment and the corresponding solutions were listed in Table S11. We anticipate that by removing the high cost, heavy workload, long experimental period, and other technical obstacles, this genetic transformation technology will be a valuable tool for routine investigation of endogenous and exogenous genes in citrus.

Data availability statement

The transcriptome data presented in the study are deposited in the NCBI repository, accession number PRJNA800116.

Author contributions

Conceptualization, HM, SD, XS; writing—original draft preparation, HM; writing—review and editing, SD, XS, FG, NW; supervision, HM, SD, XS, JL; project administration, HM, XM; transcriptome analysis, YG; experiment, XM, MW, NL, SH, ML; plants maintenance, XM, HX, KX, MW; funding acquisition, JL, ML. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (32202427 and 32002021); the Key Project for New Agricultural Cultivar Breeding in Zhejiang Province, China (2021C02066-1); The Major Science and Technology R& D Program of Jiangxi Province (20194ABC28007).

Acknowledgments

We thank Honghong Deng, Fabieli Irizarry, Ming Huang, Chen Ling, Jiaying Fang, Hujing Wang, and Qibin Yu for assistance with plant materials and laboratory activities.

Conflict of interest

JL was employed by Natural Medicine Institute of Zhejiang YangShengTang Co., LTD.

The remaining 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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1039094/full#supplementary-material

References

Beigmohamadi, M., Sharafi, A., and Jafari, S. (2019). An optimized protocol for *Agrobacterium rhizogenes*-mediated genetic transformation of *Citrullus colocynthis*. *J. Appl. Biotechnol. Rep.* 6, 113–117. doi: 10.29252/JABR.06.03.06

Bolger, A. M., Marc, L., and Bjoern, U. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Boscariol, R. L., Monteiro, M., Takahashi, E. K., Chabregas, S. M., and Mendes, B. M. J. (2006). *Attacin* a gene from *Tricloplusia ni* reduces susceptibility to *Xanthomonas axonopodis* pv. *citri* in transgenic *Citrus sinensis* `Hamlin'. *J. Am. Soc. Hortic. Sci.* 131, 530–536. doi: 10.21273/JASHS.131.4.530

Cao, D., Hou, W., Song, S., Sun, H., Wu, C., Gao, Y., et al. (2009). Assessment of conditions affecting *Agrobacterium rhizogenes*-mediated transformation of soybean. *Plant Cell Tissue Organ Culture1* 96, 45–52. doi: 10.1111/j.1745-4557.2007.00153.x

Castellanos-Arévalo, A. P., Estrada-Luna, A. A., Cabrera-Ponce, J. L., Valencia-Lozano, E., Herrera-Ubaldo, H., de Folter, S., et al. (2020). *Agrobacterium rhizogenes*-mediated transformation of grain (*Amaranthus hypochondriacus*) and leafy (*A. hybridus*) amaranths. *Plant Cell Rep.* 39, 1143–1160. doi: 10.1007/s00299-020-02553-9

Cheng, Y., Wang, X., Cao, L., Ji, J., Liu, T., and Duan, K. (2021). Highly efficient *Agrobacterium rhizogenes*-mediated hairy root transformation for gene functional and gene editing analysis in soybean. *Plant Methods* 17, 1–12. doi: 10.1186/S13007-021-00778-7

Chetty, V. J., Ceballos, N., Garcia, D., Narváez-Vásquez, J., Lopez, W., and Orozco-Cárdenas, M. L. (2013). Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* l.) cultivar micro-tom. *Plant Cell Rep.* 32, 239–247. doi: 10.1007/s00299-012-1358-1

Dai, S, Zhang, Z, and Chen, S. (2004). RF2b, a rice bZIP transcription activator, interacts with RF2a and is involved in symptom development of rice tungro disease [J]. *Proceedings of the National Academy of Sciences* 101 (2), 687–692.

Domínguez, A., Mendoza, A. H. D., Guerri, J., Cambra, M., Navarro, L., Moreno, P., et al. (2002). Pathogen-derived resistance to *Citrus tristeza virus* (CTV) in transgenic mexican lime (*Citrus aurantifolia* (Christ.) swing.) plants expressing its *p25* coat protein gene. *Mol. Breed.* 10, 1–10. doi: 10.1023/A:1020347415333

Ellison, E. E., Nagalakshmi, U., Gamo, M. E., Huang, P. J., and Voytas, D. F. (2020). Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nat. Plants* 6, 1–5. doi: 10.1038/s41477-020-0670-y

Estrada-Navarrete, G., Alvarado-Affantranger, X., Olivares, J. E., Guillén, G., Diaz-Camino, C., Campos, F., et al. (2007). Fast, efficient and reproducible genetic transformation of *Phaseolus spp*. by *Agrobacterium rhizogenes*. *Nat. Protoc.* 2, 1819–1824. doi: 10.1038/nprot.2007.259

Fagoaga, C., López, C., Mendoza, A., Moreno, P., Navarro, L., Flores, R., et al. (2006). Post-transcriptional gene silencing of the p23 silencing suppressor of *Citrus tristeza virus* confers resistance to the virus in transgenic Mexican lime. *Plant Mol. Biol.* 60, 153–165. doi: 10.1007/s11103-005-3129-7

Febres, V. J., Lee, R. F., and Moore, G. A. (2008). Transgenic resistance to *Citrus tristeza virus* in grapefruit. *Plant Cell Rep.* 27, 93–104. doi: 10.1007/s11103-005-3129-7

Fu, X. Z., Chen, C. W., Yin, W., Liu, J. H., and Moriguchi, T. (2011). Ectopic expression of *MdSPDS1* in sweet orange (Citrus sinensis osbeck) reduces canker susceptibility: involvement of H2O2 production and transcriptional alteration. *BMC Plant Biol.* 11, 1–15. doi: 10.1186/1471-2229-11-55

Gambino, G., and Gribaudo, I. (2012). Genetic transformation of fruit trees: current status and remaining challenges. *Transgenic Res.* 21, 1163–1181. doi: 10.1007/s11248-012-9602-6

Ghoshal, B., Vong, B., Picard, C. L., Feng, S., Tam, J. M., and Jacobsen, S. E. (2020). A viral guide RNA delivery system for CRISPR-based transcriptional activation and heritable targeted DNA demethylation in *Arabidopsis thaliana*. *PLos Genet.* 16, e1008983. doi: 10.1371/journal.pgen.1008983

Gutiérrez, E., M. A., Luth, D., and Moore, G. A. (1997). Factors affecting *Agrobacterium*-mediated transformation in citrus and production of sour orange (*Citrus aurantium* l.) plants expressing the coat protein gene of *citrus tristeza virus*. *Plant Cell Rep.* 16, 745–753. doi: 10.1007/s002990050313

Habibi, P., Sa, M., Silva, A., Makhzoum, A., Costa, J., Borghetti, I. A., et al. (2016). Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*. *Physiology&Molecular Biol. Plants* 22, 271–277. doi: 10.1007/s12298-016-0354-2

Hongge, J., Xiuping, Z., Vladimir, O., and Nian, W. (2019). Genome editing in citrus tree with CRISPR/Cas9. *Methods Mol. Biol.* 1917, 235–241. doi: 10.1007/978-1-4939-8991-1_17

Irigoyen, S., Ramasamy, M., Pant, S., Niraula, P., Bedre, R., Gurung, M., et al. (2020). Plant hairy roots enable high throughput identification of antimicrobials against candidatus liberibacter spp. *Nat. Commun.* 11 (1), 1–14. doi: 10.1038/s41467-020-19631-x

Ishida, Y., Hiei, Y., and Komari, T. (2007). *Agrobacterium*-mediated transformation of maize. *Nat. Protoc.* 2, 1614–1621. doi: 10.1007/s00003-007-0272-x

Jia, H., Omar, A., Orbovi, V., and Wang, N. (2021). Biallelic editing of the *LOB1* promoter via CRISPR/Cas9 creates canker-resistant 'Duncan' grapefruit. *Phytopathology* 112, 308–314. doi: 10.1094/PHYTO-04-21-0144-R

Jia, H., Zhang, Y., Orbovi, V., Xu, J., White, F. F., Jones, J. B., et al. (2017). Genome editing of the disease susceptibility gene *CsLOB1* in citrus confers resistance to citrus canker. *Wiley-Blackwell Online Open* 15, 817–823. doi: 10.1111/pbi.12677

Kaur, A., and Sah, S. K. (2014). Genetic transformation of rice: problems, progress and prospects. *Rice Res. Open Access* 3. doi: 10.4172/2375-4338.1000132

Kereszt, A., Li, D., Indrasumunar, A., Nguyen, C. D., Nontachaiyapoom, S., Kinkema, M., et al. (2007). *Agrobacterium rhizogenes*-mediated transformation of soybean to study root biology. *Nat. Protoc.* 2, 948–952. doi: 10.1038/nprot.2007.141

Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* 12, 357–360. doi: 10.1038/nmeth.3317

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. doi: 10.1186/s13059-014-0550-8

Luo, J., Li, S., Xu, J., Yan, L., Ma, Y., and Xia, L. (2021). Pyramiding favorable alleles in an elite wheat variety in one generation by CRISPR-Cas9-mediated multiplex gene editing. *Mol. Plant* 14, 847–850. doi: 10.1016/j.molp.2021.03.024

Majumdar, S., Garai, S., and Jha, S. (2011). Genetic transformation of *Bacopa monnieri* by wild type strains of *Agrobacterium rhizogenes* stimulates production of bacopa saponins in transformed calli and plants. *Plant Cell Rep.* 30, 941–954. doi: 10.1016/j.molp.2021.03.024

Ma, X., Zhang, X., Liu, H., and Li, Z. (2020). Highly efficient DNA-free plant genome editing using virally delivered CRISPR–Cas9. *Nat. Plants* 6, 773–779. doi: 10.1038/s41477-020-0704-5

Orbović, V., and Grosser, J. W. (2015). "Citrus transformation using juvenile tissue explants," in *Agrobacterium protocols* (New York, NY: Springer), 245–257. doi: 10.1007/978-1-4939-1658-0_20

Paultre, D. S. G., Gustin, M. P., Molnar, A., and Oparka, K. J. (2016). Lost in transit: long-distance trafficking and phloem unloading of protein signals in Arabidopsis homografts. *The Plant Cell* 28 (9), 2016–2025.

Peña, L. (2000). Generation of transgenic citrus plants with the tolerance-to-salinity gene *HAL2* from yeast. *J. Hortic. Sci. Biotechnol.* 75, 26–30. doi: 10.1080/14620316.2000.11511195

Peng, A., Chen, S., Lei, T., Xu, L., He, Y., Wu, L., et al. (2017). Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. *Plant Biotechnol. J.* 15, 1509–1519. doi: 10.1111/pbi.12733

Poles, L., Licciardello, C., Distefano, G., Nicolosi, E., and Malfa, S. L. (2020). Recent advances of in vitro culture for the application of new breeding techniques in citrus. *Plants* 9, 938. doi: 10.3390/plants9080938

Raineri, D., Bottino, P., Gordon, M., and Nester, E. (1990). *Agrobacterium*–mediated transformation of rice (*Oryza sativa* l.). *Bio/technology* 8, 33–38. doi: 10.1038/nbt0190-33

Richards, E., Reichardt, M., and Rogers, S. (1994). Preparation of genomic DNA from plant tissue. *Curr. Protoc. Mol. Biol.* 27, 2–3. doi: 10.1002/0471142727.mb0203s27

Roychowdhury, D., Halder, M., and Jha, S. (2017). Mediated Transformation in Medicinal Plants: Genetic Stability in Long-Term Culture[J]. *Transgenesis Secondary Metab.* 323–345. doi: 10.1007/978-3-319-28669-3_8

Stover, E., Stange, R. R., Mccollum, T. G., Jaynes, J., and Mirkov, E. (2013). Screening antimicrobial peptides in vitro for use in developing transgenic citrus resistant to huanglongbing and citrus canker. *J. Am. Soc. Horticulturalence* 138, 142–148. doi: 10.21273/JASHS.138.2.142

Talon, M., and Gmitter, G. (2008). Citrus genomics. *Int. J. Plant Genomics*, 2008. doi: 10.1155/2008/528361

Tsuro, M., Kubo, T., Shizukawa, Y., Takemoto, T., and Inaba, K. (2005). *Agrobacterium rhizogenes* is a useful transporter for introducing T-DNA of the binary plasmid into the chrysanthemum, *Dendranthema grandiflorum* kitamura, genome. *Plant Cell Tissue Organ Culture* 81, 175–181. doi: 10.1007/s11240-004-4783-1

Tzean, Y, Chang, HH, Tu, TC, et al. (2020) Engineering plant resistance to tomato yellow leaf curl Thailand virus using a phloem-specific promoter expressing hairpin RNA[J]. *Molecular Plant-Microbe Interactions* 33 (1), 87–97

Velázquez, K., Agüero, J., Vives, M. C., Aleza, P., Pina, J., Moreno, P., et al. (2016). Precocious flowering of juvenile citrus induced by a viral vector based on *Citrus leaf blotch virus*: a new tool for genetics and breeding. *Plant Biotechnol. J.* 14 (10), 1976–1985. doi: 10.1111/pbi.12555

White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P., and Nester, E. W. (1985). Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J. Bacteriol.* 164, 33–44. doi: 10.1128/jb.164.1.33-44.1985

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation* 2, 100141. doi: 10.1016/j.xinn.2021.100141

Zhang, Y. Y., Zhang, D. M., Zhong, Y., Chang, X. J., Hu, M. L., and Cheng, C. Z. (2017). A simple and efficient in planta transformation method for pommelo (*Citrus maxima*) using *Agrobacterium tumefaciens*. *Scientia Hortic.* 214, 174–179. doi: 10.1016/j.scienta.2016.11.033

frontiers | Frontiers in Genome Editing

TYPE Original Research
PUBLISHED 20 January 2023
DOI 10.3389/fgeed.2023.1124794

OPEN ACCESS

EDITED BY
Hailong Wang,
Shandong University (Qingdao), China

REVIEWED BY
Jin Wang,
Shenzhen Second People's Hospital, China
Min Li,
Gannan Normal University, China

*CORRESPONDENCE
Tong Zhang,
zhangtong@scau.edu.cn

SPECIALTY SECTION
This article was submitted to Genome Editing Tools and Mechanisms, a section of the journal Frontiers in Genome Editing

RECEIVED 15 December 2022
ACCEPTED 12 January 2023
PUBLISHED 20 January 2023

CITATION
Wang J, Huang X, Chen S, Chen J, Liang Z, Chen B, Yang X, Zhou G and Zhang T (2023), On-site and visual detection of sorghum mosaic virus and rice stripe mosaic virus based on reverse transcription-recombinase-aided amplification and CRISPR/Cas12a. *Front. Genome Ed.* 5:1124794. doi: 10.3389/fgeed.2023.1124794

On-site and visual detection of sorghum mosaic virus and rice stripe mosaic virus based on reverse transcription-recombinase-aided amplification and CRISPR/Cas12a

Junkai Wang[1], Xiuqin Huang[1], Siping Chen[1], Jiahao Chen[1], Zhengyi Liang[1], Biao Chen[1], Xin Yang[1], Guohui Zhou[1] and Tong Zhang[1,2]*

[1]Guangdong Province Key Laboratory of Microbial Signals and Disease Control, College of Plant Protection, South China Agricultural University, Guangzhou, China, [2]State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China

Rapid, sensitive and visual detection of plant viruses is conducive to effective prevention and control of plant viral diseases. Therefore, combined with reverse transcription and recombinase-aided amplification, we developed a CRISPR/Cas12a-based visual nucleic acid detection system targeting sorghum mosaic virus and rice stripe mosaic virus, which cause harm to crop production in field. When the RT-RAA products were recognized by crRNA and formed a complex with LbCas12a, the ssDNA labeled with a quenched green fluorescent molecule will be cleaved by LbCas12a, and then a significant green fluorescence signal will appear. The entire detection process can be completed within 30 min without using any sophisticated equipment and instruments. The detection system could detect samples at a dilution of 10^7, about 10^4-fold improvement over RT-PCR, so the system was successfully to detect rice stripe mosaic virus in a single leafhopper, which is the transmission vector of the virus. Finally, the CRISPR/Cas12a-based detection system was utilized to on-site detect the two viruses in the field, and the results were fully consistent with that we obtained by RT-PCR in laboratory, demonstrating that it has the application prospect of detecting important crop viruses in the field.

KEYWORDS

RT-RAA, CRISPR/Cas12a, SrMV, RSMV, visual detection, on-site detection

1 Introduction

Viruses are the most prevalent disease-causing agents in plants and pose serious threat to agriculture and food security (Jones and Naidu, 2019). Despite the significant increase of ssDNA viruses in recent years, RNA viruses cause more serious losses in crops and enormously damage to agricultural production (Zhang et al., 2018). The development of a sensitive and accurate diagnostic method is crucial to control plant viruses. A series of methods have been established, including, but not limited to, polymerase chain reaction (PCR), reverse transcription followed by PCR (RT-PCR), loop (or RT-loop) mediated isothermal amplifications (LAMP and RT-LAMP), molecular hybridization, and enzyme-linked immunosorbent assay (ELISA) (Rubio et al., 2020). PCR-based detection methods are the most widely used virus detection technology at present, especially real-time quantitative PCR

(qPCR) and (RT-qPCR), which rely on the binding of a virus sequence-specific fluorescent probe to the PCR-amplified region and are become more popular since the epidemic of SARS-CoV-2. However, qPCR and RT-qPCR detection methods require well-equipped laboratories and relatively more expensive reagents, impeding the use of these technologies at resource-limited areas for plant virus diagnosis. ELISA is another widely used technology in virus detection, which has the advantages of high sensitivity and high throughput, but needs viral specific antibodies to the virus structure protein (Clark and Adams, 1977). Most of these methods are faced with some defects such as high cost, limited accuracy, insufficient sensitivity, and inability to use in the field for on-site diagnosis in a low-resource environment. Therefore, establishment of rapid, sensitive and portable sensing of viral detection technologies will greatly promote the research, prevention and control of plant virus diseases.

The clustered regularly short palindromic repeat-associated systems (CRISPR-Cas) comprise sequence-specific RNA-directed endonuclease complexes that bind and cleave nucleic acids (Bhaya et al., 2011). In the last decade, CRISPR-Cas has been extensively exploited in eukaryotic species for genome engineering, molecular immunity, and transcriptional regulation (Doudna and Charpentier, 2014; Zhang et al., 2018; Zhang et al., 2019; Zhao et al., 2020; Zhu et al., 2020). Further, many CRISPR systems were found to unleashes indiscriminate single stranded DNase activity by target identification and cleavage (Li et al., 2018a; Chen et al., 2018). This property can be harnessed for nucleic acid detection, and several detection platforms have been established in the past years. For instance, SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing), HOLMES (an one-Hour Low-cost Multipurpose highly Efficient System), and DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) are three pioneer nucleic acid detection platforms, which employ the non-specific endonuclease activities of Cas13-ssRNA reporter or Cas12-ssDNA reporter (Gootenberg et al., 2017; Li et al., 2018b; Chen et al., 2018; Gootenberg et al., 2018; Kellner et al., 2019). These detection platforms have the advantages of high sensitivity and direct display of detection results.

Isothermal amplification techniques, such as loop mediated isothermal amplifications (LAMP) and recombinase polymerase amplification (RPA), are more and more widely used in the detection of viral nucleic acid, because they allow nucleic acid amplification at a single temperature, thus supporting their use in the field for on-site diagnosis in a low-resource environment (Notomi et al., 2000; Piepenburg et al., 2006). LAMP and RPA are further customized as RT-LAMP and RT-RPA when combined with a reverse transcriptase, that can be utilized for the detection of RNA viruses (Fukuta et al., 2003; Euler et al., 2013). In the past few years, LAMP and RPA methods (including RT-LAMP and RT-RPA) have been integrated with CRISPR/Cas system for rapid and portable detection of both DNA and RNA viruses (Kellner et al., 2019; Broughton et al., 2020; Kaminski et al., 2021).

Sorghum mosaic virus (SrMV), genus *Potyvirus*, family Potyviridae, is one of the main pathogens causing sugarcane mosaic disease, and is widely distributed in major sugarcane regions in the world at present (Lu et al., 2021). The rapid

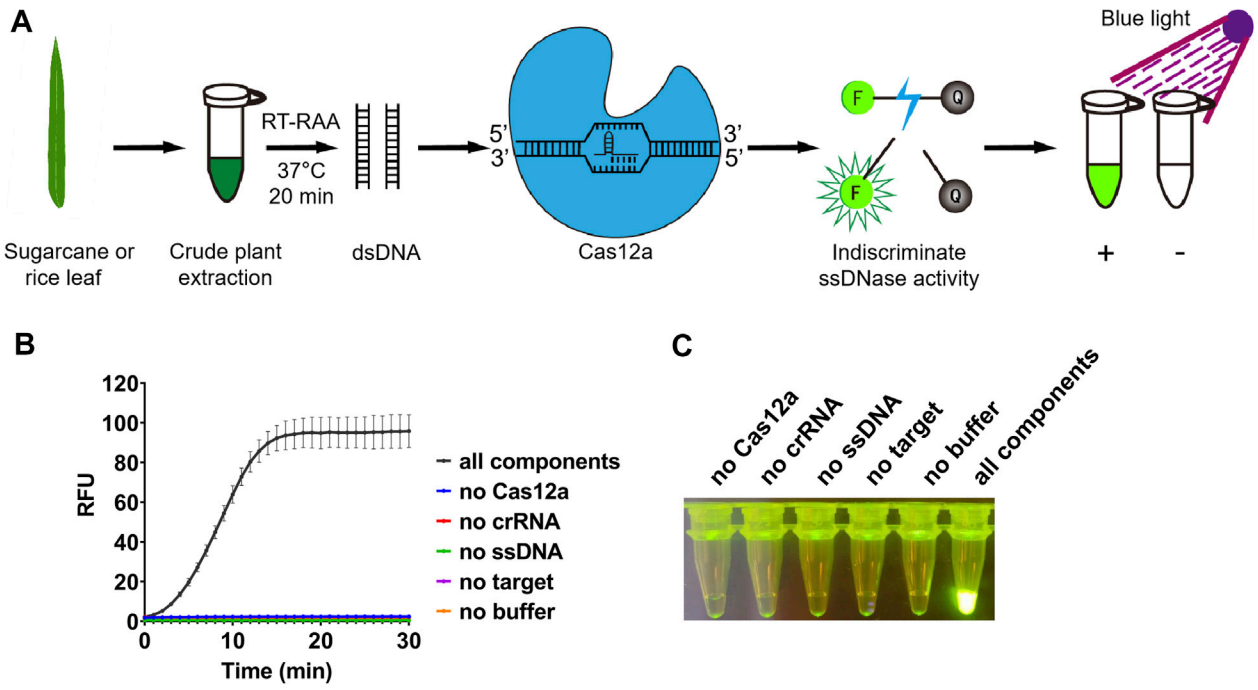


FIGURE 1
Detection system based on reverse transcription-recombinase-aided amplification and CRISPR/Cas12a. **(A)** Schematic diagram of the method for rapid and visual nucleic acid detection using RT-RAA-CRISPR/Cas12a assay. In this detection system, target gene fragments were amplified with RAA; ssDNA probes (5′-FAM/3′-BHQ1 labeled) were added, and subsequently cleaved by Cas12a to generate green fluorescence. The detection results were directly visible under blue light. **(B)** Validation of the CRISPR/Cas12a assay. In the absence of each component, the real-time fluorescence signal was continuously monitored for 30 min. **(C)** After 30 min of reaction, the tubes were irradiated with UV light for visualization. n = 3 technical replicates, and data points are shown as mean ± SD.

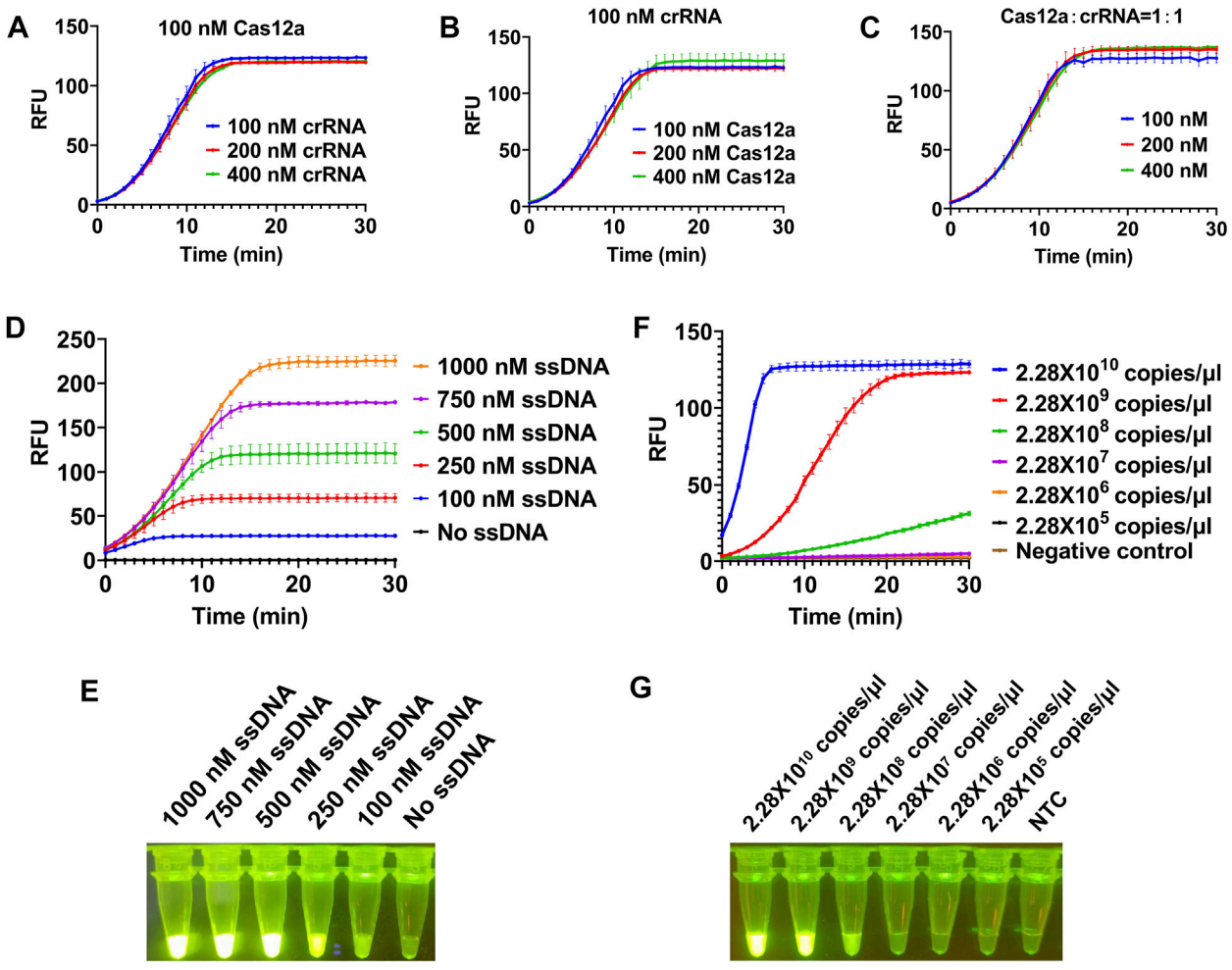


FIGURE 2
Optimization of reaction conditions for CRISPR/Cas12a-based visual detection. The SrMV RT-RAA product was cloned into vector and the plasmid was used for the optimization of the CRISPR/Cas12a system. **(A)** The concentration of Cas12a protein was constant, and the concentration of crRNA in the system was optimized. **(B)** The concentration of crRNA was constant, and the concentration of Cas12a protein in the system is optimized. **(C)** The concentration of Cas12a protein and crRNA was optimized in equal proportion. **(D)** Real-time fluorescence signal analysis of ssDNA probes at different concentrations. **(E)** Determination of optimal ssDNA concentration by visual observation. **(F)** Real-time fluorescence signal analysis of plasmid templates at different concentrations. **(G)** Determination of optimal plasmid templates concentrations by visual observation. n = 3 technical replicates, and data points are shown as mean ± SD.

detection and identification of this virus is helpful to control the sugarcane mosaic disease in the field.

Rice stripe mosaic cytorhabdovirus (RSMV) is cytoplasmic rhabdovirus damaging rice production in China (Yang et al., 2017a). RSMV infected plants exhibit distinct symptoms, including yellow stripes, mosaic patterns, and twisted leaf tips (Yang et al., 2017a; Chen et al., 2019), and it is transmitted by *Recilia dorsalis* leafhoppers in a persistent-propagative manner (Yang et al., 2017b). With the increase of the number of leafhoppers in the field in south China, the damage caused by RSMV is also increasing year by year. Therefore, it is necessary to have a method that can detect viruses in the field, and it is better to have the ability to detect viruses in vector insects.

In this study, we developed a rapid, sensitive, specific and visual method to detect SrMV and RSMV based on RT-recombinase-aided amplification (RT-RAA) and CRISPR/Cas12a system. RT-RAA is an isothermal nucleic acid amplification procedure which is similar to RT-RPA. From sample homogenization to RT-RAA followed by CRISPR/Cas12a detection, the entire detection process can be completed within 30 min without using any sophisticated equipment and instruments. The new assay is highly sensitive compared with RT-PCR and highly specific for the target viruses. Furthermore, the method was applied to detect RSMV in samples of a single vector leafhopper, demonstrating that it has the application prospect of detecting important crop viruses in the field.

2 Materials and methods

2.1 Plant and insect samples

Sugarcane leaves infected with sorghum mosaic virus (SrMV), sugarcane mosaic virus (SCMV), sugarcane yellow leaf virus (SCYLV),

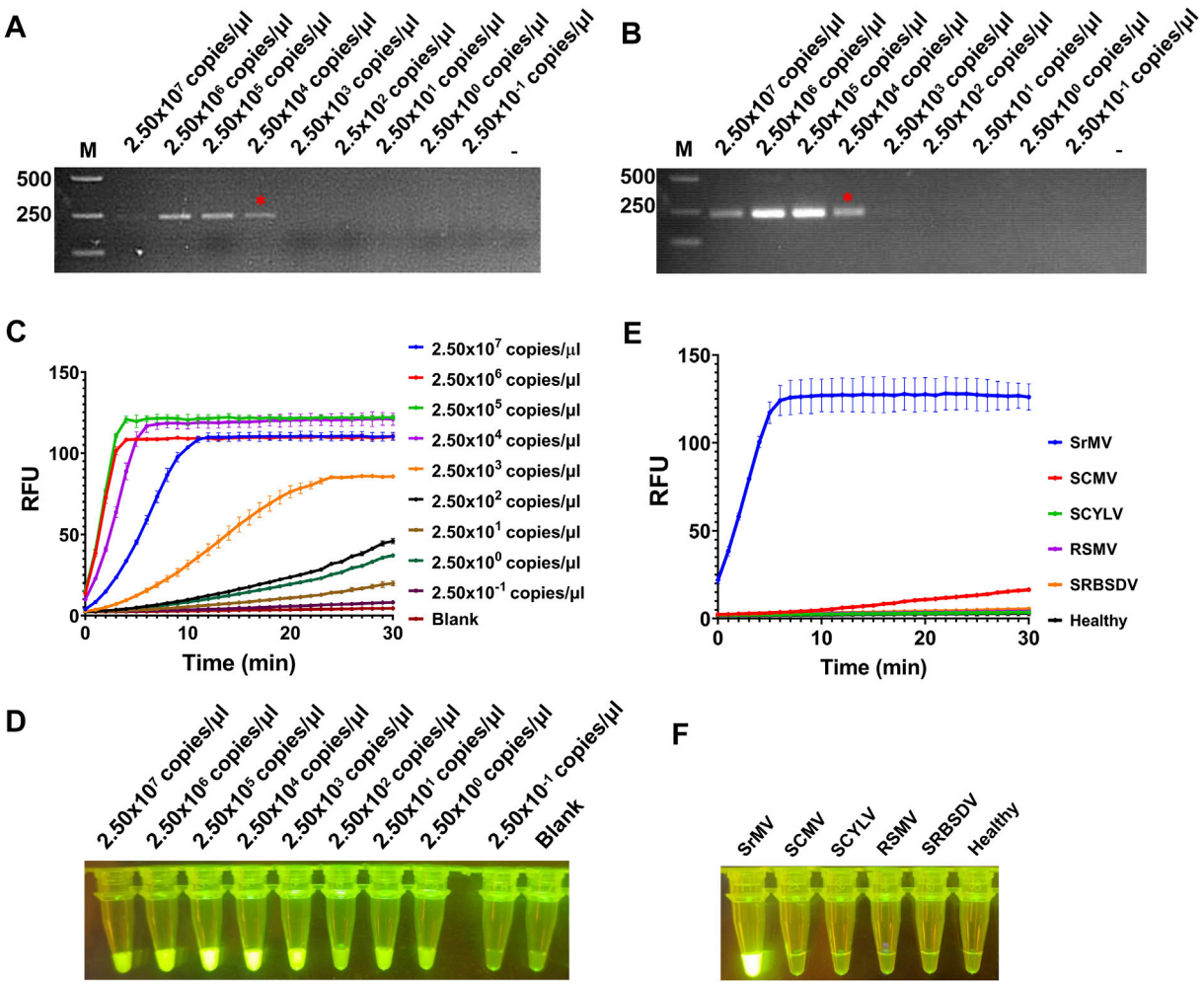


FIGURE 3
Sensitivity and specificity assay for RT-RAA-CRISPR/Cas12a detection for SrMV. **(A)** The crude extracts of SrMV infected sugarcane leaves were diluted gradiently and tested with RT-RAA. **(B)** The crude extracts were diluted gradiently and tested with RT-PCR. **(C)** The crude extracts were diluted gradiently and tested by RT-RAA-CRISPR/Cas12a detection method, and the real-time fluorescence signal was continuously monitored for 30 min. Water was used as blank control. **(D)** The gradiently diluted crude extracts were tested by RT-RAA-CRISPR/Cas12a method and followed by visual observation. **(E)** The crude extracts of plants samples infected with different viruses were tested by RT-RAA-CRISPR/Cas12a detection method, and the real-time fluorescence signal was continuously monitored for 30 min. Healthy plant sample was used as control. **(F)** The crude extracts of plants samples infected with different viruses were tested by RT-RAA-CRISPR/Cas12a method and followed by visual observation. SrMV: sorghum mosaic virus, SCMV: sugarcane mosaic virus, SCYLV: sugarcane yellow leaf virus, RSMV: rice stripe mosaic virus, SRBSDV: southern rice black-streaked dwarf virus. n = 3 technical replicates, and data points are shown as mean ± SD.

and rice plants infected with rice stripe mosaic virus (RSMV), southern rice black-streaked dwarf virus (SRBSDV), rice gall dwarf virus (RGDV), rice ragged stunt virus (RRSV), rice orange leaf phytoplasma (ROLP) were preserved in our laboratory. Leafhoppers (*Recilia dorsalis*) were reared and propagated on the RSMV-infected rice plants. RT-PCR with specific primers of each of these pathogens was conducted to verify the samples were indeed infected.

2.2 RT-RAA primer design

The sequences of 11 SrMV isolates were downloaded from NCBI (Supplementary Table S1) and compared by MegAlign (DNAStar, Madison, WI, USA), and the conserved sequences were designed as the RT-RAA primers (Supplementary Table S2). For RSMV, the RT-RAA primers were designed from the conserved region of its encoded N gene (Supplementary Table S2).

2.3 RT-RAA assay

The RT-RAA assay was performed using the RT-RAA kit (Cat: S003ZC, ZC Bio-Sci&Tech, Hangzhou, China). The reaction mixture contained 9.5 μl buffer A, 9.5 μl nuclease-free water, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 2 μl buffer B, and 2 μl RNA template. Buffer B was pre-loaded inside the lid, and the reaction tube was centrifuged briefly to ensure the reagents were well-mixed. The products were visualized by gel electrophoresis using 2% agarose gels.

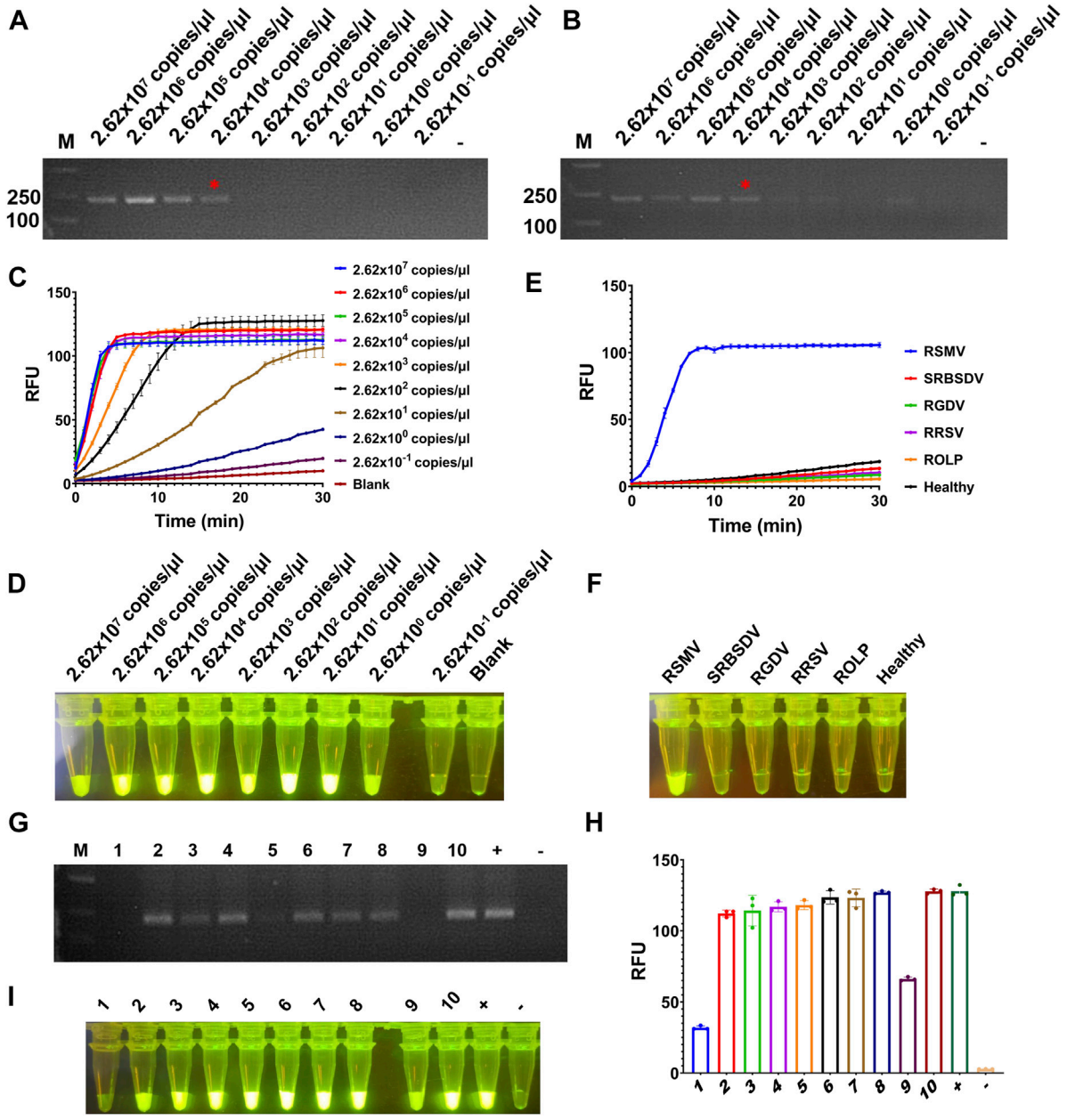


FIGURE 4
Sensitivity and specificity assay for RT-RAA-CRISPR/Cas12a detection for RSMV. **(A)** The crude extracts of RSMV infected rice leaves were diluted gradiently and tested by RT-RAA. **(B)** The crude extracts were diluted gradiently and tested by RT-PCR. **(C)** The crude extracts were diluted gradiently and tested by RT-RAA-CRISPR/Cas12a detection method, and the real-time fluorescence signal was continuously monitored for 30 min. Water was used as blank control. **(D)** The gradiently diluted crude extracts were tested by RT-RAA-CRISPR/Cas12a method and followed by visual observation. **(E)** The crude extracts of rice samples infected with different pathogens were tested by RT-RAA-CRISPR/Cas12a detection method, and the real-time fluorescence signal was continuously monitored for 30 min. Healthy plant sample was used as control. **(F)** The crude extracts of rice samples infected with different pathogens were tested by RT-RAA-CRISPR/Cas12a method and followed by visual observation. **(G)** The crude extracts of single leafhopper samples were tested by RT-PCR. **(H)** The crude extracts of single leafhopper samples were tested by RT-RAA-CRISPR/Cas12a detection method, and the fluorescence signal was quantitative monitored by microplate reader. **(I)** The crude extracts of single leafhopper samples were tested by RT-RAA-CRISPR/Cas12a detection method and followed by visual observation. RSMV: rice stripe mosaic virus, SRBSDV: southern rice black-streaked dwarf virus, RGDV: rice gall dwarf virus, RRSV: rice ragged stunt virus, ROLP: rice orange leaf phytoplasma. n = 3 technical replicates, and data points are shown as mean ± SD.

2.4 RT-PCR

Total RNA was extracted from leaf samples using the TRIzol reagent (Vazyme, Nanjing, China). RT-PCR amplification was carried out using a One-Step RNA PCR kit (Cat: RR055, Takara, Dalian, China) with the SrMV or RSMV specific RT-RAA primers. The reaction system (10 μl) contains 5 μl of 2X reaction mix, 1 μl RNA template, 0.25 μl forward primer, 0.25 μl reverse primer, and 3.5 μl ddH_2O. The procedure of PCR was as follows: 50°C for 30 min; 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. After amplification, the resulting RT-PCR products were visualized by gel electrophoresis using 2% agarose gels.

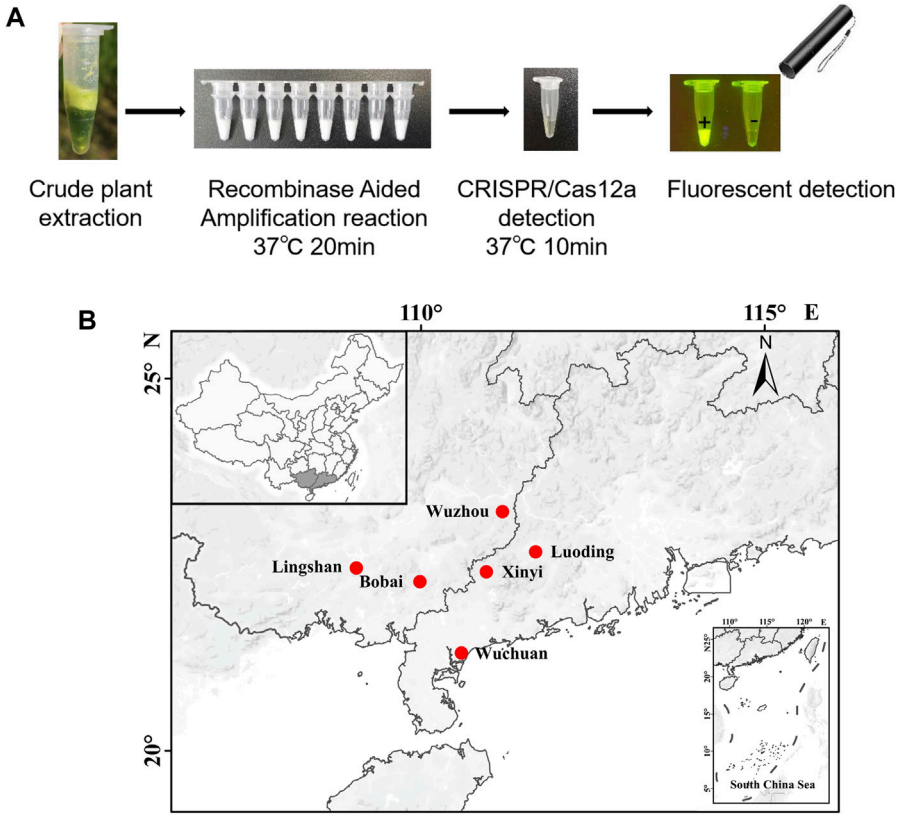


FIGURE 5
Field on-site detection of SrMV and RSMV by the RT-RAA-CRISPR/Cas12a system. **(A)** The pipeline of on-site plant virus detection based on the RT-RAA-CRISPR/Cas12a system. Take a piece of leaf from the plants and put it into a 1.5 ml tube, followed by grinding with 400 μl crude extraction buffer. Then take 2 μl crude extract into 25 μl RT-RAA reaction system, water bath at 37°C for 20 min. Finally take 2 μl RT-RAA products into 18 μl CRISPR/Cas12a detection solution, water bath at 37°C for 10 min and observed under a UV flashlight. **(B)** The sites we performed the on-site detection of SrMV and RSMV in Guangdong and Guangxi province, China.

TABLE 1 Field samples detection for SrMV by two methods.

Target virus	Location	Samples amount	RT-RAA-CRISPR-Cas12a (on-site)		RT-PCR (in laboratory)	
			No. Positive	Positive rate (%)	No. Positive	Positive rate (%)
SrMV	Luoding	13	3	23.07	2	15.38
	Xinyi	20	1	5.00	1	5.00
	Wuchuan	18	1	5.55	0	0.00
	Xindi	24	5	20.83	5	20.83
	Lingshan	20	2	10.00	1	5.00
	Bobai	16	4	25.00	4	25.00

2.5 The design and synthesis of crRNAs

The crRNAs of LbCas12a recognize a 20-nt target sequence adjacent to a 5′-TTTN-3′ site. The spacer sequence of crRNA was designed to recognize the region between the RT-RAA primers. The spacer sequence was aligned using NCBI BLAST to ensure the specificity of the crRNA target sequence (Supplementary Table S3). The crRNAs were synthesized by Sangon Biotech (Shanghai, China).

2.6 Purification of the LbCas12a protein

The LbCas12a was cloned into pET28a (Novagen) with N-terminal 6×His tagging. The plasmid was transformed into *E. coli* strain Rosetta (DE3). For protein expression, a single clone was first cultured overnight in 5-mL liquid LB and then 1% (v/v) inoculated into 200 ml of fresh liquid LB. Cells were grown with shaking at 220 rpm and 37°C until the OD600 reached 0.8, and then

TABLE 2 Field samples detection for RSMV by two methods.

Target virus	Location	Samples amount	RT-RAA-CRISPR-Cas12a (on-site)		RT-PCR (in laboratory)	
			No. Positive	Positive rate (%)	No. Positive	Positive rate (%)
RSMV	Luoding	24	10	41.67	10	41.67
	Xinyi	16	7	43.75	7	43.75
	Wuchuan	12	3	25.00	3	25.00
	Xindi	20	3	15.00	3	15.00
	Lingshan	14	6	42.86	6	42.86
	Bobai	18	4	22.22	4	22.22

0.5 mM IPTG was added followed by further culture at 37°C for 4 h. Cells were harvested and resuspended in 25 mL lysis buffer (50 mM Tris-Hcl, 600 mM NaCl, 1 mM DTT, and 5% glycerol) with 1 mM PMSF as the protease inhibitor, then were sonicated on ice. The obtained lysate was then centrifuged at 15,000 rpm for 30 min. The supernatant was mixed with 5 mL Ni-NTA beads (ThermoFisher Scientific) and softly shaken for 1 h at 4°C. The beads were washed with wash buffer (lysis buffer supplemented with 30 mM imidazole) and eluted with elution buffer (lysis buffer supplemented with 600 mM imidazole). Fractions containing LbCas12a proteins were verified by SDS-PAGE and then pooled for dialysis with dialysis buffer (50 mM Tris-HCl, 600 mM NaCl, 2 mM DTT) overnight. Finally, the protein was collected and diluted to a final concentration of 10 μM and stored at −80 °C.

2.7 The LbCas12a-based fluorescence assay for trans-cleavage activity

The total CRISPR/Cas12a reaction volume was 20 μl, and the reaction mixture contained 500 nM LbCas12a, 500 nM crRNA, 2 μl 10X Enhanced Buffer (100 mM Tris-HCl, 100 mM NaCl, 150 mM $MgCl_2$, 10 mM DTT, 5% PEG-200), 2 μl plasmid that contained the target gene, 2 μM fluorophore quencher labeled ssDNA probe (5′-FAM-CCCCCCCC-BHQ1-3′). Reactions were performed at 37°C on multi-functional microplate reader Varioskan LUX (Thermo), and the fluorescence was measured every 30 s (λex: 492 nm; λem: 520 nm). The fluorescence signals were also examined by under a UV Flashlight.

For testing the minimum detection limit of the CRISPR/Cas12a reaction, the RT-RAA products were cloned into pEASY-Blunt Zero vector (TransGen Biotech) and the obtained plasmids were verified by sequencing. Then the concentration of the plasmids was determined by Nanodrop spectrophotometry (Thermo Scientific) and the copy number was calculated. The viral copy number of crude extract was calculated by comparing the Cq number of the plasmids based on RT-qPCR data. The series of 10-fold diluted plasmids or crude extract of virus infected leaves were proceeded for CRISPR/Cas12a reaction system.

2.8 Direct virus detection from crude extracts

15–20 mg of leaf tissue or a single insect was homogenized by a hand-held tissue homogenizer in a 1.5-ml tube with 400 μl or 50 μl extraction buffer (6% PEG 200 and 20 mM NaOH), respectively. After incubation at room temperature for 3–5 min, the crude extract was directly subjected to RT-RAA without purification, followed by CRISPR/Cas12a visual detection using the above procedure.

2.9 Sensitivity and specificity assay

For sensitivity assay, a crude extract of the leaf sample was considered the 1x dilution. Further 10-fold serial dilutions, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 were made and proceed for RT-RAA-CRISPR/Cas12a detection.

For specificity assay, plants infected with different viruses were sampled for RT-RAA-CRISPR/Cas12a detection.

2.10 Field sample detection

To test the CRISPR/Cas12a-based detection system, sugarcane and rice samples were collected from different locations in Guangxi, Guangdong. The RT-RAA reaction was incubated at 37°C for 20 min, and 2 μl of the RT-RAA product was used for the CRISPR/Cas12a-based detection as described above. Moreover, to verify the accuracy of the CRISPR/Cas12a-based detection system, the samples were taken back to the laboratory and analyzed by using RT-PCR method.

3 Results

3.1 Optimization of the conditions for RT-RAA

The RT-RAA-CRISPR/Cas12a RNA virus detection platform is shown in Figure 1A. The first step is RT-RAA amplification on the crude extract of the sample. So we optimized the conditions of RT-RAA by using SrMV infected sugarcane leaf samples. We designed multiple RT-RAA primer sets against the conserved region of SrMV genome (Supplementary Table S2). 6 out of 8 sets of primers were effective in the RT-RAA reaction, and the third set (RAA-SrMV-F3 and RAA-SrMV-R3) was more active (Supplementary Figure S1A). We thus proceeded with this primer set in later SrMV RT-RAA experiments. Then we tested the effect of different primers concentration, reaction temperature, and reaction time for the RT-RAA efficiency. The results showed that primer concentration between 0.4–0.8 μM, reaction

temperature between 32°C–42°C, and reaction for 20–40 min was suitable for RT-RAA reaction (Supplementary Figure S1B-D). To facilitate and save reagents for the RT-RAA reaction, we set the primer concentration at 0.4 μM, reaction temperature at 37°C, and reaction time for 20 min for further experiments.

3.2 Optimization of the conditions for CRISPR/Cas12a-based visual detection

After amplification, the RT-RAA product will be used for detection assay with CRISPR/Cas12a. When the RT-RAA products were recognized by crRNA and formed a complex with LbCas12a, the ssDNA labeled with a quenched green fluorescent molecule will be cleaved by LbCas12a, and then a significant green fluorescence signal will appear (Figure 1A). We purified LbCas12a which was expressed in *Escherichia coli*, and obtained the protein with correct size (Supplementary Figure S2). The detection results can be observed directly under blue light after reacting for approximately 10 min (Figure 1B). In contrast, no fluorescence signal will be produced if the system lacks LbCas12a, crRNA, ssDNA, RT-RAA product, or buffer (Figures 1B, C).

To achieve a better detection effect, we optimized the concentration of each component in the system using plasmid as template. We first investigated the dosage of LbCas12a and crRNA in this reaction (100, 200, 400 nM). The results showed that no matter the concentration of LbCas12a or crRNA increased alone or that of both LbCas12a and crRNA was increased, there was no significant improve on the trans cleavage activity of LbCas12a (Figures 2A–C). Next we tested the dosage of ssDNA in this reaction, and a series of concentrations of ssDNA (0, 100, 250, 500, 750, and 1000 nM) were applied. With the increase of the concentration of ssDNA added, the fluorescence signal is gradually enhanced (Figure 2D), and clear green fluorescence was produced when the concentration reached 500 nM (Figure 2E). Together, final concentrations of 100 nM for LbCas12a, 100 nM for crRNA, and 500 nM for ssDNA were used for further experiments.

After determining the optimal concentration of each component in CRISPR/Cas12a system, we subsequently tested the minimum detection limit of this system. We cloned the RT-RAA product into a clone vector and got the concentration of 2.28×10^{10} copies/μl plasmid. When the plasmid was diluted to 2.28×10^{8} copies/μl, the fluorescence signal could be detected but weak, and when diluted to 2.28×10^{7} copies/μl, no obvious fluorescence signal can be detected (Figures 2F,G), indicating the detection limit of our CRISPR/Cas12a system was 2.28×10^{8} copies/μl.

3.3 Sensitivity and specificity assay for RT-RAA-CRISPR/Cas12a detection for SrMV

Here we combined our RT-RAA assay and CRISPR/Cas12a detection system together to set up a visual detection for SrMV. To test the sensitivity of the detection method, the crude extract of SrMV infected sugarcane leaves was serially diluted by 10-fold and quantified by RT-qPCR (2.50×10^{7} to 2.50×10^{-1} copies/μl). The diluted samples were used as templates of RT-RAA or RT-PCR, and then the amplification products were proceeded for electrophoretic detection or CRISPR/Cas12a detection. The results showed that for both RT-RAA and RT-PCR followed by electrophoretic detection, only the samples containing more than 2.50×10^{4} copies/μl viral RNA can be detected (Figures 3A, B). In contrast, in the RT-RAA followed by CRISPR/Cas12a detection, fluorescence can still be observed after the crude extract was diluted to 2.50 copies/μl viral RNA (Figures 3C, D). Therefore, the combination of RT-RAA and CRISPR/Cas12a significantly enhanced the sensitivity in virus detection.

Next, we tested the specificity of the RT-RAA-CRISPR/Cas12a method for SrMV detection. We used the crude extracts of plants infected with different viruses as templates to carry out experiments. The results showed that only the samples infected with SrMV could produce strong fluorescence signals, and the others had no obvious fluorescence emitted (Figures 3E, F), indicating the detection method based on RT-RAA and CRISPR/Cas12a was specific.

3.4 The development of the CRISPR/Cas12a-based visual detection for RSMV

RSMV is a new emerging rice virus in South China and is lacking rapid on-site detection system (Yang et al., 2017a). We therefore developed the RT-RAA-CRISPR/Cas12a detection system for RSMV. We designed multiple RT-RAA primer sets against the RSMV genome (Supplementary Table S2). 7 out of 8 sets of primers were effective in the RT-RAA reaction, and the first set (RAA-RSMV-F1 and RAA-RSMV-R1) was more active (Supplementary Figure S3A). We thus proceeded with this primer set in later RSMV detection. Then we tested the effect of different primers concentration, reaction temperature, and reaction time for the RT-RAA efficiency. Similar as we did for SrMV detection, primer concentration between 0.1 and 0.4 μM, reaction temperature between 30°C–42°C, and reaction for 20–45 min was suitable for RT-RAA reaction for RSMV (Supplementary Figure S3B-D), and we set the primer concentration at 0.4 μM, reaction temperature at 37°C, and reaction time for 20 min for further experiments.

3.5 Sensitivity and specificity assay for RT-RAA-CRISPR/Cas12a detection for RSMV

To test the sensitivity of the detection for RSMV, the crude extract of RSMV infected rice leaves was serially diluted by 10-fold and quantified by RT-qPCR (2.62×10^{7} to 2.62×10^{-1} copies/μl). The diluted samples were used as templates of RT-RAA or RT-PCR, and then the amplification products were proceeded for electrophoretic detection or CRISPR/Cas12a detection. The results showed that for both RT-RAA and RT-PCR followed by electrophoretic detection, similar as we did for SrMV, only the samples containing more than 2.62×10^{4} copies/μl viral RNA can be detected (Figures 4A, B), but the detection limit of RT-RAA-CRISPR/Cas12a for RSMV go to 2.62 copies/μl viral RNA (Figures 4C, D). We also tested the specificity of the RT-RAA-CRISPR/Cas12a method for RSMV detection. We used the crude extracts of rice plants infected with four different viruses or one phytoplasma as templates to carry out experiments. The results showed that only the samples infected with RSMV could produce strong fluorescence signals, and the others had no obvious fluorescence emitted (Figures 4E, F), indicating this RSMV detection method had high specificity.

RSMV is transmitted by leafhopper vector, and the detection of vector viruliferous rate is the basis for disease occurrence prediction and control

(Wang et al., 2021). Hence, we tested the RT-RAA-CRISPR/Cas12a detection for RSMV in leafhopper samples. We picked 10 leafhoppers fed on rice plants infected with RSMV, crude extracted, and detected by RT-PCR or the RT-RAA-CRISPR/Cas12a methods. 7 out of 10 samples showed strong positive signal by both methods, but the last three samples (#1, #5, and #9) only showed obvious signal by RT-RAA-CRISPR/Cas12a system (Figures 4G–I). The results indicate that the RT-RAA-CRISPR/Cas12a visual detection method can effectively detect the leafhopper vector with RSMV, and its sensitivity is higher than that of traditional RT-PCR method.

The application of RT-RAA-CRISPR/Cas12a detection in field.

According to the above results, we can get a field visual detection process for SrMV and RSMV: 1, Take a piece of leaf from the plants with suspected disease symptoms and put it into 1.5 ml tubes, followed by grinding with 400 μl crude extraction buffer; 2, Take 2 μl crude extract into 25 μl RT-RAA reaction system, water bath at about 37°C for 20 min; 3, Take 2 μl RT-RAA product into 18 μl CRISPR/Cas12a detection system, water bath at about 37°C for 10 min and then observed under a UV flashlight (Figure 5A).

Using this system, we detected over 100 samples for each virus in 6 field locations in South China (Figure 5B). The sugarcane samples were on-site detected for SrMV and the rice samples for RSMV, the infection rates were ranging from 5%–25% of SrMV (Table 1) and 15%–43% of RSMV (Table 2) in different locations. We also take the samples from the same plants back to laboratory for RT-PCR detection, and the detection rate was almost the same (Supplementary Table S4), indicating the RT-RAA-CRISPR/Cas12a on-site visual detection can be effectively applied in the field.

4 Discussion

Sugarcane diseases caused by SrMV infection are widely distributed worldwide, and rice diseases caused by RSMV infection are becoming more serious and spreading year by year (Yang et al., 2018; Lu et al., 2021). Rapid and accurate detection of these viruses is helpful for forecasting and preventing the epidemics. However, current diagnostics for SrMV and RSMV can be performed only in equipped laboratory, causing a delay between field sample collection and detection results informing. Here, we developed a method for visual detection of RNA virus based on CRISPR/LbCas12a system, including RT-RAA reaction and CRISPR/LbCas12a detection. In this visual detection system, the ssDNA probe, labeled with 5′-FAM and 3′-BHQ1, was added and could be cleaved by the ssDNase activity of LbCas12a to produce green fluorescence. The method is portable and economical, by which the detection results can be observed directly by nacked-eye under blue light. Therefore, this method has a promising application prospect in field on-site detection.

Compared with other plant virus detection methods, this technology shows significant advantages in three aspects. First, the RT-RAA-CRISPR/Cas12a detection system has much higher sensitivity. In our sensitivity assay of RT-RAA-CRISPR/Cas12a system, for both SrMV and RSMV detection had 10^4 times higher sensitivity than direct RT-RAA or RT-PCR methods. Interestingly, the 10^2 and 10^3 diluted crude extract samples showed stronger fluorescence signal than 10^0 and 10^1 diluted samples (Figures 3C, D; 4C, D). We speculate that this may be due to the composition of the crude extract buffer affecting the reaction of CRISPR/Cas12a detection system, and suggest diluting the crude extract 100 to 1000 times in use to obtain more sensitive results. Second, the RT-RAA-CRISPR/Cas12a detection system has higher time efficiency. The RT-RAA reaction can be completed within 20 min, and the CRISPR/Cas12a fluorescence display takes only 10 min to observe the results. Therefore, with simple sample collection, homogenization and reagent addition, it only takes about 30 min to get the virus diagnosis results on site. Last but not the least, the RT-RAA-CRISPR/Cas12a detection method has low requirement for equipment and instruments. Unlike traditional PCR assays, RAA reaction does not require repeated heating and cooling process, and the detection results are directly fluorescent displayed in the reaction tube by the CRISPR/LbCas12a system. Thus, this detection system only needs a single temperature water bath (even just a cup of warm water, the RAA reaction does not need accurate temperature) and a UV flashlight.

So far, isothermal amplification combined with CRISPR/Cas12a nucleic acid recognition system has been broadly employed to detect various viruses (Broughton et al., 2020; Duan et al., 2022). Notably, the risk of aerosol contamination during the detection procedure should not be ignored. To avoid transferring the amplified products which easily causes aerosol contamination thus leading to false positive risk, some one-pot methods integrating nucleic acid amplification and CRISPR detection into a single reaction tube have been exploited (Li et al., 2019; Aman et al., 2020; Ding et al., 2020). However, these one-pot methods generally suffer from poor sensitivity due to the cross interference between the reagents of isothermal amplification and CRISPR/Cas12a detection system. Here the detection platform we established was mainly used for on-site virus detection in the field, where has less aerosol contamination than in the laboratory, so a two-step approach was adopted to get best sensitivity of the detection.

In summary, a rapid and sensitive method for visual detection of SrMV and RSMV has been developed. This method for RNA virus detection is highly efficient and its detection limit is much lower than that of RT-PCR. This system has been successfully applied in the field for on-site detection, and the detection rate is almost the same or even higher with this technology compared with RT-PCR detection in the laboratory.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

TZ conceived the project; JW, XH, SC, JC, ZL, and BC performed the experiments; JW, XH, XY, GZ, and TZ analyzed and interpreted the data; JW and TZ wrote the manuscript.

Funding

This study was supported by the grants from the National Natural Science Foundation of China (32072388, 32222071), Guangdong Special Branch Plan for Young Talent with Scientific and Technological Innovation (2019TQ05N158), Natural Science Foundation of Guangdong Province (2022A1515010770), the Postdoctoral Science Foundation of China (2021M691083), Science and Technology Base and Talent Special Project of Guangxi Province (GuikeAD22035012), Guangdong Basic and Applied Basic Research Foundation (2021A1515110363), and Guangdong Provincial Innovation

Team for General Key Technologies in Modern Agricultural Industry (2019KJ133).

Acknowledgments

We thank Yongwen Qi and Zilin Wu (Institute of Nanfan & Seed Industry, Guangdong Academy of Science, China) for providing SrMV infected sugarcane samples.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgeed.2023.1124794/full#supplementary-material

References

Aman, R., Mahas, A., Marsic, T., Hassan, N., and Mahfouz, M. M. (2020). Efficient, rapid, and sensitive detection of plant RNA viruses with one-pot RT-RPA–CRISPR/cas12a assay. *Front. Microbiol.* 11, 610872. doi:10.3389/fmicb.2020.610872

Bhaya, D., Davison, M., and Barrangou, R. (2011). CRISPR-cas systems in bacteria and archaea: Versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* 45 (1), 273–297. doi:10.1146/annurev-genet-110410-132430

Broughton, J. P., Deng, X., Yu, G., Fasching, C. L., Servellita, V., Singh, J., et al. (2020). CRISPR–Cas12-based detection of SARS-CoV-2. *Nat. Biotechnol.* 38 (7), 870–874. doi:10.1038/s41587-020-0513-4

Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., et al. (2018). CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* 360 (6387), 436–439. doi:10.1126/science.aar6245

Chen, S., Li, W., Huang, X., Chen, B., Zhang, T., and Zhou, G. (2019). Symptoms and yield loss caused by rice stripe mosaic virus. *Virology J.* 16 (1), 145. doi:10.1186/s12985-019-1240-7

Clark, M. F., and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. General Virology* 34 (3), 475–483. doi:10.1099/0022-1317-34-3-475

Ding, X., Yin, K., Li, Z., Lalla, R. V., Ballesteros, E., Sfeir, M. M., et al. (2020). Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. *Nat. Commun.* 11 (1), 4711. doi:10.1038/s41467-020-18575-6

Doudna, J. A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346 (6213), 1258096. doi:10.1126/science.1258096

Duan, X., Ma, W., Jiao, Z., Tian, Y., Ismail, R. G., Zhou, T., et al. (2022). Reverse transcription-recombinase-aided amplification and CRISPR/Cas12a-based visual detection of maize chlorotic mottle virus. *Phytopathology Res.* 4 (1), 23. doi:10.1186/s42483-022-00128-y

Euler, M., Wang, Y., Heidenreich, D., Patel, P., Strohmeier, O., Hakenberg, S., et al. (2013). Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *J. Clin. Microbiol.* 51 (4), 1110–1117. doi:10.1128/JCM.02704-12

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., et al. (2003). Detection of Japanese yam mosaic virus by RT-LAMP. *Archives Virology* 148 (9), 1713–1720. doi:10.1007/s00705-003-0134-5

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., and Zhang, F. (2018). Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* 360, 439–444. doi:10.1126/science.aaq0179

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., et al. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 356 (6336), 438–442. doi:10.1126/science.aam9321

Jones, R. a. C., and Naidu, R. A. (2019). Global dimensions of plant virus diseases: Current status and future perspectives. *Annu. Rev. Virol.* 6 (1), 387–409. doi:10.1146/annurev-virology-092818-015606

Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F., and Collins, J. J. (2021). CRISPR-based diagnostics. *Nat. Biomed. Eng.* 5 (7), 643–656. doi:10.1038/s41551-021-00760-7

Kellner, M. J., Koob, J. G., Gootenberg, J. S., Abudayyeh, O. O., and Zhang, F. (2019). Sherlock: Nucleic acid detection with CRISPR nucleases. *Nat. Protoc.* 14 (10), 2986–3012. doi:10.1038/s41596-019-0210-2

Li, L., Li, S., Wu, N., Wu, J., Wang, G., Zhao, G., et al. (2019). HOLMESv2: A CRISPR-cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synth. Biol.* 8 (10), 2228–2237. doi:10.1021/acssynbio.9b00209

Li, S-Y., Cheng, Q-X., Liu, J-K., Nie, X-Q., Zhao, G-P., and Wang, J. (2018a). CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA. *Cell Res.* 28 (4), 491–493. doi:10.1038/s41422-018-0022-x

Li, S-Y., Cheng, Q-X., Wang, J-M., Li, X-Y., Zhang, Z-L., Gao, S., et al. (2018b). CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discov.* 4 (1), 20. doi:10.1038/s41421-018-0028-z

Lu, G., Wang, Z., Xu, F., Pan, Y-B., Grisham, M. P., and Xu, L. (2021). Sugarcane mosaic disease: Characteristics, identification and control. *Microorganisms* 9 (9), 1984. doi:10.3390/microorganisms9091984

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28 (12), e63. doi:10.1093/nar/28.12.e63

Piepenburg, O., Williams, C. H., Stemple, D. L., and Armes, N. A. (2006). DNA detection using recombination proteins. *PLOS Biol.* 4 (7), e204. doi:10.1371/journal.pbio.0040204

Rubio, L., Galipienso, L., and Ferriol, I. (2020). Detection of plant viruses and disease management: Relevance of genetic diversity and evolution. *Front. Plant Sci.* 11, 1092. doi:10.3389/fpls.2020.01092

Wang, Z., Chen, B., Zhang, T., Zhou, G., and Yang, X. (2021). Rice stripe mosaic disease: Characteristics and control strategies. *Front. Microbiol.* 12, 715223. doi:10.3389/fmicb.2021.715223

Yang, X., Chen, B., Zhang, T., Li, Z., Xu, C., and Zhou, G. (2018). Geographic distribution and genetic diversity of rice stripe mosaic virus in southern China. *Front. Microbiol.* 9 (3068), 3068. doi:10.3389/fmicb.2018.03068

Yang, X., Huang, J., Liu, C., Chen, B., Zhang, T., and Zhou, G. (2017a2140). Rice stripe mosaic virus, a novel cytorhabdovirus infecting rice via leafhopper transmission. *Front. Microbiol.* 7, 2140. doi:10.3389/fmicb.2016.02140

Yang, X., Zhang, T., Chen, B., and Zhou, G. (2017b). Transmission biology of rice stripe mosaic virus by an efficient insect vector Recilia dorsalis (Hemiptera: Cicadellidae). *Front. Microbiol.* 8 (2457), 2457. doi:10.3389/fmicb.2017.02457

Zhang, T., Zhao, Y., Ye, J., Cao, X., Xu, C., Chen, B., et al. (2019). Establishing CRISPR/Cas13a immune system conferring RNA virus resistance in both dicot and monocot plants. *Plant Biotechnol. J.* 17 (7), 1185–1187. doi:10.1111/pbi.13095

Zhang, T., Zheng, Q., Yi, X., An, H., Zhao, Y., Ma, S., et al. (2018). Establishing RNA virus resistance in plants by harnessing CRISPR immune system. *Plant Biotechnol. J.* 16 (8), 1415–1423. doi:10.1111/pbi.12881

Zhao, Y., Yang, X., Zhou, G., and Zhang, T. (2020). Engineering plant virus resistance: From RNA silencing to genome editing strategies. *Plant Biotechnol. J.* 18 (2), 328–336. doi:10.1111/pbi.13278

Zhu, H., Li, C., and Gao, C. (2020). Publisher Correction: Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nat. Rev. Mol. Cell Biol.* 21, 712. doi:10.1038/s41580-020-00304-y

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 20 April 2023
DOI 10.3389/fpls.2023.1179394

OPEN ACCESS

EDITED BY
Tong Zhang,
South China Agricultural University, China

REVIEWED BY
Hui Zhang,
Shanghai Normal University, China
Mulan Zhu,
National Key Laboratory of Plant Molecular Genetics, Center for Excellence in Molecular Plant Sciences (CAS), China

*CORRESPONDENCE
Xiangqing Ma
lxymxq@126.com
Qiang Zhu
zhuqiang@fafu.edu.cn

SPECIALTY SECTION
This article was submitted to Plant Biotechnology, a section of the journal Frontiers in Plant Science

RECEIVED 04 March 2023
ACCEPTED 27 March 2023
PUBLISHED 20 April 2023

CITATION
Ye S, Ding W, Bai W, Lu J, Zhou L, Ma X and Zhu Q (2023) Application of a novel strong promoter from Chinese fir (*Cunninghamia lanceolate*) in the CRISPR/Cas mediated genome editing of its protoplasts and transgenesis of rice and poplar. *Front. Plant Sci.* 14:1179394. doi: 10.3389/fpls.2023.1179394

Application of a novel strong promoter from Chinese fir (*Cunninghamia lanceolate*) in the CRISPR/Cas mediated genome editing of its protoplasts and transgenesis of rice and poplar

Shanwen Ye, Wensha Ding, Weiyuan Bai, Jiaao Lu, Linying Zhou, Xiangqing Ma* and Qiang Zhu*

College of Forestry, Fujian Agriculture and Forestry University, Fuzhou, China

Novel constitutive promoters are essential for plant biotechnology. Although in angiosperms, a number of promoters were applied in monocots or dicots genetic engineering, only a few promoters were used in gymnosperm. Here we identified two strong promoters (*Cula11* and *Cula08*) from Chinese fir (*C. lanceolate*) by screening the transcriptomic data and preliminary promoter activity assays in tobacco. By using the newly established Chinese fir protoplast transient expression technology that enables *in vivo* molecular biology studies in its homologous system, we compared the activities of *Cula11* and *Cula08* with that of the commonly used promoters in genetic engineering of monocots or dicots, such as *CaM35S*, *CmYLCV*, and *ZmUbi*, and our results revealed that *Cula11* and *Cula08* promoters have stronger activities in Chinese fir protoplasts. Furthermore, the vector containing *Cas* gene driven by *Cula11* promoter and sgRNA driven by the newly isolated *CulaU6b polyIII* promoters were introduced into Chinese fir protoplasts, and CRISPR/Cas mediated gene knock-out event was successfully achieved. More importantly, compared with the commonly used promoters in the genetic engineering in angiosperms, *Cula11* promoter has much stronger activity than *CaM35S* promoter in transgenic poplar, and *ZmUbi* promoter in transgenic rice, respectively, indicating its potential application in poplar and rice genetic engineering. Overall, the novel putative constitutive gene promoters reported here will have great potential application in gymnosperm and angiosperm biotechnology, and the transient gene expression system established here will serve as a useful tool for the molecular and genetic analyses of Chinese fir genes.

KEYWORDS

Chinese fir, promoter, protoplast, transient expression, genome editing, CRISPR/Cas

Introduction

Strong gene promoters that drive the high levels of constitutive gene expression are one of the key elements for plant biotechnology applications. Several well-known gene promoters were widely used for transgene expression in plants. The cauliflower mosaic virus *(CaMV) 35S* gene promoter is the most commonly used in dicotyledonous plants, as it strongly and constitutively drives transgene expression (Odell et al., 1985). In monocots, various promoters such as *ZmUbi1* from maize (Christensen et al., 1992), and *Act1* (McElroy et al., 1990), *OsCc1* (Jang et al., 2002), *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5* from rice (Park et al., 2010), are regarded as constitutive gene promoters in crop biotechnology applications. However, in gymnosperm, limited reports showed that the *CaM35S* constitutive promoter was commonly used for gene-overexpression, but its activity tends to be low in conifers (Lin et al., 2005; Song et al., 2020). Moreover, despite *CaM35S* promoter works in several conifers, there is currently a shortage of efficient promoters for high-level constitutive gene expression, which may satisfy performing multiple transgenes in a single vector that require different strong promoters in gymnosperm, as well as CRISPR/Cas mediated gene editing technology.

CRISPR/Cas mediated gene editing technology facilitates the fundamental research and molecular breeding in plants (Zhu et al., 2020; Gao, 2021). In the CRISPR/Cas system, the expression of *Cas* is generally driven by an *RNA polymerase II* (*Pol II*) promoter, while the *Pol III* promoters of small nuclear RNA (snRNA) genes, such as *U3* and *U6* promoters, are commonly used to drive sgRNA expression in plants (Zhang et al., 2017; Scheben and Edwards, 2018). Therefore, the choice of promoter is a crucial factor for *gRNA* and *Cas* gene expression in plant cells, thereby affecting the editing efficiencies (Manghwar et al., 2019; Hassan et al., 2021). Constitutive promoters sustained high expression activities in all cell types, and *CaM35S* promoter and *Ubiquitin* promoter were mostly used in the mutagenesis in dicots and monocots, respectively (Rahman et al., 2022). In gymnosperm, *CaM35S* promoter was used in-planta genome editing technologies in three conifer species, namely *P.radiata* (Poovaiah et al., 2021), *C.japonica* (Nanasato et al., 2021), and *P.glauca* (Cui et al., 2021). However, their efficiencies are quite low, probably due to the relatively low activity of *CaM35S* promoter. Optimizing those elements of CRISPR/Cas system is perquisite for the successful genome editing in plants, and identifying stronger promoters that are highly expressed in gymnosperm is crucial for its genome editing.

Since generating stable genome-edited plants is time-consuming and complex, it is necessary to develop a simple and rapid system for testing, selecting and verifying the activity of candidate promoters in its homologous system. Protoplasts transient expression assay provides a versatile tool for performing genomics, transcriptomics, metabolic and epigenetics studies (Xu et al., 2022). It nicely bypasses the difficult stable transformation procedure, and enables obtaining results within several hours or days. At present, it was widely used in studying the molecular mechanisms controlling plant growth development, plant hormone signaling, gene expression regulation, as well as other physiological processes (Eeckhaut et al., 2013; Lin et al., 2017; Priyadarshani et al., 2018; Wang et al., 2021b; Xu et al., 2022). By coupling protoplast transient expression experiments with the high-resolution imaging technology, scientists can simply, rapidly and efficiently analyze and characterize gene functions and regulatory network, such as protein subcellular localization, protein-protein interaction, transcriptional regulatory networks, as well as gene response to various external cues (Poddar et al., 2020; Wang et al., 2021a; Wang et al., 2022). To date, protoplast isolation and transfection system have been successfully used in the molecular studies in many angiosperms, such as rice (Yu et al., 2014b), switchgrass (Burris et al., 2016), barley (Yu et al., 2014a), grapevine (Zhao et al., 2016), wheat (Liao et al., 2010), ryegrass (Yu et al., 2017), Arabidopsis (Sang-Dong et al., 2007; Wu et al., 2009), maize (Sheen, 2001), tobacco (Locatelli et al., 2003), populus (Guo et al., 2012; Tan et al., 2013), cucumber (*Cucumis sativus*) (Huang et al., 2013) and pineapple (Priyadarshani et al., 2018). Mutagenesis in protoplasts can be achieved by CRISPR/Cas gene editing in several plant species (Malnoy et al., 2016; Lin et al., 2017; Liu et al., 2018; Ye et al., 2020). Whereas in gymnosperm, including Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook), the protoplast isolation and transformation techniques are still difficult.

Chinese fir, which belongs to the Cupressaceae family, covers the largest plantation area and becomes the most important timber species in China (Cao et al., 2022). With the development of modern biology, researchers are more and more interested in unraveling the molecular mechanisms as well as identifying the important genes that control wood formation, growth and development, nutrient absorption, and abiotic or biotic stress response. Currently, a large number of bioinformatical data was generated (Wan et al., 2012; Lin et al., 2020), however, few studies reported on its gene functional analysis. And until now, no native genes were transiently expressed in Chinese fir, mainly due to the lack of efficient transient and stable gene expression system. In Chinese fir, the use of native protoplast transient expression system is important as it can perform gene expression analysis without the hard technique and time-consuming problems in obtaining transgenic plants and callus, and provides an efficient, precise and consistent way for revealing molecular mechanisms. At present, a few studies reported the isolation of Chinese fir protoplasts (GeQiang and Guoning, 2012; Tang et al., 2018), which contributes to its molecular study. However, the derived protoplasts are always low yield and quality (shrunken protoplast and fractured), probably that's why few protoplast transformation studies were reported in Chinese fir. And a more stable and easily reproducible protocol is required gene functional characterization, promoter activity assays, as well as optimization of the CRISPR/Cas elements that are suitable for its gene editing.

In this study, we identified two new promoters (*Cula08* and *Cula11*) from Chinese fir based on the transcriptomic data. To quickly check their activities natively, we first established a stable and repeatable protoplast isolation and transformation protocol enabling promoter activity assays, protein subcellular localization,

protein-protein interaction, and Ribonucleoprotein (RNP)-mediated gene editing studies. With this system, we showed that promoters that commonly used in monocots and dicots transformation, such as *CmYLCV*, *ZmUbi* and *CaM35S* promoters also work in Chinese fir protoplasts. Whereas *Cula08* and *Cula11* exhibited stronger activities. More importantly, *Cula11* promoter showed much higher activity than *CaM35S* promoters in transgenic poplar, and *ZmUBI* promoter in transgenic rice, suggesting its potential application in poplar and rice genetic engineering. By using *Cula 11* promoter for the expressions of *Cas* genes (*SpRY* and *SpG*) and the newly isolated *CulaU6b* promoter for sgRNA expression, gene editing was successfully achieved in Chinese fir protoplast. The promoter identified here will contribute to the gene expression analysis in Chinese fir, as well as rice and poplar, and the protoplast-mediated transient expression system will be useful tools for molecular studies in Chinese fir.

Materials and methods

Plant materials and growth conditions

The Chinese fir Yangkou-020, which was widely planted in China, was used for micro-propagation. Shoot explants were excised from *in vitro* grown seedlings, and germinated on MS plus BA 0.3 mg/L, NAA 0.2 mg/L, sucrose 30 g/L and agar 6 g/L. The pH value was adjusted to 5.8. The explants grow at 26°C under 1500 lx light and 16 h/8 h photoperiod. The plantlets were sub-cultured monthly.

Plasmid construction

For the promoter activity assays in tobacco, around 2.5-kb upstream of ATG were cloned with the primers listed in Supplemental Table 2. The PCR products were inserted into the upstream of *pPLV04* plasmid using in-fusion cloning methods (TaKaRa, Japan) (De Rybel et al., 2011), which enable that promoters drive the expression of the downstream eGFP. The resulted plasmids were used for premilitary promoter activity tests in tobacco.

For promoter activity assays in Chinese fir protoplasts. *35S:NLS-TdTomato* plasmid was used as the backbone (Liu et al., 2017), and the coding sequence for *TdTomato* was first replaced with eGFP between *BamHI* and *StuI* sites to generate the *35S:NLS-eGFP* plasmid. The promoters of *Cula 11*, *Cula 08*, *CmYLCV*, and *ZmUbi* were cloned by using the primers listed in Supplemental Table 2, and used for replacing the *CaM35S* promoter. The generated plasmids containing *Cula 11: NLS-eGFP*、*Cula 08: NLS-eGFP*, *YLCV: NLS-eGFP*, and *ZmUbi: NLS-eGFP* cassette were used for Chinese fir protoplast transformation, respectively.

For BiFC experiments in Chinese fir protoplast, the *CulaSCL3* and *CulaDELLA* genes were cloned with the primers listed in Supplemental Table 2, and fused into the *PENTRY-EN01* entry vector (Sugano et al., 2018). The fragments were further ligated into *pB4cYGW* and *pB4nYGW* vectors by gateway cloning method (Kamigaki et al., 2016), which were used for protoplast transformation.

For optimizing CRISPR/Cas elements suitable in Chinese fir protoplast, a plasmid expressing a mutant version of RFP (*muRFP*) with a base insertion at 14th position was created by PCR with the *muRFP-F* and *muRFP-R* primers listed in Supplemental Table 2, and the resulted *muRFP* fragment was used to replace the *TdTomato* fragment at *BamHI* and *StuI* sites. A CRISPR vector containing sgRNA targeting the insertion sites of *muRFP* was designed, and was driven by Chinese fir native U6b promoter. To construct the *Cas* nucleases and *gRNA* expression plasmids, the codon-optimized *SpRY* or *SpG* gene cassettes, *35S:HPTII* expression cassettes and *Cula11* promoter were cloned with the primers listed in Supplemental Table 2 (Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2016). These fragments were integrated into the *pGGP AG* vector (Decaestecker et al., 2019) to generate the intermediate plasmid containing *SpG/SpRY-eGFP-35S:HPTII* cassette by using goldengate cloning method (Sarrion-Perdigones et al., 2013). Then the *pCulaU6b-gRNA-pCula11* fragment containing the *NcoI* restriction enzyme recognition site was cloned by using sgRNA-Cas9 expression vector as templet (Zhang et al., 2014) with the primers listed in Supplemental Table 2. The resulted fragment was integrated into the above intermediated plasmid at *NcoI* site with in-fusion cloning method (De Rybel et al., 2011).

For constructing plasmid simultaneously expressing *RFP* and *CFP*, the *RFP-P2A-CFP* fusion gene were amplified by overlapping PCR (Fang et al., 2005) with the primers listed in the Supplemental Table 2, and the resulted fusion fragments were cloned into *35S:NLS-TdTomato* plasmid by replacing the *TdTomato* fragments as we described above.

For plasmids used for poplar transformation, the promoters of *CaM35S* and *Cula11* were fused into pCambia1301 vector (Yan et al., 2012) between the *HindIII* and *NcoI* restriction enzyme sites, respectively, and resulting the vectors containing promoter: *GUS* expression cassette. The sequences of all plasmids were confirmed by DNA sequencing.

Protoplast isolation and transformation

We provided a detailed protocol on Chinese fir protoplast isolation, transient transfection, as well as RNP-mediated gene editing in the Supplemental Method.

Protoplast counting

Protoplast numbers were counted with a hemocytometer (QiuJing, Shanghai, China). Forty microliters of protoplast solutions were put on the surface of the hemocytometer, and then laid the cover slide carefully. The number of intact protoplasts in the four corners of the grid was counted under microscope as described before (Wang et al., 2021a). The protoplast numbers (mL^{-1}) were calculated based on the formula: the average number of the intact cells in the four corners of the gridx10^4.

Poplar and rice transformation

Aspen hybrid clones Yinzhong (*P. alba* x *P. berolinensis*) was used for transformation as described previously (Wang et al., 2011). Briefly, leaf explants excised from 3-week-old *in vitro* grown plants were inoculated with *Agrobacterium* strain EHA105 for 10 min, and co-cultured on MS with 0.1 mg/L NAA + 0.2 mg/L 6-BA + 50 mg/L AS for 2 days. The bacterial was removed by washing in sterilized distilled waters, and the explants were cultured on MS with 0.1 mg/L NAA + 0.2 mg/L 6-BA + 0.01 mg/L TDZ + 500 mg/L cefotaxime + 50 mg/L kanamycin. The medium should be changed every month until the kanamycin-resistant shoots were regenerated. Transgenic shoots were excised and transplanted into MS with 0.1 mg/L NAA + 500 mg/L cefotaxime + 25 mg/L kanamycin to induce the root. The whole processes were proceeded in the tissue culture room with the light intensity of around 200 μmol m^{-2} s^{-1} and photoperiod of 12 h light/12 h dark. The temperature was kept at about 25°C for the whole transformation process. After selection and molecular verification, a total of 5 independent transgenic lines were obtained for further analysis.

Rice transformation was performed following the *Agrobacterium*-mediated transformation protocol established previously (Sahoo et al., 2011). Briefly, the sterilized rice seeds were placed on the callus induction media (CIM) containing casein hydrolase (300 mg/L), proline (560 mg/L), maltose (36g/L), 2-4-D (2.5 mg/L), BAP (0.25 mg/L), and Phytagel (3 g/L) with a PH of 5.8. The induced embryogenic calli were infected with *Agrobacterium* culture and kept in co-cultivation media for 48 h. After washing, the calli were kept on selection medium containing hygromycin (50 mg/L) and cefotaxime (250 mg/L), and actively growing calli were then transferred to regeneration medium for regeneration, rooting and hardening.

Gus staining

Histochemical staining for GUS activity in transgenic poplar and rice was performed as the protocol described in (Jefferson et al., 1987). At least 3 independent transgenic lines were used for this experiment.

Tobacco infiltration

Tobacco infiltration was performed based on the established protocol (Yang et al., 2000). Briefly, the overnight culture of *Agrobacterium* EHA 105 containing promoter: GFP plasmids as well as the transformation helper plasmid *pSoup* was diluted and cultured to OD_{600} 0.6. The agrobacteria was harvested by centrifugation and resuspended in 10 mM MES (pH5.5) plus MS basal medium (Murashige and Skoog, 1962), and then adjusted to OD_{600} 0.5 with the acetosyringone concentration of 150 μM. The bacterial suspensions were incubated 2 h at 25°C, and then were infiltrated into leaves of 5-week-old *N. benthamiana* plants using a needless syringe. After agroinfiltration, tobacco plants were maintained in a growth chamber at 22°C under 16 h light for 48 h.

Co-focal imaging

The incubated protoplasts were centrifuged at 900 rpm for 2 min, the supernatant was carefully removed with around 100 μL leftover. Protoplasts were resuspended with blunt tips, and transferred to slide with a cover glass for observation. Observations were used a fluorescence confocal microscope (Leica TCS SP8X DLS), adjusting for different excitation wavelengths and filter types at the 493-520 nm for GFP, 519-540 nm for YFP, 587-638 nm for RFP and 450-460nm for DAPI. The GFP fluorescence was recorded, and the green GFP signal intensities were quantified relative to using the ImageJ software (n>20).

Genomic DNA extraction and PCR/restriction enzyme assay

Protoplasts samples were pooled and collected 2 days after plasmid or RNP transfection, the protoplast genomic DNA was extracted using TIANamp Micro DNA kit (TIANGEN, Shanghai), and PCR-amplified with high fidelity DNA polymerase using primers spanning the cutting site with the primers listed in Supplemental Table 2. The amplicons were then digested with a restriction enzyme that cut the wildtype sequences while not the mutant sequence that caused by CRISPR. Alternatively, *T7E1* enzyme assay were also performed for this verification (Liang et al., 2018). The products were visualized by agarose gel electrophoresis. Uncut bands were purified with TIANgel Midi Purification Kit (TIANGEN, Shanghai) according to the manufacturer's instructions, and used as the DNA templet for the second round PCR. The 2^{nd} round restriction enzyme assay was performed again as described previously (Wang et al., 2014). The uncut bands were purified and the mutated sequences were identified by cloning and sequencing plasmids isolated from single colonies.

Results

Identification of novel constitutively expressed genes in Chinese fir

To screen for genes that are constitutively expressed in Chinese fir, the expression profiling was performed using the public the transcriptomic data from public database was downloaded from NCBI (SRX2586190, SRX2586189, SRX2586188, and SRX139598) (Li et al., 2017; Wang et al., 2020). These analyses identified 24 genes that were expressed in all tissues at high levels (Supplemental Table S1). All 24 putative promoters (~2.5 kb upstream of ATG) were cloned and inserted into the *pPLV04* vector (De Rybel et al., 2011), which resulted in constructs with each promoter linked to the green

fluorescent protein gene (GFP) fused with nuclear localization signal (NLS). To make the preliminary screening easier, we first transiently expressed those constructs in tobacco. And our results showed only 4 promoters produced GFP fluorescence, *Cula11*、*Cula08*、and *Cula04* promoters have 10.8-、9.9-、and 1.5- times higher than the putative *Cula Actin* (*Cula12*) promoter based on the fluorescence signal intensities (Supplemental Figure 1). By checking their expressions from various tissues and growth stages, including leaves, roots, shoots, as well as seedlings at indicated days after germination, we found that the activities of *Cula11* and *Cula08* genes are constitutively expressed (Supplemental Figure 2). Based on the annotation, *Cula11* gene encodes a putative ubiquitin gene, and *Cula08* gene encodes a pathogenesis-related protein, whereas, their molecular functions remain to be elucidated. In summary, *Cula11* and *Cula08* promoters that might have strong activities were screened and used for the following further analysis.

Establishment of a stable and repeatable protoplast isolation and transient expression system in Chinese fir

Promoter activity assays are usually performed by analyzing the transgenic plants expressing promoter::reporter constructs. However, as for Chinese fir, it is well-known for its difficult or recalcitrant to transformation, and few promoters have been characterized in Chinese fir. Thus, alternative methods, such as protoplast-based expression system is required for functional genomics studies, including promoter activity assays. Protoplast isolation has some common features, and the enzymes used to digest the cell wall matrix usually consist of various mixtures of cellulase (Cellulase R-10 or Cellulase RS), hemicellulase, and pectinase (Macerozyme R-10). However, in different plant species, the protocols are always different (Eeckhaut et al., 2013; Lin et al., 2014). We attempted to isolate protoplasts using the reported protocols (GeQiang and Guoning, 2012; Tang et al., 2018), but found that the obtained protoplasts are always low yield and quality (shrunken and fractured) (Supplemental Figure 3), and not always reproducible. Then we systematically investigated various tissues including calluses, cell suspension culture, cotyledons, leaves, roots, wood-forming cells from stem and seedlings, and found that fresh aerial part of 90-day-old tissue culture plants is most suitable starting materials for its protoplast isolation (Supplemental Figure 4). In our lab, the enzyme recipe for releasing protoplast in Chinese fir is: using 0.4 g fresh aerial part of 1.5-month old tissue culture derived Chinese fir seedlings, incubating with 2.0% (w/v) Cellulase-RS, 1.5% (w/v) Macerozyme R-10, and 0.3% (w/v) Pectinase Y-23 for 2 h (Supplemental Figure 5; Supplemental Method). Four key steps for Chinese fir protoplast isolation and transient transformation: firstly, selection of the right starting material is crucial (Supplemental Figure 4; Supplemental Method); secondly, after transformation, a modified W5 solution with equal osmotic pressure to the MMG solution should be used, as it substantially avoids obtaining shrunken and fractured protoplasts (Supplemental Figure 3; Supplemental Method); thirdly, transient heat shock treatment (40°C for 2 min) is necessary for the successful transformation (Supplemental Figure 5); fourthly, Chinese fir protoplasts are quite sensitive to osmotic potential, and minute difference in the concentration of mannitol will result in different yield of intact protoplast. Under our experimental conditions, 0.5 mM mannitol provides the best results (Supplemental Figure 5). Compared with angiosperms, the current protoplast isolation ($1.5x10^5$/g FW) and transformation (~25%-45%) efficiencies are not so high (Figure 1A; Supplemental Figure 5), but easy to operate and highly repeatable. More importantly, the protoplasts derived based on our protocol remain intact and healthy, instead of shrink and fractured shape as commonly showed in the previous protocols (Supplemental Figure 3), which satisfied the following gene functional studies.

To test if *CaM35S* promoter works in Chinese fir protoplasts, and the utility of the transient expression system in protein subcellular localization analysis, organelle-markers tagged with GFP or RFP, including plasma membrane marker *GFP-PEN1* (Assaad et al., 2004), endoplasmic reticulum marker *Calnexin-mRFP* (Gao et al., 2012), peroxisome marker *px-gb* and mitochondria marker *mt-rk* (Nelson et al., 2007) driven by *CaM35S* promoter were introduced into Chinese fir protoplasts, and observed under co-focal microscope. The results showed positive protoplasts display predicted organelle patterns with low background fluorescence levels (Figure 1B), indicating the protoplast-mediated transient expression system is applicable in analyzing protein subcellular localization in Chinese fir.

To show this transient expression system is repeatable and stable, we performed protein interaction assays. In Arabidopsis, *SCARECROW-LIKE 3 (SCL3)* protein interacts with DELLA, and acts as a positive regulator in gibberellin signaling pathway (Zhang et al., 2011). To check if the homologues of SCL3 and DELLA from Chinese fir also have interactions, *CulaSCL3* and *CulaDELLA* genes were cloned and linked with C- and N- terminal of *YFP* for bimolecular fluorescence complementation (BiFC) analysis in its protoplasts. Positive signals appear after co-transforming plasmids harboring *CulaSCL3-cYFP* and *CulaDELLA-nYFP*, while the negative controls gave no fluorescence (Figure 1C), supporting that SCL3 and DELLA have conserved binding ability in Chinese fir, and this transient expression system could be used for protein-protein interaction assays.

To check if this system could be used for expressing ribonucleoprotein (RNP), Cas9 protein and the *sgRNA* targeting putative *4-coumarate:CoA ligase* (*4CL*) gene (Figure 1D) were introduced to Chinese fir protoplasts. Protoplasts were pooled and used for PCR/restriction enzyme (RE) assay as described previously (Shan et al., 2014). The digestion-resistant bands in the RNP-treated samples were detected by PCR/RE assay (Figure 1E). Further cloning and sequencing of these uncut bands revealed the existence of various mutations (Figures 1F, G), indicating the successful native gene knock-out in Chinese fir protoplasts.

In summary, we showed that the protoplast-based expression system established here is stable and repeatable, and could be used for many molecular biology applications.

10.3389/fpls.2023.1179394

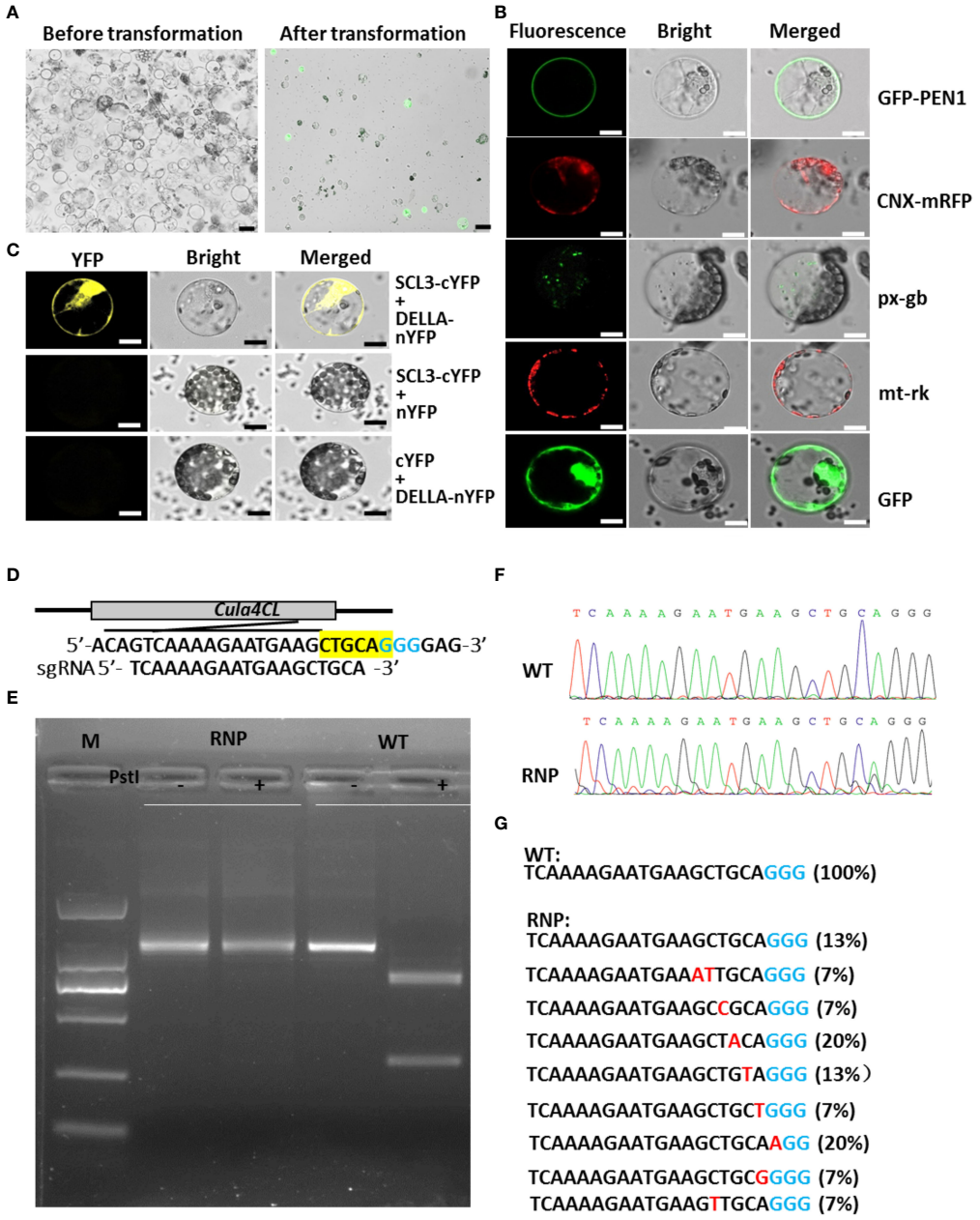


FIGURE 1
Protoplast isolation, transfection and gene function study in Chinese fir. **(A)** Status of Chinese fir protoplasts before (left) and after (right) transformation. Scale bar: 10 μm. **(B)** Subcellular localization of various cell organelle markers on plasma membrane (*PEN1-GFP*), ER (*CNX-mRFP*), peroxisome (*px-gb*), mitochondria (*mt-rk*) and control (*35S:GFP*) in Chinese fir protoplasts. Scale bar: 10 μm. **(C)** BiFC assay showing the interaction between *CulaSCL3* and *CulaDELLA* in Chinese fir protoplasts. Scale bar: 10 μm. **(D)** Schematic of the *Cula4CL* gene with the RNP targets (bottom). The corresponding PAMs (blue) and *PstI* site were highlighted (yellow). **(E)** PCR/RE assay to detect engineered RNP-induced mutations in protoplasts. **(F)** Sequencing of the PCR products from wild-type and RNP-delivered samples. **(G)** Mutation types and frequencies of RNP-delivered protoplasts. PAM (blue) and indels (Red) are shown; the ratios of certain indel type are shown in brackets.

Cula11 and *Cula08* promoter activity assay in Chinese fir protoplast

To investigate promoter activity in the Chinese fir protoplast, the well-known promoters in the genetic engineering of dicots and monocots, including *CaM35S*, *CmYLCV* (Stavolone et al., 2003), and *ZmUbi*, as well as the newly isolated *Cula11* and *Cula08* promoters, were fused with *35S:NLS-eGFP* plasmid (Liu et al., 2017), and introduced to the Chinese fir protoplast (Figure 2A). Our results showed that all the promoters tested here work and sufficiently drive the downstream gene expression work and sufficiently drive downstream gene expressions in Chinese fir protoplasts; however, the activities of *Cula11* and *Cula08* promoters are substantially higher than that of the promoters commonly used in angiosperms (Figures 2B, C). Those results suggested that *Cula11* and *Cula08* promoters have strong strength in Chinese fir protoplasts, and they might be suitable for driving high levels of gene expression in stable transformants of Chinese fir in the future.

10.3389/fpls.2023.1179394

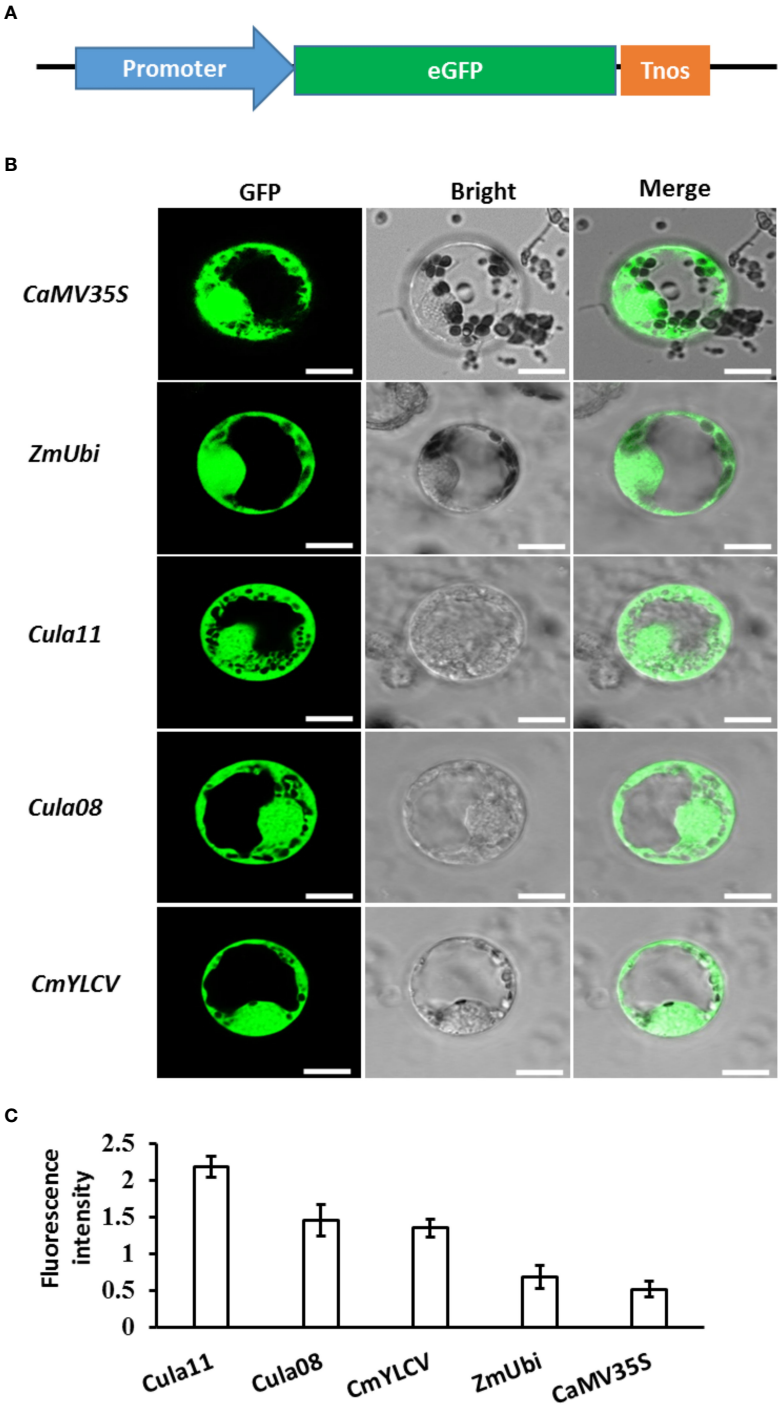


FIGURE 2
Activities of various promoters in Chinese fir protoplasts. **(A)** Schematic representation of the vector used for protoplast transformation in Chinese fir. **(B)** Represented Chinese fir protoplasts transfected with the plasmid expressing GFP driven by various promoters. Scale bar: 10 μm. **(C)** Promoter activity assays. The fluorescence of the positive protoplasts were measured using ImageJ, and the mean ± s.d for n=25 individual protoplasts for each sample were calculated. These experiments were repeated three times.

Cula11 and *CulaU6b* promoter contribute to the CRISPR/Cas mediated gene editing in Chinese fir protoplast

CRISPR/Cas technology becomes revolutionary tool for plant molecular breeding (Zhu et al., 2020). However, no gene editing was achieved in Chinese fir. To select the proper CRISPR/Cas elements that function in its gene editing, a mutant RFP version (*muRFP*) containing 1-bp "Guanine" insertion and giving no fluorescence was artificially constructed (Figure 3A). The CRISPR/Cas elements composed of plant codon-optimized *SpRY* or *SpG* gene (Ren et al., 2021) driven by the *Cula11*-strong promoter, and the sgRNA driven

10.3389/fpls.2023.1179394

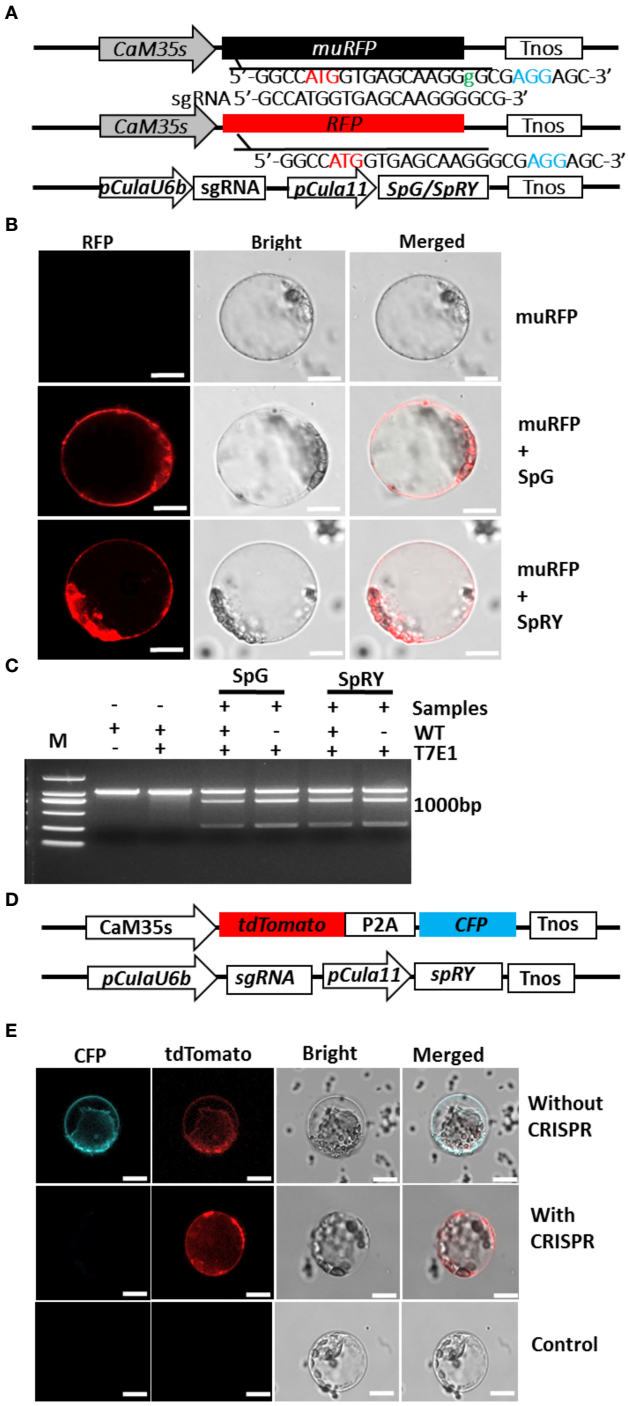


FIGURE 3
Application of *Cula11* promoter in the CRISPR/Cas mediated genome editing in Chinese fir protoplasts. **(A)** Strategies for verifying CRISPR/Cas constructs in Chinese fir protoplasts. Constructs expressing non-functional *muRFP* (Top) and optimized CRISPR elements (Bottom) were co-transformed, and *muRFP* will be corrected to *RFP* once the CRISPR elements work (Middle). **(B)** Representative Chinese fir protoplasts expressing *muRFP* (Top), and co-transfected with *SpG* and *SpRY* expressing constructs that reproducibly emitting fluorescence signals (Middle and Bottom),Scale bar: 10 μm. **(C)** Detecting of target mutations by T7 endonuclease (*T7E1*) assay. **(D)** Alternative strategy for verifying *CRISPR/Cas* constructs in Chinese fir protoplasts. The *tdTomato* and *CFP* simultaneously expressed under *CaM35S* promoters (Top), and CRISPR plasmid which *sgRNA* was driven by *pCulaU6b* and *SpRY* driven by *pCula11*. **(E)** Detection of gene mutation in Chinese fir protoplasts. Representative protoplasts simultaneously expressing *CFP* and *tdTomato* without CRISPR plasmid (Top), and CFP signal disappeared when optimized CRISPR elements work (Middle), protoplasts that give no fluorescence were used as control (Bottom). Scale bar: 10 μm.

by *CulaU6b* promoter (Supplemental Figure 6) were co-transformed with this plasmid, and once CRISPR/Cas system works in Chinese fir, RFP function might be restored by deleting the additional "Guanine" in *muRFP* sequence (Figure 3A). After co-transformation, RFP signals appeared in both *SpRY* and *SpG* expressing protoplasts (Figure 3B), indicating the CRISPR/Cas

elements work in Chinese fir protoplasts and *muRFP* sequence was successfully edited to *RFP* coding sequence. Further PCR/RE assay confirmed the effectiveness of these CRISPR elements in Chinese fir (Figure 3C). Since the current protoplast isolation and transformation efficiencies are not so high, and to make the test more convenient, a plasmid simultaneously expressing *tdTomato* and *CFP* that produce both red and blue signals was co-transformed with CRISPR/Cas plasmid targeting the CDS of *CFP*, a small proportion of protoplasts gave only red but not blue fluoresce were observed under confocal microscopy (Figures 3D, E), further confirmed that the *Cula11* promoter could be used for CRISPR/Cas mediated gene editing in Chinese fir protoplast.

Cula11 promoter showed stronger activity in transgenic poplar and rice

To check if *Cula11* also works in other plant species, we generated transgenic poplar expressing *35S::GUS* and *Cula11 promoter::GUS*, and compared their activities by GUS staining. The results showed that *Cula11* promoters have much stronger activities in both leaves and roots compared with *35S* promoter, as revealed by GUS staining signal (Figure 4A). Moreover, to check if it also works in monocot such as rice, we also generated GUS expressing lines driven by *ZmUBI*, *ACT1* and *Cula11* promoters, and compared their activities in transgenic rice. Our results also supported that *Cula11* promoter has much stronger activities than the commonly used *ZmUBI* and *ACT1* promoter (Figure 4B). In summary, the *Cula11* promoter is useful as a promoter for ubiquitous expression of transgene in both monocots and dicots.

Discussion

Despite isolating numerous promoters from numerous varieties of plants, only a few of them are commonly used in plant genetic engineering. Moreover, the promoter that could be used in gymnosperm is still limited. Up to now, only a few reports have shown that *CaM35S* promoter works in conifers (Tzfira et al., 1996; Liu et al., 2020; Poovaiah et al., 2021), and isolating novel promoters that are potentially used for its genetic engineering is important. In this report, we screened two strong promoters from Chinese fir, and by using the newly established protoplast mediated transient gene expression system, we showed its strong activities natively. More importantly, *Cula11* promoter has much stronger activity in transgenic poplar and rice, suggesting its potential broad usage in plant genetic engineering.

Due to the technical obstacles and the long time required in generating stable transgenic Chinese fir, an easy and repeatable transient expression system is urgently required for accelerating plant genomic study, including promoter activity analysis. In this report, we showed that generating high-quality protoplasts depends on the proper shoot tip materials, the composition of the enzyme solution, as well as the compositions of modified W5 solution for incubation (Supplemental Method). In the preliminary experiment, we tested the protoplast separation efficiency from various tissues of Chinese fir, including cotyledon, leaves, and callus. However, quite low efficient and low-quality protoplasts were obtained in most cases when compared with the fresh aerial part of 90-day old tissue culture plants. This protocol can be used in different Chinese fir varieties that were widely planted in China, including Chinese fir 020 and 061. Notably, we found that the Chinese protoplasts are quite sensitive to the osmotic stress. Protoplasts are easily shrunken under high osmotic potential, while burst under low osmotic potential. The compositions of modified W5 solution, which has equal osmotic pressure with the following MMG solution, is quite important to keep the Chinese fir protoplast intact. Moreover, we showed that the protoplasts prepared from this method are healthy and competent for transformation, and successful transformation not only depends on the PEG concentration and the amount of DNA, but also heat-shock treatment (Supplemental Figure 5). The protocol provided here will be a convenient technique for promoter function validation, as well as many other molecular biology studies in Chinese fir.

With the advance in DNA sequencing technology, the bulk of transcriptomic data of Chinese fir were generated, providing good chance for studying the gene expression patterns of different tissues and screening genes that have high expression in all tissues. In this study, 24 promoters were cloned for the preliminary promoter activity assays in tobacco (Supplemental Figure 1). Further test in Chinese fir protoplasts showed that both *Cula11* and *Cula08* promoters have strong activities, much higher than the commonly used promoter in monocots and dicots, such as *CaM35S*, *ZmUBI*, or *CmYLCV* (Figure 1). And those two promoters, especially *Cula11* that with stronger activity than *Cula08*, have the great potential for Chinese fir transformation. At present, although the *Cula11* gene was annotated as ubiquitin, its molecular functions remain unknown. In the future, this gene should be characterized at the cellular level. Several ubiquitin promoters that derived from multiple plant species, including Arabidopsis, potato, sunflower, rice or maize, were isolated, but most of them were not frequently applied (Sharma and Sharma, 2009). The most widely applied constitutive promoter utilized for genetic engineering were *ZmUBI* from maize (Cornejo et al., 1993). To the best of our knowledge, *Cula11* promoter is the only one that was isolated from gymnosperm, and has the potential for gymnosperm and angiosperms genetic engineering.

CRISPR/Cas mediated genome editing technology provides a powerful tool for molecular study and breeding in Chinese fir. High levels expression of *Cas* gene is perquisite for the successful gene editing in Chinese fir. By using the newly isolated *Cula11* promoter, we showed that CRISPR/Cas mediated gene editing was successfully achieved (Figure 3), indicating its potential usage in future genetic engineering.

With the progress of gene stacking technology (Zhu et al., 2017), a battery of different promoters are needed to avoid homology-dependent gene silencing that commonly happens in transgenic plants with multiple copies of the same promoter (Potenza et al., 2004). Here we showed that the novel gene promoters from Chinese fir confer high levels of gene expression in transgenic rice and poplar, suggesting its potential alternative to *35S* promoter and *ZmUBI* promoters for the high-level expression of genes in monocots and dicots. In the future, expression patterns of these genes during the

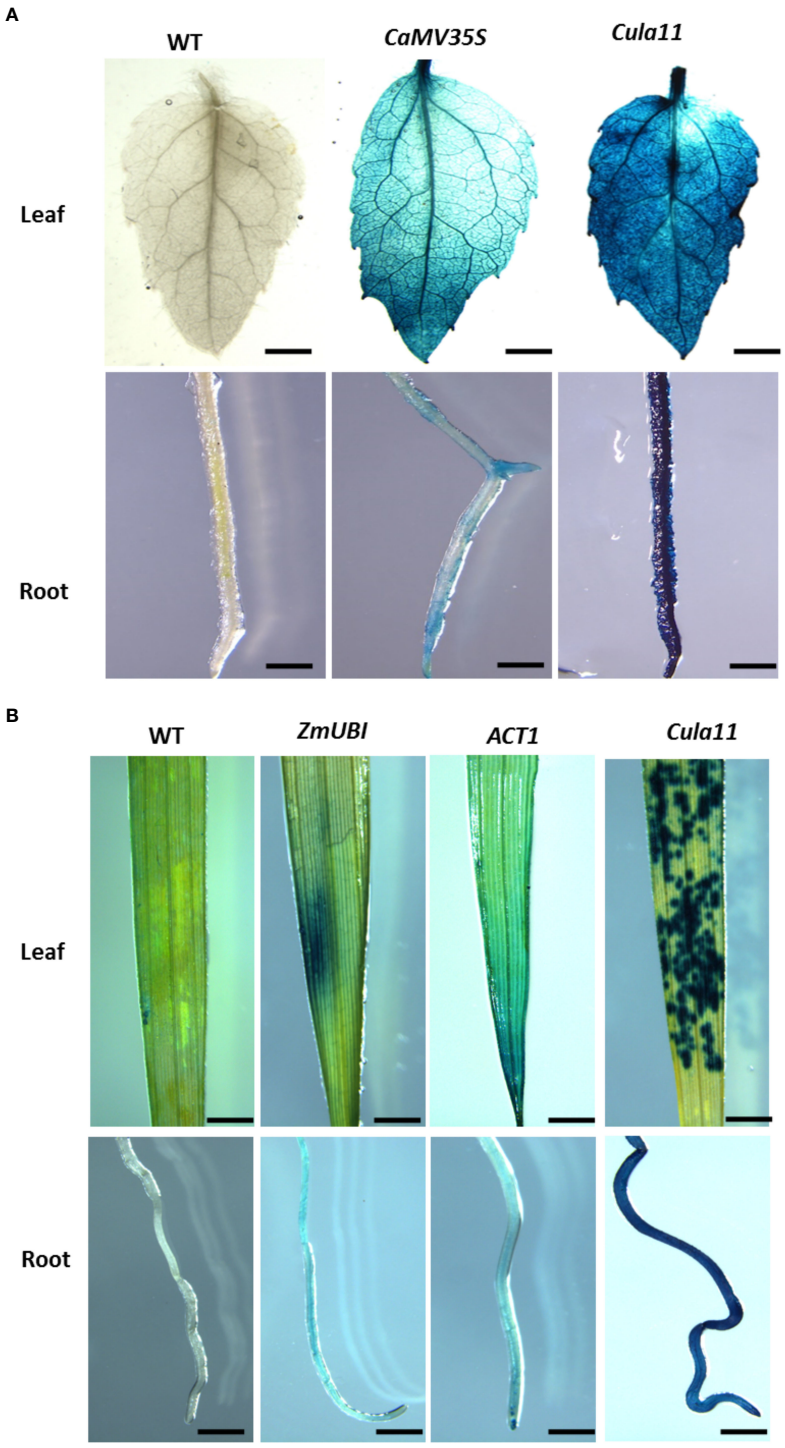


FIGURE 4
Cula11 promoter activity assay in the root and shoot of the *promoter:GUS* transgenic poplars and rice. **(A)** Transgenic poplars harboring the *Cula11 promoter:GUS* were analyzed in comparison with the transgenic lines containing *35S:GUS* constructs. Roots (UP panel) and shoots (Bottom Panel) from at least 3 independent transgenic lines were analyzed, and represented images were shown here. Scale bar: 2 cm; **(B)** Transgenic rice harboring *Cula11 promoter:GUS* were analyzed in comparison with the transgenic lines driven by *ZmUbi : GUS* and *Act1:GUS*. Roots (UP panel) and shoots (Bottom Panel) from at least 3 independent transgenic lines were analyzed, and represented images were shown here. Scale bar: 1 cm.

whole spectrum of transgenic plant growth, especially in rapidly dividing cells, should be functionally analyzed.

In conclusion, we reported two useful promoters for high-level expression of genes in gymnosperm such as Chinese fir, as well as in angiosperms such as rice and poplar. We established a repeatable protocol and highlighted the tips for protoplast isolation and transfection in Chinese fir, and for the first time we showed that this transient expression system satisfied its gene functional studies in Chinese fir protoplast, including protein localization, protein-protein interaction assays and Ribonucleoprotein (RNP) - mediated

gene editing studies. Moreover, we showed the activities of several well-known promoters in Chinese fir protoplasts, and highlighted two new native stronger promoters that potentially be used for the genetic engineering of both poplar and Chinese fir in the future. With the newly isolated *Cula11* and *U6b* promoters, CRISPR/Cas-mediated gene editing was successfully achieved in its protoplast. The technology provided here will facilitate genomic study and genetic engineering in Chinese fir in the 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

XM and QZ conceived this project, designed experiments, and interpreted the results. SY, WD, WB, JL and LZ performed the experiments and analyzed the data. All authors read and approved the submission of this manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Forestry Science and technology program of Fujian Province (2022FKJ06), National Natural Science Foundation of China (32071847), and the Foundation for the Development of Forestry Research in Fujian Agriculture and Forestry University (118/72202200201). The funding bodies were not involved in the design of the study or in any aspect of the data collection, analysis and interpretation of data and in paper writing.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1179394/full#supplementary-material

References

Assaad, F. F., Qiu, J. L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., et al. (2004). The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. *Mol. Biol. Cell* 15, 5118–5129. doi: 10.1091/mbc.e04-02-0140

Burris, K. P., Dlugosz, E. M., Collins, A. G., Stewart, C. N., and Lenaghan, S. C. (2016). Development of a rapid, low-cost protoplast transfection system for switchgrass (panicum virgatum l.). *Plant Cell Rep.* 35, 693–704. doi: 10.1007/s00299-015-1913-7

Cao, S., Deng, H., Zhao, Y., Zhang, Z., Tian, Y., Sun, Y., et al. (2022). Metabolite profiling and transcriptome analysis unveil the mechanisms of red-heart Chinese fir [Cunninghamia lanceolata (Lamb.) hook] heartwood coloration. *Front. Plant Sci.* 13, 854716. doi: 10.3389/fpls.2022.854716

Christensen, A. H., Sharrock, R. A., and Quail, P. H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* 18, 675–689. doi: 10.1007/BF00020010

Cornejo, M.-J., Luth, D., Blankenship, K. M., Anderson, O. D., and Blechl, A. E. (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Mol. Biol.* 23, 567–581. doi: 10.1007/BF00019304

Cui, Y., Zhao, J., Gao, Y., Zhao, R., Zhang, J., and Kong, L. (2021). Efficient multi-sites genome editing and plant regeneration *via* somatic embryogenesis in picea glauca. *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.751891

Decaestecker, W., Buono, R. A., Pfeiffer, M. L., Vangheluwe, N., Jourquin, J., Karimi, M., et al. (2019). CRISPR-TSKO: A technique for efficient mutagenesis in specific cell types, tissues, or organs in arabidopsis. *Plant Cell* 31, 2868–2887. doi: 10.1105/tpc.19.00454

De Rybel, B., Van Den Berg, W., Lokerse, A., Liao, C. Y., Van Mourik, H., Moller, B., et al. (2011). A versatile set of ligation-independent cloning vectors for functional studies in plants. *Plant Physiol.* 156, 1292–1299. doi: 10.1104/pp.111.177337

Eeckhaut, T., Lakshmanan, P. S., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Progress in plant protoplast research. *Planta* 238, 991–1003. doi: 10.1007/s00425-013-1936-7

Fang, J., Qian, J.-J., Yi, S., Harding, T. C., Tu, G. H., Vanroey, M., et al. (2005). Stable antibody expression at therapeutic levels using the 2A peptide. *Nat. Biotechnol.* 23, 584–590. doi: 10.1038/nbt1087

Gao, C. (2021). Genome engineering for crop improvement and future agriculture. *Cell* 184, 1621–1635. doi: 10.1016/j.cell.2021.01.005

Gao, C., Yu, C. K., Qu, S., San, M. W., Li, K. Y., Lo, S. W., et al. (2012). The golgi-localized arabidopsis endomembrane protein12 contains both endoplasmic reticulum export and golgi retention signals at its c terminus. *Plant Cell* 24, 2086–2104. doi: 10.1105/tpc.112.096057

GeQiang, Z., and Guoning, Q. (2012). Establishment of cell suspension culture and isolation of protoplast of Chinese fir. *For. Res.* 5, 628–632.

Guo, J., Morrell-Falvey, J. L., Labbé, J. L., Muchero, W., Kalluri, U. C., Tuskan, G. A., et al. (2012). Highly efficient isolation of populus mesophyll protoplasts and its application in transient expression assays. *PLoS One* 7, e44908. doi: 10.1371/journal.pone.0044908

Hassan, M. M., Zhang, Y., Yuan, G., De, K., Chen, J. G., Muchero, W., et al. (2021). Construct design for CRISPR/Cas-based genome editing in plants. *Trends Plant Sci.* 26, 1133–1152. doi: 10.1016/j.tplants.2021.06.015

Huang, H., Wang, Z., Cheng, J., Zhao, W., Xin, L., Wang, H., et al. (2013). An efficient cucumber (cucumis sativus l.) protoplast isolation and transient expression system. *Scientia Hortic.* 150, 206–212. doi: 10.1016/j.scienta.2012.11.011

Jang, I.-C., Choi, W.-B., Lee, K.-H., Song, S. I., Nahm, B. H., and Kim, J.-K. (2002). High-level and ubiquitous expression of the rice cytochrome c gene OsCc1 and its promoter activity in transgenic plants provides a useful promoter for transgenesis of monocots. *Plant Physiol.* 129, 1473–1481. doi: 10.1104/pp.002261

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907. doi: 10.1002/j.1460-2075.1987.tb02730.x

Kamigaki, A., Nito, K., Hikino, K., Goto-Yamada, S., Nishimura, M., Nakagawa, T., et al. (2016). Gateway vectors for simultaneous detection of multiple protein-protein interactions in plant cells using bimolecular fluorescence complementation. *PLoS One* 11, e0160717. doi: 10.1371/journal.pone.0160717

Li, M., Su, S.-S., Wu, P.-F., Cameron, K. M., Zhang, Y., Chen, W.-T., et al. (2017). Transcriptome characterization of the Chinese fir (Cunninghamia lanceolata (Lamb.) hook.) and expression analysis of candidate phosphate transporter genes. *Forests* 8, 420. doi: 10.3390/f8110420

Liang, Z., Chen, K., Zhang, Y., Liu, J., Yin, K., Qiu, J. L., et al. (2018). Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 *in vitro* transcripts or ribonucleoproteins. *Nat. Protoc.* 13, 413–430. doi: 10.1038/nprot.2017.145

Liao, J. M., Wang, B. C., Wang, Y. C., and Tian, J. Q. (2010). Optimization conditions of arabidopsis mesophyll protoplast isolation. *Acta Botanica Boreali-Occidentalia Sin.* 30, 1271–1276. doi: 10.3724/SP.J.1206.2010.00266

Lin, C. S., Hsu, C. T., Yang, L. H., Lee, L. Y., Fu, J. Y., Cheng, Q. W., et al. (2017). Application of protoplast technology to CRISPR/Cas9 mutagenesis: From single cell mutation detection to mutant plant regeneration. *Plant Biotechnol. J.* 16, 1295–1310. doi: 10.1111/pbi.12870

Lin, Y. C., Li, W., Chen, H., Li, Q., Sun, Y. H., Shi, R., et al. (2014). A simple improved-throughput xylem protoplast system for studying wood formation. *Nat. Protoc.* 9, 2194–2205. doi: 10.1038/nprot.2014.147

Lin, X., Zhang, W., Takechi, K., Takio, S., Ono, K., and Takano, H. (2005). Stable genetic transformation of larix gmelinii l. by particle bombardment of zygotic embryos. *Plant Cell Rep.* 24, 418–425. doi: 10.1007/s00299-005-0955-7

Lin, E., Zhuang, H., Yu, J., Liu, X., Huang, H., Zhu, M., et al. (2020). Genome survey of Chinese fir (Cunninghamia lanceolata): Identification of genomic SSRs and demonstration of their utility in genetic diversity analysis. *Sci. Rep.* 10, 4698. doi: 10.1038/s41598-020-61611-0

Liu, S., Ma, J., Liu, H., Guo, Y., Li, W., and Niu, S. (2020). An efficient system for agrobacterium-mediated transient transformation in pinus tabuliformis. *Plant Methods* 16, 52. doi: 10.1186/s13007-020-00594-5

Liu, Y., Merrick, P., Zhang, Z., Ji, C., Yang, B., and Fei, S. Z. (2018). Targeted mutagenesis in tetraploid switchgrass (Panicum virgatum l.) using CRISPR/Cas9. *Plant Biotechnol. J.* 16, 381–393. doi: 10.1111/pbi.12778

Liu, K. H., Niu, Y., Konishi, M., Wu, Y., Du, H., Sun Chung, H., et al. (2017). Discovery of nitrate-CPK-NLP signalling in central nutrient-growth networks. *Nature* 545, 311–316. doi: 10.1038/nature22077

Locatelli, F., Vannini, C., Magnani, E., Coraggio, I., and Bracale, M. (2003). Efficiency of transient transformation in tobacco protoplasts is independent of plasmid amount. *Plant Cell Rep.* 21, 865–871. doi: 10.1007/s00299-003-0593-x

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., et al. (2016). DNA-Free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Front. Plant Sci.* 7, 1904. doi: 10.3389/fpls.2016.01904

Manghwar, H., Lindsey, K., Zhang, X., and Jin, S. (2019). CRISPR/Cas system: Recent advances and future prospects for genome editing. *Trends Plant Sci.* 24, 1102–1125. doi: 10.1016/j.tplants.2019.09.006

McElroy, D., Zhang, W., Cao, J., and Wu, R. (1990). Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell* 2, 163–171. doi: 10.2307/3868928

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Nanasato, Y., Mikami, M., Futamura, N., Endo, M., Nishiguchi, M., Ohmiya, Y., et al. (2021). CRISPR/Cas9-mediated targeted mutagenesis in Japanese cedar (Cryptomeria japonica d. don). *Sci. Rep.* 11, 16186. doi: 10.1038/s41598-021-95547-w

Nelson, B. K., Cai, X., and Nebenfuhr, A. (2007). A multicolored set of *in vivo* organelle markers for co-localization studies in arabidopsis and other plants. *Plant J.* 51, 1126–1136. doi: 10.1111/j.1365-313X.2007.03212.x

Odell, J. T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 313, 810–812. doi: 10.1038/313810a0

Park, S. H., Yi, N., Kim, Y. S., Jeong, M. H., Bang, S. W., Choi, Y. D., et al. (2010). Analysis of five novel putative constitutive gene promoters in transgenic rice plants. *J. Exp. Bot.* 61, 2459–2467. doi: 10.1093/jxb/erq076

Poddar, S., Tanaka, J., Cate, J. H. D., Staskawicz, B., and Cho, M. J. (2020). Efficient isolation of protoplasts from rice calli with pause points and its application in transient gene expression and genome editing assays. *Plant Methods* 16, 151. doi: 10.1186/s13007-020-00692-4

Poovaiah, C., Phillips, L., Geddes, B., Reeves, C., Sorieul, M., and Thorlby, G. (2021). Genome editing with CRISPR/Cas9 in pinus radiata (D. don). *BMC Plant Biol.* 21, 363. doi: 10.1186/s12870-021-03143-x

Potenza, C., Aleman, L., and Sengupta-Gopalan, C. (2004). Targeting transgene expression in research, agricultural, and environmental applications: Promoters used in plant transformation. *In Vitro Cell. Dev. Biol. - Plant* 40, 1–22. doi: 10.1079/IVP2003477

Priyadarshani, S., Hu, B., Li, W., Ali, H., Jia, H., Zhao, L., et al. (2018). Simple protoplast isolation system for gene expression and protein interaction studies in pineapple (Ananas comosus l.). *Plant Methods* 14, 95. doi: 10.1186/s13007-018-0365-9

Rahman, F., Mishra, A., Gupta, A., and Sharma, R. (2022). Spatiotemporal regulation of CRISPR/Cas9 enables efficient, precise, and heritable edits in plant genomes. *Front. Genome Ed* 4, 870108. doi: 10.3389/fgeed.2022.870108

Ren, Q., Sretenovic, S., Liu, S., Tang, X., Huang, L., He, Y., et al. (2021). PAM-less plant genome editing using a CRISPR–SpRY toolbox. *Nat. Plants.* 7, 25–33. doi: 10.1038/s41477-020-00827-4

Sahoo, K. K., Tripathi, A. K., Pareek, A., Sopory, S. K., and Singla-Pareek, S. L. (2011). An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant Methods* 7, 49. doi: 10.1186/1746-4811-7-49

Sang-Dong, Y., Young-Hee, C., and Jen, S. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2, 1565–1572. doi: 10.1038/nprot.2007.199

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palaci, J., Castelijns, B., Forment, J., Ziarsolo, P., et al. (2013). GoldenBraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. *Plant Physiol.* 162, 1618–1631. doi: 10.1104/pp.113.217661

Scheben, A., and Edwards, D. (2018). Towards a more predictable plant breeding pipeline with CRISPR/Cas-induced allelic series to optimize quantitative and qualitative traits. *Curr. Opin. Plant Biol.* 45, 218–225. doi: 10.1016/j.pbi.2018.04.013

Shan, Q., Wang, Y., Li, J., and Gao, C. (2014). Genome editing in rice and wheat using the CRISPR/Cas system. *Nat. Protoc.* 9, 2395–2410. doi: 10.1038/nprot.2014.157

Sharma, A. K., and Sharma, M. K. (2009). Plants as bioreactors: recent developments and emerging opportunities. *Biotechnol. Adv.* 27, 811–832. doi: 10.1016/j.biotechadv.2009.06.004

Sheen, J. (2001). Signal transduction in maize and arabidopsis mesophyll protoplasts. *Plant Physiol.* 127, 1466–1475. doi: 10.1104/pp.010820

Song, Y., Bai, X., Dong, S., Yang, Y., Dong, H., Wang, N., et al. (2020). Stable and efficient agrobacterium-mediated genetic transformation of larch using embryogenic callus. *Front. Plant Sci.* 11, 584492. doi: 10.3389/fpls.2020.584492

Stavolone, L., Kononova, M., Pauli, S., Ragozzino, A., De Haan, P., Milligan, S., et al. (2003). Cestrum yellow leaf curling virus (CmYLCV) promoter: a new strong constitutive promoter for heterologous gene expression in a wide variety of crops. *Plant Mol. Biol.* 53, 663–673. doi: 10.1023/B:PLAN.0000019110.95420.bb

Sugano, S. S., Nishihama, R., Shirakawa, M., Takagi, J., Matsuda, Y., Ishida, S., et al. (2018). Efficient CRISPR/Cas9-based genome editing and its application to conditional genetic analysis in marchantia polymorpha. *PloS One* 13, e0205117. doi: 10.1371/journal.pone.0205117

Tan, B., Xu, M., Chen, Y., and Huang, M. (2013). Transient expression for functional gene analysis using populus protoplasts. *Plant Cell Tissue Organ Culture* 114, 11–18. doi: 10.1007/s11240-013-0299-x

Tang, J., Lin, E., Huang, H., Tong, Z., and Lou, X. (2018). Isolation and total RNA extraction of leaf protoplasts in Chinese fir. *Scientia Silvae Sinicae* 54, 38–48. doi: 10.11707/j.1001-7488.20180405

Tzfira, T., Yarnitzky, O., Vainstein, A., and Altman, A. (1996). Agrobacterium rhizogenes-mediated DNA transfer inPinus halepensis mill. *Plant Cell Rep.* 16, 26–31. doi: 10.1007/BF01275443

Vazquez-Vilar, M., Bernabe-Orts, J. M., Fernandez-Del-Carmen, A., Ziarsolo, P., Blanca, J., Granell, A., et al. (2016). A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. *Plant Methods* 12, 10. doi: 10.1186/s13007-016-0101-2

Wan, L.-C., Wang, F., Guo, X., Lu, S., Qiu, Z., Zhao, Y., et al. (2012). Identification and characterization of small non-coding RNAs from Chinese fir by high throughput sequencing. *BMC Plant Biol.* 12, 146. doi: 10.1186/1471-2229-12-146

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., et al. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951. doi: 10.1038/nbt.2969

Wang, D., Hao, Z., Long, X., Wang, Z., Zheng, X., Ye, D., et al. (2020). The transcriptome of cunninghamia lanceolata male/female cone reveal the association between MIKC MADS-box genes and reproductive organs development. *BMC Plant Biol.* 20, 508. doi: 10.1186/s12870-020-02634-7

Wang, H., Wang, C., Liu, H., Tang, R., and Zhang, H. (2011). An efficient agrobacterium-mediated transformation and regeneration system for leaf explants of two elite aspen hybrid clones populus alba x p. berolinensis and populus davidiana x p. bolleana. *Plant Cell Rep.* 30, 2037–2044. doi: 10.1007/s00299-011-1111-1

Wang, J., Wang, Y., Lu, T., Yang, X., Liu, J., Dong, Y., et al. (2022). An efficient and universal protoplast isolation protocol suitable for transient gene expression analysis and single-cell RNA sequencing. *Int. J. Mol. Sci.* 23, 3419. doi: 10.3390/ijms23073419

Wang, X., Xu, L., Liu, X., Xin, L., Wu, S., and Chen, X. (2021b). Development of potent promoters that drive the efficient expression of genes in apple protoplasts. *Hortic. Res.* 8, 211. doi: 10.1038/s41438-021-00646-4

Wang, Q., Yu, G., Chen, Z., Han, J., Hu, Y., and Wang, K. (2021a). Optimization of protoplast isolation, transformation and its application in sugarcane (Saccharum spontaneum l). *Crop J.* 9, 133–142. doi: 10.1016/j.cj.2020.05.006

Wu, F. H., Shen, S. C., Lee, L. Y., Lee, S. H., Chan, M. T., and Lin, C. S. (2009). Tape-arabidopsis sandwich - a simpler arabidopsis protoplast isolation method. *Plant Methods* 5, 16. doi: 10.1186/1746-4811-5-16

Xu, Y., Li, R., Luo, H., Wang, Z., Li, M. W., Lam, H. M., et al. (2022). Protoplasts: small cells with big roles in plant biology. *Trends Plant Sci.* 27, 828–829. doi: 10.1016/j.tplants.2022.03.010

Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X., et al. (2012). Effective small RNA destruction by the expression of a short tandem target mimic in arabidopsis. *Plant Cell* 24, 415–427. doi: 10.1105/tpc.111.094144

Yang, Y., Li, R., and Qi, M. (2000). *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J.* 22, 543–551. doi: 10.1046/j.1365-313x.2000.00760.x

Ye, S., Chen, G., Kohnen, M. V., Wang, W., Cai, C., Ding, W., et al. (2020). Robust CRISPR/Cas9 mediated genome editing and its application in manipulating plant height in the first generation of hexaploid ma bamboo (Dendrocalamus latiflorus Munro). *Plant Biotechnol. J.* 18, 1501–1503. doi: 10.1111/pbi.13320

Yu, G., Cheng, Q., Xie, Z., Xu, B., Huang, B., and Zhao, B. (2017). An efficient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1. *Plant Methods* 13, 46. doi: 10.1186/s13007-017-0196-0

Yu, B., Ning, H., Wu, J., Yang, Y., Wang, J., Zhu, M., et al. (2014a). A transient gene expression system using barley protoplasts to evaluate microRNAs for post-transcriptional regulation of their target genes. *Plant Cell Tissue Organ Culture* 119, 211–219. doi: 10.1007/s11240-014-0527-z

Yu, C., Wang, L., Chen, C., He, C., Hu, J., Zhu, Y., et al. (2014b). Protoplast: A more efficient system to study nucleo-cytoplasmic interactions. *Biochem. Biophys. Res. Commun.* 450, 1575–1580. doi: 10.1016/j.bbrc.2014.07.043

Zhang, Z. L., Ogawa, M., Fleet, C. M., Zentella, R., Hu, J., Heo, J. O., et al. (2011). Scarecrow-like 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2160–2165. doi: 10.1073/pnas.1012232108

Zhang, H., Zhang, J., Lang, Z., Botella, J. R., and Zhu, J.-K. (2017). Genome editing–principles and applications for functional genomics research and crop improvement. *Crit. Rev. Plant Sci.* 36, 291–309. doi: 10.1080/07352689.2017.1402989

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., et al. (2014). The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12, 797–807. doi: 10.1111/pbi.12200

Zhao, F. L., Li, Y. J., Hu, Y., Gao, Y. R., Zang, X. W., Ding, Q., et al. (2016). A highly efficient grapevine mesophyll protoplast system for transient gene expression and the study of disease resistance proteins. *Plant Cell Tissue Organ Culture* 125, 43–57. doi: 10.1007/s11240-015-0928-7

Zhu, H., Li, C., and Gao, C. (2020). Applications of CRISPR-cas in agriculture and plant biotechnology. *Nat. Rev. Mol. Cell Biol.* 21, 661–677. doi: 10.1038/s41580-020-00288-9

Zhu, Q., Yu, S., Zeng, D., Liu, H., Wang, H., Yang, Z., et al. (2017). Development of "Purple endosperm rice" by engineering anthocyanin biosynthesis in the endosperm with a high-efficiency transgene stacking system. *Mol. Plant* 10, 918–929. doi: 10.1016/j.molp.2017.05.008

frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 30 August 2023
DOI 10.3389/fpls.2023.1247707

OPEN ACCESS

EDITED BY
Tong Zhang,
South China Agricultural University, China

REVIEWED BY
Yi Xu,
Nanjing Agricultural University, China
Zhi Gang Meng,
Biotechnology Research institute of CAAS, China

*CORRESPONDENCE
Xiangchun Qu
jlsnkyqxc@126.com
Wenyuan Hao
wenyuan_h@163.com

†These authors share first authorship

RECEIVED 26 June 2023
ACCEPTED 28 July 2023
PUBLISHED 30 August 2023

CITATION
Yao D, Zhou J, Zhang A, Wang J, Liu Y, Wang L, Pi W, Li Z, Yue W, Cai J, Liu H, Hao W and Qu X (2023) Advances in CRISPR/Cas9-based research related to soybean [*Glycine max* (Linn.) Merr] molecular breeding.
Front. Plant Sci. 14:1247707.
doi: 10.3389/fpls.2023.1247707

Advances in CRISPR/Cas9-based research related to soybean [*Glycine max* (Linn.) Merr] molecular breeding

Dan Yao[1,2†], Junming Zhou[1†], Aijing Zhang[3], Jiaxin Wang[1], Yixuan Liu[1], Lixue Wang[1], Wenxuan Pi[1], Zihao Li[1], Wenjun Yue[1], Jinliang Cai[1], Huijing Liu[1], Wenyuan Hao[4*] and Xiangchun Qu[2*]

[1]College of Life Science, Jilin Agricultural University, Changchun, Jilin, China, [2]Institute of Crop Resources, Jilin Provincial Academy of Agricultural Sciences, Gongzhuling, Jilin, China, [3]College of Agronomy, Jilin Agricultural University, Changchun, China, [4]Jilin Provincial Academy of Agricultural Sciences, Changchun, Jilin, China

Soybean [*Glycine max* (Linn.) Merr] is a source of plant-based proteins and an essential oilseed crop and industrial raw material. The increase in the demand for soybeans due to societal changes has coincided with the increase in the breeding of soybean varieties with enhanced traits. Earlier gene editing technologies involved zinc finger nucleases and transcription activator-like effector nucleases, but the third-generation gene editing technology uses clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). The rapid development of CRISPR/Cas9 technology has made it one of the most effective, straightforward, affordable, and user-friendly technologies for targeted gene editing. This review summarizes the application of CRISPR/Cas9 technology in soybean molecular breeding. More specifically, it provides an overview of the genes that have been targeted, the type of editing that occurs, the mechanism of action, and the efficiency of gene editing. Furthermore, suggestions for enhancing and accelerating the molecular breeding of novel soybean varieties with ideal traits (e.g., high yield, high quality, and durable disease resistance) are included.

KEYWORDS

CRISPR/Cas9, soybean, molecular breeding, gene editing, application

1 Introduction

Soybean is a significant source of vegetable proteins for humans and an important oilseed crop, making it a commercially valuable plant (Zhang A, et al., 2023). More than 90% of the soybean plants cultivated in the three main soybean-producing countries (USA, Brazil, and Argentina) are genetically modified varieties generated using gene editing technology (Fang et al., 2023). In terms of sustainable food production, the demand for soybeans has continued to increase because of the scarcity of arable land. In the field of

molecular breeding, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has emerged as a commonly used third-generation gene editing technology (Nakamori, 2023). Thus, many new and desirable soybean traits have been developed using gene editing technology, which is currently a hot topic in scientific research (Osakabe and Osakabe, 2017; Chen et al., 2022; Zhou et al., 2023a).

In recent years, CRISPR/Cas9 gene editing technology has been used by plant molecular breeders to improve various plant traits (Ma et al., 2016; Zhang et al., 2017; Rao et al., 2022). Because it can simply, effectively, and precisely edit target genes responsible for specific characteristics, CRISPR/Cas9 has replaced previously used gene editing techniques (Zheng et al., 2021; Impens et al., 2022; Liu H. et al., 2022). Several crop traits, including yield, quality, stress tolerance, disease resistance, and herbicide resistance, can be improved using CRISPR/Cas9 systems. This can lead to the development of novel germplasm with superior traits as well as significant advancements in plant molecular breeding (Yin et al., 2017; Hussain et al., 2018; Wada et al., 2020; Gan and Ling, 2022; Qi et al., 2023).

The limitations of early genome editing methods included the inability to explore the relationships between several related genes (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). These previous methods were mostly employed to edit individual genes. Because soybean is a paleotetraploid, it has many homologous and redundant genes, which makes the functional characterization of soybean genes challenging (Tran and Mochida, 2010; Du et al., 2023). The CRISPR/Cas9 system has recently been used to edit multiple genes in the soybean genome. This has considerably decreased the effects of redundant genes on the efficient editing of specific genes for breeding soybean varieties with desirable traits (Bao et al., 2020; Xu H. et al., 2020; Baek et al., 2022; Guan et al., 2022; Rasheed et al., 2022a).

This review describes the recent improvements in soybean traits via the application of the CRISPR/Cas9 gene editing technology. It also presents information regarding the target genes and their mechanism of action, while providing a brief overview of transformation efficiency and gene editing efficiency. Furthermore, suggestions for future CRISPR/Cas9 development and use in soybean molecular breeding programs are included.

2 Application of CRISPR/Cas9 gene editing technology in soybean molecular breeding

There has recently been an increase in the use of CRISPR/Cas9 to edit genes in soybean, corn, wheat, rice, cotton, and other crops (Figure 1, Table 1). The creation of new soybean germplasm with many excellent traits using various transformation methods (e.g., *Agrobacterium*-mediated transformation) has laid the foundation for further improving CRISPR/Cas9 gene editing technology for soybean molecular breeding (Figure 2).

2.1 Enhancement of soybean resistance to abiotic stresses

During different soybean developmental stages, many genetic and biochemical processes control how soybean perceives and responds to abiotic stresses, including salinity and drought. One of the primary objectives of molecular breeding research is improving stress tolerance (Deshmukh et al., 2014; Amoanimaa-Dede et al., 2022; Cadavid et al., 2023). Osmotic stress in plant cells is typically caused by abiotic factors (e.g., drought or excessive

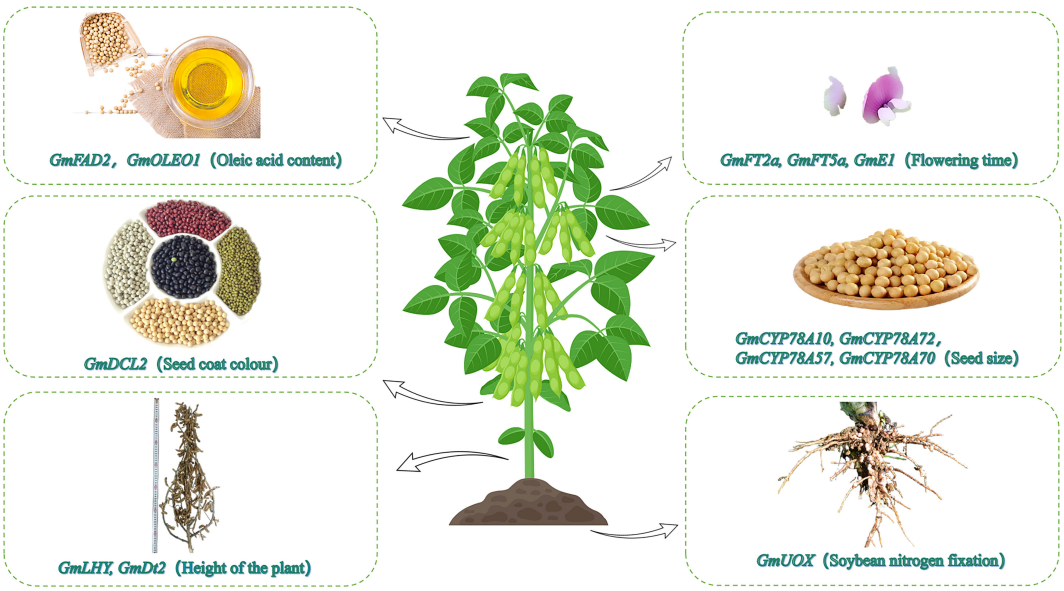


FIGURE 1
Utility of CRISPR/Cas9 for editing soybean functional genes. The CRISPR/Cas9 gene editing technology has recently been used to modify soybean genes affecting the oil content, photoperiodic flowering, seed coat color, seed size, plant height, and nodulation.

TABLE 1 Applications of CRISPR/Cas9 in five major agricultural crops.

Specie	Gene Name	Gene function	Gene editing method	Edit Type	Editing efficiency	Transformation method	Research significance	Reference
Soybean	*GmFAD2*	Soybean oleic acid content	Single target	Deletion And Insertion	40%-85%	*Agrobacterium*-mediated method	Creation of high oleic acid soybeans	(Zhou et al., 2023b)
	GmTAP1	Regulation of soybean resistance to soybean blast	Single target	Deletion And Insertion	Around 50%	*Agrobacterium*-mediated method	Creation of blast-resistant soybean germplasm	(Liu T. F. et al., 2023)
	GmVPS8a	Regulation of soybean phenotype	Single target	Deletion	81.25%	*Agrobacterium*-mediated method	Verify that the gene is a multifunctional gene	(Kong et al., 2023)
	GmPDCT	Regulation of soybean oil synthesis	Dual Target	Deletion And Insertion	46.7%	*Agrobacterium*-mediated method	Creation of high oleic acid soybean germplasm	(Li et al., 2023b)
	GmSPL2b	Regulation of heat tolerance in soybean during flowering	Dual Target	Deletion	–	*Agrobacterium*-mediated method	Creation of heat-resistant soybean varieties	(Ding et al., 2023)
Rice	*Wx/OsBADH9*	Reduced straight-chain starch content and improved aroma	Dual Target	Deletion	Around 55%	*Agrobacterium*-mediated method	Improving the edible quality of hybrid rice	(Tian et al., 2023)
	OsHPPD	Herbicide resistance	Single target	Deletion And Insertion	Around 44%	*Agrobacterium*-mediated method	Creation of herbicide-resistant rice	(Wu et al., 2023)
	OsHPP04	Anti-parasitic nematode	Dual Target	Deletion And Insertion	Around 30%	*Agrobacterium*-mediated method	Creation of parasitic nematode resistant rice germplasm	(Huang et al., 2023)
	OsLCD	Reduction of cadmium accumulation in rice seeds	Dual Target	Deletion And Insertion	–	*Agrobacterium*-mediated method	Creation of low cadmium rice germplasm	(Chen H. M., et al., 2023)
	OsC1	Regulation of the phenotype of rice purple leaf sheath	Single target	Deletion	–	*Agrobacterium*-mediated method	Creation of purple sheath deficient phenotype rice germplasm	(Chin et al., 2016)
Maize	*ZmPLA*	Induced haploid germplasm in maize	Triple target	Deletion and Replace	1.04%	Gene gun transformation method	Creation of double haploid germplasm resources of maize	(Rangari et al., 2023)
	ZmG6PDH1	Regulation of cold stress tolerance in maize	Dual Target	Deletion	63%-75%	*Agrobacterium*-mediated method	Creation of cold-stress tolerant maize germplasm	(Li et al., 2023a)
	ZmChSK1	Regulation of southern leaf blight susceptibility in corn	Dual Target	Deletion And Insertion	13.1%	*Agrobacterium*-mediated method	Creation of southern leaf blight resistant maize germplasm	(Chen C., et al., 2023)
	ZmbHLH121		Dual Target		–			

(Continued)

10.3389/fpls.2023.1247707

TABLE 1 Continued

Specie	Gene Name	Gene function	Gene editing method	Edit Type	Editing efficiency	Transformation method	Research significance	Reference
		Regulation of cortical gas formation in maize roots		Deletion And Insertion		*Agrobacterium*-mediated method	Creation of maize germplasm for elimination of cortical aerial traits in the root system	(Schneider et al., 2023)
	ZmCals12	Gene encoding callose synthase	Dual Target	Deletion And Insertion	–	*Agrobacterium*-mediated method	Creation of maize germplasm with male sterile traits	(Niu et al., 2023)
Wheat	*TaTFL1-5*	Regulation of flowering time and inflorescence structure in rice	Single、Dual、Triple target	Deletion And Insertion	Around 40%	*Agrobacterium*-mediated method	Verification that the regulation of tiller and spikelet formation in wheat has some similar molecular mechanisms	(Sun et al., 2023)
	TaDCL4、*TaDCL5*、*TaRDR6*	Regulation of male sterility in wheat	Single target	Deletion And Insertion	70%-75%	*Agrobacterium*-mediated method	Creation of male sterile wheat lines	(Zhang R. Z., et al., 2023)
	TaHRC、*Tsn9*	Regulation of disease resistance in wheat	Dual Target	Deletion And Insertion	33%	*Agrobacterium*-mediated method	Creation of wheat germplasm with disease resistance	(Karmacharya et al., 2023)
	TaPpd	Regulation of wheat flowering time	Dual Target	Deletion And Insertion	2%	*Agrobacterium*-mediated method	Confirmation that this gene regulates wheat spike structure and grain morphological characteristics	(Errum et al., 2023)
	TraesFLD1D01G005600、*TraesFLD1B01G010600*	Regulating the quality of wheat consumption	Single target	Deletion And Insertion	–	*Agrobacterium*-mediated method	Creation of high quality edible wheat germplasm	(Liu et al., 2023)
Cotton	*GhEMS1*	Regulation of male sterility traits in cotton	Dual Target	Deletion And Insertion	3%	*Agrobacterium*-mediated method	Creation of male sterile cotton germplasm with necrosis-like black spots on anthers	(Zhang J., et al., 2023)
	GhCLA1	Regulation of Cotton Whitening Phenotype	Dual Target	Deletion And Insertion	66.7-100%	*Agrobacterium*-mediated method	Achieving multiple gene editing in polyploid crops	(Chen et al., 2021b)
	GhALARP	Encodes an alanine-rich protein	Single target	Deletion And Insertion	71.4-100%	*Agrobacterium*-mediated method	Validation of the gene function	(Zhu et al., 2018)
	GhFAD2	Regulation of lipid synthesis function	Dual Target	Deletion And Insertion	68.42%-73.68%	*Agrobacterium*-mediated method	Creation of high oleic acid cotton germplasm	(Chen et al., 2021b)
	GhGPAT12/25	Regulation of anther cuticle and pollen assembly	Dual Target	Deletion And Insertion	–	*Agrobacterium*-mediated method	Creation of male sterile cotton germplasm	(Zhang et al., 2021)

10.3389/fpls.2023.1247707

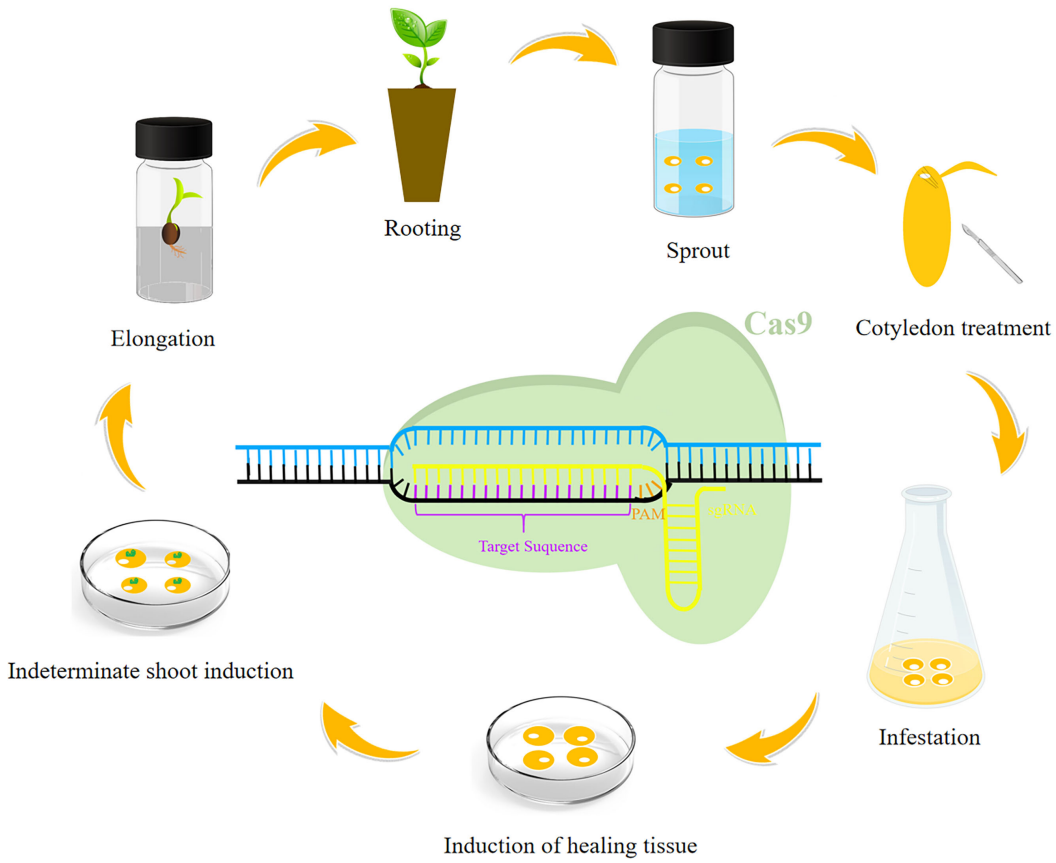


FIGURE 2
General soybean genetic transformation process. The following steps are generally included in the genetic transformation of soybean: sprouting, cotyledon treatment, infestation, induction of healing tissue, indeterminate shoot induction, elongation, and rooting. A schematic diagram is provided to show how the CRISPR/Cas9 system cleaves the target genomic segment.

salinity). Analyses of the sequences of the related genes revealed the regulatory effects of various plant cellular components, such as sensors, receptors, phytohormones, transcription factors, kinases, phosphatases, and microRNAs, on abiotic stress response-related pathways (Ramesh et al., 2019; Mangena, 2020; Staniak et al., 2023).

Water deficiency substantially restricts soybean growth and development, which can decrease the soybean yield by up to 40% (Khan, 2018). Thus, there is a critical need for exploring the mechanism underlying soybean drought resistance and generating new drought-resistant soybean germplasm (Ramlal et al., 2022). By deleting miR398c in soybean, Zhou et al. (2020) increased the expression of *GmCSD1a/b*, *GmCSD2a/b/c*, and *GmCCS* (relative to the corresponding levels in over-expression strains), thereby increasing the capacity to scavenge O^{2-} (Zhou et al., 2020). In 2021, Xiao et al. identified 112 *GmPLA* family genes in the soybean genome and used CRISPR/Cas9 technology to knock out two homologous genes (*GmpPLA-II* epsilon and zeta). Knocking out one or both genes affected the root response to phosphorus deficiency, with some mutant lines exhibiting increased resistance to flooding and drought conditions (compared with the control) (Xiao et al., 2021). Additionally, in 2021, Yu et al. reported that the *GmNF-YC14* deletion mutant created using CRISPR/Cas9 technology is more susceptible to drought stress than wild-type soybean, implying *GmNF-YC14* may be useful for increasing soybean drought tolerance (Yu et al., 2021). By comparing the agronomic features of soybean plants over-expressing *sHSP26* with those of soybean plants in which *sHSP26* had been edited, Liu S. Y., et al. (2022) revealed that *sHSP26* may considerably increase soybean drought tolerance and yield (Liu S. Y., et al., 2022). In 2022, Yang et al. edited the soybean transcription factor gene *GmNAC12*, which decreased the survival of the transgenic plants exposed to drought stress by at least 12%. They concluded that *GmNAC12* is a key gene that positively regulates soybean tolerance to drought conditions (Yang C.F., et al., 2022).

Salinity can severely decrease the seed yield and quality of soybean, which is a salt-sensitive crop species (Phang et al., 2008; Cai et al., 2022; Feng et al., 2023). In addition to accelerating the development of salt-tolerant soybean varieties to increase grain yield, research on salt stress tolerance can also optimize the use of saline farmland (Chen et al., 2018; Jin et al., 2021). In 2021, Niu et al. clarified the effects of knocking down and over-expressing *lncRNA77580* on the expression of nearby protein-coding genes linked to the soybean response to salt stress. Additionally, increases in the length of the DNA fragment deleted from *lncRNA77580* via the application of CRISPR/Cas9 technology increased the changes in the expression of *lncRNA77580* and nearby genes (Niu et al.,

2021). By simultaneously targeting six *GmAITR* genes using a CRISPR/Cas9 system, Wang et al. (2021) produced a Cas9-free *GmAITR3* and *GmAITR6* double mutant and a *GmAITR2 GmAITR3 GmAITR4 GmAITR5 GmAITR6* quintuple mutant. They determined that salt tolerance was more pronounced in the higher-order mutants, suggesting that mutating *GmAITR* genes can enhance soybean salt tolerance (Wang et al., 2021). Zhang M.H. et al. (2022) produced three soybean mutants in which *GmSOS1* was edited and observed that Na^+ accumulated significantly more in the mutants than in the control. Accordingly, this gene is essential for soybean salt tolerance because it helps maintain Na^+ homeostasis (Zhang M.H., et al., 2022).

The adaptation of soybean to severe drought and salt stresses involves the activation of overlapping pathways at the morphological, physiological, and molecular levels. Drought tolerance and salt tolerance are polygenic traits (Chen et al., 2018; Kofsky et al., 2018; Mammadov et al., 2018). Additionally, the perception of stress and its effects on soybean growth or development are similar among the abiotic stress factors. In an earlier study by Du et al. (2018), soybean plants in which the transcription factor gene *GmMYB118* was silenced were more susceptible to drought and saline conditions than soybean plants over-expressing *GmMYB118*. Moreover, the decreased production of minor heat shock proteins increased the resistance of plants to drought, cold, and salt stresses (Du et al., 2018). However, when Zhang M.H. et al. (2022) knocked out *GmHsps_p23*, which encodes a minor heat shock protein in soybean, the transgenic plants were highly susceptible to salt and drought conditions. Future research will need to focus on the use of several gene editors to simultaneously target and regulate the expression of functional genes mediating drought and salinity tolerance to produce novel soybean genotypes with superior traits (Zhang Y.Z., et al., 2022).

2.2 Enhance disease and insect resistance in soybean

Tobacco ringspot virus, soybean dwarf virus, soybean vein necrosis virus, soybean mosaic virus (SMV), bean pod mottle virus, and alfalfa mosaic virus are only a few of the viruses that can infect soybean (Liu et al., 2016; Widyasari et al., 2020; Lin et al., 2022). Multiple viruses can simultaneously infect soybean plants, causing more harm than an infection by a single virus. Hence, the use of gene editing tools to target genes that control soybean disease resistance and improve disease resistance-related traits has become a major objective in soybean molecular breeding programs (Chang et al., 2016; Chandra et al., 2022; Zhao et al., 2023).

Several non-homologous end-joining and homology directed repair-mediated gene replacement mutants were produced by Fang et al. (2015), who targeted the soybean blast fungal pathogenicity gene *Avr4/6*. These mutants were more resistant to diseases caused by oomycetes than the controls (Fang and Tyler, 2016). Ochola et al. (2020) edited the usual effector genes of the soybean root pathogen *Phytophthora sojae*. They observed that disease resistance was affected by the *Avr* gene expression level in soybean (Ochola et al., 2020). In 2020, Ma et al. confirmed that *GmLMM2* deficiencies increased the resistance to *P. sojae* by increasing tetrapyrrole biosynthesis, but decreased the chlorophyll content by disrupting tetrapyrrole biosynthesis. The elimination of *GmLMM2* expression resulted in the appearance of necrotic regions in the growing leaves of the CRISPR/Cas9-edited mutants (Ma et al., 2020). Zhang P.P, et al. (2020) targeted *GmF3H1*, *GmF3H2*, and *GmFNSII-1* in soybean plants (including the hairy roots) using a CRISPR/Cas9-mediated multiple gene editing system. They detected a significant increase in the isoflavone content and a significant decrease in the SMV coat protein content (approximately 33% decrease) in the mutants, indicating that the increased isoflavone content enhanced the leaf resistance to SMV (Zhang P.P., et al., 2020). Three crucial genes in the soybean *Rsc4* gene family (*Rsc4-1, Rsc4-2, and Rsc4-3*) were modified by CRISPR/Cas9 in 2021 to alter soybean resistance to SMV (Yin et al., 2021). To investigate the effector gene *Avr1b-1* in the soybean pathogen *Blastomyces* in terms of its function as well as the underlying mechanism. Gu et al. (2021) created target locus-specific knockout and knock-in mutants. All selected knockout mutants were virulent on plants expressing *Rps1b*, whereas the infection of plants lacking *Rps1b* was unaffected. When a sgRNA-resistant variant of *Avr1b-1* was re-introduced into the *Avr1b-1* locus of the mutants in which *Avr1b* was knocked out, the resulting knock-in transformants expressing *Avr1b-1* were unable to infect soybean plants carrying *Rps1b* (Gu et al., 2021). Compared with the RNAi and over-expression strains, the soybean plants in which *GmDRR1* was knocked down (in 2022) were considerably less resistant to *Blastomyces* infections (Yu et al., 2022). By altering the coding region of the soybean transcription factor gene *GmTCP19L*, Fan et al. (2022) produced a mutant with a 2 bp deletion. This mutant soybean germplasm resource exhibited increased susceptibility to blast molds (Fan et al., 2022).

Plants that are resistant to *Rps* gene products can perceive certain pathogen effectors encoded by Avr genes. By deleting *Avr45a*, Arsenault-Labrecque et al. (2022) produced novel soybean plants resistant to *Rps8* (Arsenault-Labrecque et al., 2022). In 2022, Zhang et al. identified *Glyma.07g110300* (LOC100775351) as a quantitative trait locus (QTL)-M marker gene encoding the UDP-glycosyltransferase (UGT) primarily responsible for soybean resistance to leaf-chewing insects. Using a CRISPR/Cas9 system, they enhanced the resistance of soybean to *Helicoverpa armigera* and *Spodoptera litura* via the following two mutation types: large fragment deletion and single base insertion. Zhang Y.X., et al. (2022) confirmed that *GmUGT* confers resistance to leaf-chewing insects by changing the flavonoid content and the expression of genes related to flavonoid biosynthesis and defense (Zhang Y.X., et al., 2022). By editing the soybean 14-3-3 gene (*Glyma05g29080*) via large fragment insertions and deletions and producing transgenic plants with increased susceptibility to hard tick infestations and decreased nodulation, Zhang Y.F., et al. (2023) showed *Glyma05g29080* contributes to nodulation and defense responses (Zhang Y.F., et al., 2023). Using a CRISPR/Cas9 gene editing method, Liu et al. (2023b) silenced *GmTAP1* in soybean, which resulted in increased resistance to *P. sojae* strains P231, P233, and P234. An analysis of reactive oxygen species revealed that a loss-of-function mutation to *GmTAP1* does not substantially alter plant basal immunity (Liu T.F., et al., 2023).

The soybean cyst nematode (SCN) is responsible for the soybean disease associated with the largest economic losses (Bent, 2022). By altering two functional genes (*Glyma.12G194800* and *Glyma.16G154200*) in the syntaxin family of SCN resistance genes, Dong et al. (2020) produced SCN-resistant soybean cultivars (Dong et al., 2020). In 2021, Butler et al. demonstrated that *Glyma.15G191200* of cqSCN-006, which encodes gamma-SNAP, influences SCN resistance. Additionally, using CRISPR/Cas9 gene editing technology to disrupt the cqSCN-006 allele decreased the SCN resistance of the transgenic roots (Butler et al., 2021). In 2022, Zhang et al. mutated *Glyma.07g110300* by introducing a CRISPR/Cas9 expression vector into the Tianlong 1 soybean variety to increase the resistance to *S. litura* and *H. armigera* (Zhang Y.X., et al., 2022).

2.3 Improvement of seed quality in soybean

Soybean is used as a source of food for animals, including humans (Medic et al., 2014). It has the highest protein content of any crop and is a significant source of edible oils (Gupta and Manjaya, 2022; Zaaboul et al., 2022; Song et al., 2023). In the past few years, several studies have employed CRISPR/Cas9 gene editing technology to enhance the protein and oleic acid contents of soybean.

Using germinal root transformation technology, Li et al. altered the soybean seed storage protein-encoding genes *Glyma.20g148400*, *Glyma.03g163500*, and *Glyma.19g164900* to increase soybean seed protein contents (Li et al., 2019a). By simultaneously modifying the soybean genes *GmFAD2-1A* and *GmFAD2-1B*, Do et al. (2019) managed to increase the oleic acid content by more than 80%, while also decreasing the linoleic acid level by 1.3%–1.7% (Do et al., 2019). Zhang et al. (2019) silenced the soybean phospholipase *D1*-encoding gene, which increased the oil content and germination rate of the mutant seeds (compared with the wild-type seeds) at high temperatures and high humidity levels (Zhang et al., 2019). In 2021, Qu et al. analyzed the oleic acid contents of soybean plants over-expressing *Gm15G117700* and soybean plants in which the gene was edited; the oleic acid content increased in the gene-edited plants by 3.49% (Qu et al., 2021). Zhou et al. (2023a) recently edited five important enzyme-encoding genes in the *GmFAD2* family and analyzed the associated effects on soybean oil synthesis. Editing *GmFAD2-1A* increased the oleic acid content by 91.49% (Zhou et al., 2023a). In another recent study, Li et al. (2023) edited two target genes by altering the conserved PAP2 structural domain-encoding sequences of *GmPDCT1* and *GmPDCT2*. The decrease in phosphatidylcholine-derived diacylglycerol contents via the knockdown of *GmPDCT* prevented the entry of phosphatidylcholine-modified polyunsaturated fatty acids into the triacylglycerol biosynthesis pathway (Li et al., 2023b).

In addition to increasing the protein and oleic acid contents, researchers have attempted to enhance other soybean characteristics. Phytic acid (PA) is an anti-nutrient in grains that prevents humans from absorbing trace minerals (e.g., iron and zinc). In soybean, *GmIPK1* encodes an enzyme that converts inositol 1,3,4,5,6-pentaphosphate to inositol 1,2,3,4,5,6-hexaphosphate (Alkarawi and Zotz, 2014; Sarkhel and Roy, 2022). Using the CRISPR/Cas9 system, Song et al. (2022) edited the *GmIPK1* gene and sgRNA to introduce mutations to create soybean lines with low PA levels. The decreased PA levels in the T_2 generation mutant seeds were not accompanied by defective growth or seed development (Song et al., 2022).

Flavor is an important soybean quality-related attribute. Accordingly, CRISPR/Cas9 technology has been exploited to develop soybean germplasm with superior flavor-related traits (Fernandez-Marin et al., 2014). Because soybean proteins are allergens, decreasing the abundance of allergenic proteins will likely increase the utility of soybean as a source of protein (e.g., in processed food) (Cordle, 2004; L'Hocine and Boye, 2007; Gharibzahedi et al., 2022; Gracio et al., 2023). In 2020, Sugano et al. simultaneously targeted and edited *GmBd28k* and *GmBd30K* to eliminate two allergenic proteins in the Japanese soybean cultivars Enrei and Kariyutaka (Sugano et al., 2020). Soybean flavor and quality are influenced by three lipoxygenases (LOX1, LOX2, and LOX3). By editing three genes in the soybean *GmLox* family (*GmLox1*, *GmLox2*, and *GmLox3*), Wang J., et al. (2020) improved the edibility of soybean oil and protein products. Editing these genes decreased soybean odors (Wang J., et al., 2020). The raffinose oligosaccharide (RFO) family members are the main soluble carbohydrates in soybean seeds, but they are anti-nutritional seed components because they typically cause gas and indigestion, while also decreasing energy efficiency (Salvi et al., 2022). In 2021, Le et al. decreased the soybean seed RFO content by knocking down two galactinol synthase-encoding genes, namely *GmGOLS1A* and its homolog *GmGOLS1B* (Le et al., 2020). To decrease the RFO content in mature seeds, Cao et al. (2022) used a CRISPR/Cas9 multi-gene editing method to delete the *RS2* and *RS3* genes in soybean and cottonseed (Cao et al., 2022). Qian et al. (2022) mutated *GmBADH2* and confirmed this gene contributes to soybean odors (Qian et al., 2022). In addition, Bai et al. (2022) used CRISPR/Cas9 gene editing technology to produce two multi-gene mutants, one lacking the 7S subunit and the other lacking the 11S subunit. Both of these mutations enhanced the flavor of soybean meal (Bai et al., 2022).

2.4 Improvement of phenotype in soybean

One of the key factors influencing the development of high-yielding soybean cultivars is the appropriate regulation of plant structural features (e.g., plant height, number of nodes, number of pods, internode length, number of branches, and number of grains) (Hu and Wiatrak, 2012; Kuzbakova et al., 2022). In recent years, soybean phenotype-related genes have been edited using CRISPR/Cas9 gene editing technology to produce soybean germplasm with a variety of improved features.

Using the CRISPR/Cas9 system, Bao et al. (2019) mutated four *SPL9* family genes that encode *SQUAMOSA* promoter-binding protein-like (SPL) transcription factors. The higher-order mutant plants with different combinations of mutations had more nodes and branches on the main stem (compared with the control plants),

resulting in varying numbers of nodes per plant (Bao et al., 2019). In 2019, Cheng et al. used four gRNAs to alter four late elongated hypocotyl (LHY)-encoding *GmLHY* genes in soybean. Phenotypic analyses showed that the quadruple mutant plants had relatively short internodes and exhibited dwarfism (Cheng et al., 2019). In the Tianlong 9 variety, Jia et al. (2020) knocked out two copies of the soybean *DCL2* gene, which altered the color of the soybean seed coat from yellow to brown (Jia et al., 2020). To increase soybean production, Cai et al. (2021) modified the low-latitude spring soybean variety Huachun 6 using a CRISPR/Cas9 multi-gene editing technique. Specifically, they targeted *GmJAG*, which affects the number of seeds per pod (Cai et al., 2021). In 2022, Mu et al. targeted six *GmBIC* genes in soybean using CRISPR/Cas9 technology. The single, double, and quadruple mutants were shorter than normal (Mu et al., 2022). In another recent study, Zhong et al. (2022) edited the soybean *GmHdz4* gene, which increased the total root length, root surface area, and number of root tips (compared with the mutant lines over-expressing *GmHdz4*) (Zhong et al., 2022). Furthermore, Zhang Z. et al. (2023) silenced the soybean *GmNSS* gene, which resulted in the production of abnormally small seeds. (Zhang Z. et al., 2023).

Abscisic acid is an essential phytohormone that controls various processes related to plant growth, development, and stress responses (Nguyen et al., 2023). Using a CRISPR/Cas9 system, Zhang Z. H. et al. (2022) mutated *GmPYL17*, *GmPYL18*, and *GmPYL19*. Compared with the wild-type plants, the mutants were taller, had more branches, and were less sensitive to abscisic acid during the seed germination stage (Zhang Z. H. et al., 2022).

The shattering of soybean pods can significantly decrease yield. By altering the *GmPDH* gene family in soybean variety Huachun 6, Zhang Z. et al. (2022) showed that the *PDH1* mutation dramatically increases pod shatter resistance without modifying other important agronomic parameters (Zhang Z. et al., 2022).

2.5 Regulation of nitrogen fixation by nodules

Rhizobia can produce a symbiotic nitrogen-fixation system with legumes that increases plant output without damaging the local ecosystem (Chakraborty et al., 2022; Hawkins and Oresnik, 2022). More than 65% of the nitrogen fixation is due to the symbiotic interaction between rhizobia and legumes (Fields et al., 2021; Jimenez-Guerrero et al., 2022). Soybean converts free nitrogen in the air to chemosynthetic nitrogen that can be absorbed and used by the plant via nitrogen-fixing nodules. This process yields soybean seeds with a high protein content, thereby increasing the nutritional value of soybean (Dadnia, 2011; Meng et al., 2015; Igiehon et al., 2021).

Xu et al. (2021) promoted soybean nodulation by using CRISPR/Cas9 technology to knock down miR9c (Xu et al., 2021). By deleting the soybean *RFG193* gene, Fan et al. (2020) generated transgenic plants with mature nitrogen-fixing nodules on purple or red roots, which produced anthocyanins, whereas nodules were undetectable on the non-transgenic roots (Fan et al., 2020). In 2021, Yang et al. reported that a loss-of-function mutation to *GmHSP17.9* significantly affects soybean plant growth and seed yield through the associated changes to the number of root nodules, nodule fresh weight, nitrogenase activity, poly-hydroxybutyrate vesicles, and urea and total nitrogen contents (Yang Z.W., et al., 2022). Nguyen et al. (2021) silenced *GmUOX* in a soybean mutant, which exhibited nitrogen deficit atrophy and early nodule senescence as revealed by decreased nitrogenase (acetylene reduction) activities in the nodules, a greenish-white hue inside the nodules, and a decreased root protein output (Nguyen et al., 2021). Gao et al. (2021) investigated the role of the PIN protein during the nitrogen fixation by soybean nodules. More specifically, they produced a triple mutant (*GmPIN1*-abc family) (Gao et al., 2021). The modification of the soybean *Rfg1* allele by Fan et al. (2022) revealed *Rfg1* mediates the resistance to *Sinorhizobium fredii* and *Bradyrhizobium japonicum* strains, leading to broad-spectrum resistance to nodulation in transgenic plants (Fan et al., 2017). After knocking down *GmNN1*, Li et al. (2022) detected yellowing leaves as well as decreased nitrogen contents and decreased nodulation (compared with the wild-type control plants) (Li et al., 2022). By silencing *GmNAC039* and *GmNAC018* as well as the four target genes *GmCYP35*, *GmCYP37*, *GmCYP39*, and *GmCYP4*, Yu et al. (2023) showed that the transcription factors encoded by *GmNAC039* and *GmNAC018* directly increase the expression of *GmCYP* genes to induce root tumor senescence (Yu et al., 2023).

2.6 Regulation of flowering time in soybean

Because soybean is a short-day (SD) plant, it blooms more quickly during SD conditions than during long-day (LD) conditions (Weller and Ortega, 2015; Lin et al., 2021; Xia et al., 2021). Modulating the blooming time and minimizing the sensitivity to sunshine duration through molecular breeding can increase soybean adaptability and production by mitigating photoperiodic responses (Zhang L.X. et al., 2020; Zhang M. et al., 2022; Du et al., 2023).

Cai et al. (2018a) edited the soybean genes *GmFT2a* and *GmFT9a* and discovered that both mutants in the T_2 generation exhibited a late-blooming phenotype (Cai et al., 2018a). Using a double sgRNA design and CRISPR/Cas9 technology, Cai et al. (2018b) deleted specific DNA fragments in *GmFT2a (Glyma16g26660)* and *GmFT5a (Glyma16g04830)*. The homozygous *GmFT2a* mutants (1,618 bp deletion) in the T_2 generation flowered late (Cai et al., 2018b). Two QTL regions that respectively included *GmFT2a* and *GmFT5a* were identified by Cai et al. (2020b). They were linked to various genetic effects on flowering during various photoperiods. Under LD and SD conditions, the flowering times of transgenic plants over-expressing *GmFT2a* or *GmFT5a*, *GmFT2a* mutants, *GmFT5a* mutants, and *GmFT2a* and *GmFT5a* double mutants were examined. There was no overlap between *GmFT2a* and *GmFT5a*, which cooperatively control the blooming time, but *GmFT2a* has a greater effect than *GmFT5a* under SD conditions, while *GmFT5a* has a greater effect than *GmFT2a* under LD conditions (Cai et al., 2020a). Wang L. W. et al. (2020) mapped QTLs and identified

GmPRR37 as a functional gene encoding a regulator of soybean flowering. A natural mutation to *GmPRR37* results in early flowering, thereby enabling the cultivation of soybean plants at high latitudes (Wang L. W., et al., 2020). Li et al. (2020) used CRISPR/Cas9 technology to knock out *GmPRR3b*. The resulting soybean mutant exhibited retarded growth and a delayed transition to the flowering stage (Li et al., 2020). In 2020, Chen et al. modified the soybean *GmAP1* gene in a quadruple mutant. The observed increase in plant height was associated with delayed flowering, altered flower shapes, and increases in the number of nodes and the internode length. In contrast, under SD conditions, the over-expression of *GmAP1* led to early flowering and dwarfism (Chen et al., 2020). Li et al. (2021) edited four *LNK2* genes using a CRISPR/Cas9 system to produce a quadruple mutant lacking transgenes. This mutant flowered earlier than the wild-type control under LD conditions. In addition, the *LNK2* transcript level was lower in the quadruple mutant than in the wild-type plants (Li et al., 2021). Zhao et al. (2022) mutated *GmPHYA* or *GmPHYB* using CRISPR/Cas9 technology. The phenotypic changes due to the mutations to *GmPHYA2* and *GmPHYA3*, which have redundant and additive roles in seedling responses to daylight, indicated *GmPHYB1* is primarily responsible for daylight-induced photomorphogenesis (Zhao et al., 2022). In 2022, Zhai et al. suggested that *GmMDE* and *GmFT2a*/*GmFT5a* contribute to a positive feedback regulatory loop that promotes flowering in soybean. Knocking down the soybean *E1* gene induces *GmMDE* expression. Moreover, the over-expression of *GmMDE06* increases the expression of *GmFT2a* and *GmFT5a*, which regulate flowering (Zhai et al., 2022). In 2023, Wan et al. investigated the relationship between the dominant *E1* gene and photoperiodic regulation via the CRISPR/Cas9-mediated targeted mutation of *E1* in soybean variety Tianlong 1. Four mutations were introduced into the *E1* coding region. The significant structural changes in the generated mutants included the commencement of terminal flowering, the creation of distinct stems, and a decrease in the number of branches (Wan et al., 2022).

2.7 Creation of male sterile soybean germplasm resources

Because soybean is a self-pollinated plant that has small flower organs, artificial cross-breeding is both difficult and ineffective (Li et al., 2019b; Chen G. M., et al., 2021). Furthermore, differences in flowering times among varieties originating from various geographical regions frequently further restrict the exchange of genes, resulting in a limited genetic base for soybean breeding and genetic modifications (Li et al., 2019b). Accordingly, methods for increasing the genetic diversity of soybean varieties are needed (Bohra et al., 2016). In particular, for sexually reproducing crops, male sterility is a crucial precondition for hybrid seed generation and crop reproduction (Jiang et al., 2011; Yang et al., 2014). Male sterile lines can increase the quality of hybrids, lower the cost of hybrid seed production, and even broaden the utility of hybrids. The scarcity of adequate male sterile lines has limited the commercial use of soybean accessions (Li et al., 2016; Ramlal et al., 2022).

To create stable male sterile soybean lines, Chen et al. (2021) targeted *AMS* homologs using CRISPR/Cas9 technology. Although editing *GmAMS2* failed to produce a male sterile line, editing *GmAMS1* yielded plants with a male sterile phenotype. *GmAMS1* contributes to the development of pollen walls as well as the regulation of soybean tapetum degeneration (Chen et al., 2021a). Jiang et al. (2021) modified *Glyma.13G114200* using a CRISPR/Cas9 system; the phenotypes of two gene-edited lines were consistent with the male sterility of the *MS1* mutant (Jiang et al., 2021). By eliminating *GmSPL2b*, Ding et al. (2023) decreased the heat tolerance of a soybean cytoplasmic male sterility-based recovery line during flowering (Ding et al., 2023).

2.8 Application of other CRISPR gene editing technology in soybean

Compared with Cas9, the CRISPR family member Cas12a is more practical and effective. Hence, CRISPR/Cas12a can effectively edit multiple genes because of the specific way that CRISPR RNA (crRNA) functions (Bandyopadhyay et al., 2020; Paul and Montoya, 2020; Zhou et al., 2023b). In 2017, Jiang et al. used CRISPR/Cas12a to achieve editing in the soybean *FAD2* gene for the first time (Jiang et al., 2017). In addition, large chromosomal segments of the target genome were deleted by Duan et al. (2021) using CRISPR/Cas12a, with an editing efficiency of 91.7% (Duan et al., 2021). In 2023, Liang et al. produced CRISPR/Cas12a-edited soybeans in just 45 days, with transformation and gene editing efficiencies of 30% and 50%, respectively (Liang et al., 2023). To produce gene-edited soybeans with better traits, CRISPR/Cas12a-based multi-gene editing methods will increasingly be used to modify the soybean genome.

Because they enable the replacement of a single base via RNA editing without introducing DNA double-strand breaks or requiring donor templates, base editor tools created using the CRISPR/Cas9 system are especially useful for plant molecular breeding (Molla et al., 2021; Yang et al., 2021; Hua et al., 2022). A CRISPR/Cas9-mediated base editing tool was designed by Cai et al. (2020a) to alter individual bases in the soybean genome. A base editor was developed by combining Cas9n (D10A), rat cytosine deaminase (APOBEC1), and a uracil glycosylase inhibitor. This base editor was then cloned into the pTF101.1 vector. The targeted genes were *GmFT2a* and *GmFT4a*, which were under the control of the 2× CaMV 35S promoter. There were two types of base substitutions (C to T and C to G), both of which occurred within the target sequence (Cai et al., 2020a). Single nucleotide polymorphisms, which influence phenotypic diversity and are linked to many significant agronomic parameters, are abundant in the soybean genome. Future genetic improvement and breeding of soybean can greatly benefit from the application of base editing technology (Bharat et al., 2020; Xu R. F., et al., 2020).

3 Discussion and prospect

Because of increases in the global population and living standards, CRISPR/Cas9 technology must be exploited to quickly develop high-yielding, high-quality soybean varieties (Khan et al., 2018; Zhang and Showalter, 2020). Field tests of high-oleic soybean varieties produced using CRISPR/Cas9 gene editing technology in the US have produced positive results, with potential implications for soybean molecular breeding. There have been considerable advances in the molecular breeding of soybean since the development of CRISPR/Cas9 gene editing technology, which has decreased concerns about the safety of products made from genetically modified soybeans, leading to the gradual acceptance of genetically modified crops. The CRISPR/Cas9 system, which continues to be refined and enhanced, has largely outperformed the older technologies involving zinc finger nucleases and transcription activator-like effector nucleases in terms of gene editing efficiency and convenience (Samanta et al., 2016; Demirci et al., 2018; Farooq et al., 2018). Researchers will use CRISPR/Cas9 gene editing systems to develop soybean lines with improved features as more functional soybean genes are identified and characterized.

However, there are certain limitations to the utility of CRISPR/Cas9 for soybean breeding. Unanswered questions include the following: (i) How can genome editing tools be efficiently delivered to soybean plants? (ii) How can the functional redundancy in gene families be rapidly and precisely determined? (iii) How can the editing of multiple genes be exploited to modify various traits? (iv) How can base editing, prime editing, and government regulations regarding genome-edited crops further increase the effectiveness of gene editing? Despite encouraging results, many obstacles must be overcome before CRISPR/Cas9 can be widely used for soybean breeding.

Additionally, numerous sgRNAs for different plant genomes have been assembled into CRISPR editing vectors. Moreover, sgRNA pooling techniques have made it possible to mutate multiple genes. The diversity in the sequences that PAM can detect has increased, leading to improved gene editing, because of the creation of Cas9 homologs, such as StCas9 and SaCas9, for plant molecular breeding. The highly efficient editing of plant genomes has been achieved using the nCas9-mediated single-base editing system, while the saturation mutagenesis of plant genomes and optimal gene editing efficiencies have been attained via the two-base editing method. The CRISPR/Cas9 gene editing method will be applied to soybean molecular breeding more effectively, conveniently, and broadly in the future, thereby facilitating increasingly precise molecular breeding and accelerating soybean molecular breeding.

Author contributions

DY and JZ performed the manuscript writing; AZ, JW, YL, LW, WP, ZL summarized the literature reports; WY and JC carried out the production of pictures; HL performed the organization of the table; WH and XQ reviewed and proofread the manuscript. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Key Research and Development Program of Science and the Technology of Jilin Province (No. 20210202006NC), and the Key Laboratory of Crop Genetic Resources and Germplasm Creation in Northeast China, Ministry of Agriculture and Rural Affairs (KYJF2023DX015-3).

Acknowledgments

We thank Liwen Bianji (Edanz) (www.liwenbianji.cn) for editing the English text of a draft of this manuscript.

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.

Publisher's note

References

Alkarawi, H. H., and Zotz, G. (2014). Phytic acid in green leaves. *Plant Biol.* 16, 697–701. doi: 10.1111/plb.12136

Amoanimaa-Dede, H., Su, C. T., Yeboah, A., Zhou, H., Zheng, D., and Zhu, H. (2022). Growth regulators promote soybean productivity: a re1 view. *Peerj* 10, e12556. doi: 10.7717/peerj.12556

Arsenault-Labrecque, G., Santhanam, P., Asselin, Y., Cinget, B., Lebreton, A., Labbé, C., et al. (2022). Rxlr effector gene avr3a from *Phytophthora Sojae* is recognized by rps8 in soybean. *Mol. Plant Pathol.* 23, 693–706. doi: 10.1111/mpp.13190

Baek, D., Chun, H. J., and Kim, M. C. (2022). Genome editing provides a valuable biological toolkit for soybean improvement. *Plant Biotechnol. Rep.* 16, 357–368. doi: 10.1007/s11816-022-00778-6

Bai, M. Y., Yuan, C. C., Kuang, H. Q., Sun, Q., Hu, X. C., Cui, L. N., et al. (2022). Combination of two multiplex genome-edited soybean varieties enables customization of protein functional properties. *Mol. Plant* 15, 1081–1083. doi: 10.1016/j.molp.2022.05.011

Bandyopadhyay, A., Kancharla, N., Javalkote, V. S., Dasgupta, S., and Brutnell, T. P. (2020). CRISPR/Cas12a (cpf1): a versatile tool in the plant genome editing toolbox for agricultural advancement. *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.584151

Bao, A. L., Chen, H. F., Chen, L. M., Chen, S. L., Hao, Q. N., Guo, W., et al. (2019). CRISPR/Cas9-mediated targeted mutagenesis of gmspl9 genes alters plant architecture in soybean. *BMC Plant Biol.* 19 (1), 131. doi: 10.1186/s12870-019-1746-6

Bao, A., Tran, L. P., and Cao, D. (2020). CRISPR/Cas9-based gene editing in soybean. *Methods Mol. Biol. (Clifton N.J.)* 2107, 349–364. doi: 10.1007/978-1-0716-0235-5_19

Bent, A. F. (2022). Exploring soybean resistance to soybean cyst nematode. *Annu. Rev. Phytopathol.* 60, 379–409. doi: 10.1146/annurev-phyto-020620-120823

Bharat, S. S., Li, S. Y., Li, J. Y., Yan, L., and Xia, L. Q. (2020). Base editing in plants: current status and challenges. *Crop J.* 8, 384–395. doi: 10.1016/j.cj.2019.10.002

Bohra, A., Jha, U. C., Adhimoolam, P., Bisht, D., and Singh, N. P. (2016). Cytoplasmic male sterility (cms) in hybrid breeding in field crops. *Plant Cell Rep.* 35, 967–993. doi: 10.1007/s00299-016-1949-3

Butler, K. J., Fliege, C., Zapotocny, R., Diers, B., Hudson, M., Bent, A. F., et al. (2021). Soybean cyst nematode resistance quantitative trait locus cqscn-006 alters the expression of a gamma-snap protein. *Mol. Plant-Microbe Interact.* 34, 1433–1445. doi: 10.1094/MPMI-07-21-0163-R

Cadavid, I. C., Balbinott, N., and Margis, R. (2023). Beyond transcription factors: more regulatory layers affecting soybean gene expression under abiotic stress. *Genet. Mol. Biol.* 46, e20220166. doi: 10.1590/1678-4685-GMB-2022-0166

Cai, Y. P., Chen, L., Liu, X. J., Guo, C., Sun, S., Wu, C. X., et al. (2018a). CRISPR/Cas9-mediated targeted mutagenesis of gmft2a delays flowering time in soya bean. *Plant Biotechnol. J.* 16, 176–185. doi: 10.1111/pbi.12758

Cai, Y. P., Chen, L., Sun, S., Wu, C. X., Yao, W. W., Jiang, B. J., et al. (2018b). CRISPR/Cas9-mediated deletion of large genomic fragments in soybean. *Int. J. Mol. Sci.* 19 (12), 3835. doi: 10.3390/ijms19123835

Cai, Y. P., Chen, L., Zhang, Y., Yuan, S., Su, Q., Sun, S., et al. (2020a). Target base editing in soybean using a modified CRISPR/Cas9 system. *Plant Biotechnol. J.* 18, 1996–1998. doi: 10.1111/pbi.13386

Cai, X. X., Jia, B. W., Sun, M. Z., and Sun, X. L. (2022). Insights into the regulation of wild soybean tolerance to salt-alkaline stress. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.1002302

Cai, Y. P., Wang, L. W., Chen, L., Wu, T. T., Liu, L. P., Sun, S., et al. (2020b). Mutagenesis of *GmFT2a* and *GmFT5a* mediated by CRISPR/Cas9 contributes for expanding the regional adaptability of soybean. *Plant Biotechnol. J.* 18, 298–309. doi: 10.1111/pbi.13199

Cai, Z. D., Xian, P. Q., Cheng, Y. B., Ma, Q. B., Lian, T. X., Nian, H., et al. (2021). CRISPR/Cas9-mediated gene editing of *GmJAGGED1* increased yield in the low-latitude soybean variety huachun 6. *Plant Biotechnol. J.* 19, 1898–1900. doi: 10.1111/pbi.13673

Cao, L., Wang, Z. R., Ma, H. Y., Liu, T. F., Ji, J., Duan, K. X., et al. (2022). Multiplex CRISPR/Cas9-mediated raffinose synthase gene editing reduces raffinose family oligosaccharides in soybean. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.1048967

Chakraborty, S., Valdes-Lopez, O., Stonoha-Arther, C., and Ane, J. M. (2022). Transcription factors controlling the rhizobium-legume symbiosis: integrating infection, organogenesis and the abiotic environment. *Plant Cell Physiol.* 63, 1326–1343. doi: 10.1093/pcp/pcac063

Chandra, S., Choudhary, M., Bagaria, P. K., Nataraj, V., Kumawat, G., Choudhary, J. R., et al. (2022). Progress and prospectus in genetics and genomics of phytophthora root and stem rot resistance in soybean (*Glycine max* L.). *Front. Genet.* 13, 939182. doi: 10.3389/fgene.2022.939182

Chang, H. X., Lipka, A. E., Domier, L. L., and Hartman, G. L. (2016). Characterization of disease resistance loci in the usda soybean germplasm collection using genome-wide association studies. *Phytopathology* 106, 1139–1151. doi: 10.1094/PHYTO-01-16-0042-FI

Chen, Y. Z., Fu, M. C., Li, H., Wang, L. G., Liu, R. Z., Liu, Z. J., et al. (2021b). High-oleic acid content, nontransgenic allotetraploid cotton (*Gossypium hirsutum* L.) Generated by knockout of *GhFAD2* genes with CRISPR/Cas9 system. *Plant Biotechnol. J.* 19, 424–426. doi: 10.1111/pbi.13507

Chen, J. L., Li, S. Y., He, Y. B., Li, J. Y., and Xia, L. Q. (2022). An update on precision genome editing by homology-directed repair in plants. *Plant Physiol.* 188, 1780–1794. doi: 10.1093/plphys/kiac037

Chen, H. T., Liu, X. Q., Zhang, H. M., Yuan, X. X., Gu, H. P., Cui, X. Y., et al. (2018). Advances in salinity tolerance of soybean: genetic diversity, heredity, and gene identification contribute to improving salinity tolerance. *J. Integr. Agric.* 17, 2215–2221. doi: 10.1016/S2095-3119(17)61864-1

Chen, L. Y., Nan, H. Y., Kong, L. P., Yue, L., Yang, H., Zhao, Q. S., et al. (2020). Soybeanap1homologs control flowering time and plant height. *J. Integr. Plant Biol.* 62, 1868–1879. doi: 10.1111/jipb.12988

Chen, X., Yang, S. X., Zhang, Y. H., Zhu, X. B., Yang, X. J., Zhang, C. B., et al. (2021a). Generation of male-sterile soybean lines with the CRISPR/Cas9 system. *Crop J.* 9, 1270–1277. doi: 10.1016/j.cj.2021.05.003

Chen, H. M., Ye, R., Liang, Y., Zhang, S. C., Liu, X. L., Sun, C. J., et al. (2023). Generation of low-cadmium rice germplasms via knockout of oslcd using CRISPR/Cas9. *J. Environ. Sci.* 126, 138–152. doi: 10.1016/j.jes.2022.05.047

Chen, C., Zhao, Y. Q., Tabor, G., Nian, H. Q., Phillips, J., Wolters, P., et al. (2023). A leucine-rich repeat receptor kinase gene confers quantitative susceptibility to maize southern leaf blight. *New Phytol.* 238, 1182–1197. doi: 10.1111/nph.18781

Chen, G. M., Zhou, Y. Z., Kishchenko, O., Stepanenko, A., Jatayev, S., Zhang, D. B., et al. (2021). Gene editing to facilitate hybrid crop production. *Biotechnol. Adv.* 46, 107676. doi: 10.1016/j.biotechadv.2020.107676

Cheng, Q., Dong, L. D., Su, T., Li, T. Y., Gan, Z. R., Nan, H. Y., et al. (2019). CRISPR/Cas9-mediated targeted mutagenesis of gmlhy genes alters plant height and internode length in soybean. *BMC Plant Biol.* 19 (1), 562. doi: 10.1186/s12870-019-2145-8

Chin, H. S., Wu, Y. P., Hour, A. L., Hong, C. Y., and Lin, Y. R. (2016). Genetic and evolutionary analysis of purple leaf sheath in rice. *Rice* 9 (1), 8. doi: 10.1186/s12284-016-0080-y

Cordle, C. T. (2004). Soy protein allergy: incidence and relative severity. *J. Nutr.* 134, 1213S–1219S. doi: 10.1093/jn/134.5.1213S

Dadnia, M. R. (2011). Effect of different formulations of rhizobium japonicum on yield and yield attributes of soybean (*Glycine max* L.). *Res. On Crops* 12, 413–416.

Demirci, Y., Zhang, B. H., and Unver, T. (2018). CRISPR/Cas9: an rna-guided highly precise synthetic tool for plant genome editing. *J. Cell. Physiol.* 233, 1844–1859. doi: 10.1002/jcp.25970

Deshmukh, R., Sonah, H., Patil, G., Chen, W., Prince, S., Mutava, R., et al. (2014). Integrating omic approaches for abiotic stress tolerance in soybean. *Front. Plant Sci.* 5, 244. doi: 10.3389/fpls.2014.00244

Ding, X. L., Guo, J. F., Lv, M. L., Wang, H. J., Sheng, Y., Liu, Y., et al. (2023). The mi R156b-*GmSPL2b* module mediates male fertility regulation of cytoplasmic male sterility-based restorer line under high-temperature stress in soybean. *Plant Biotechnol. J.* 21 (8), 1542–1559. doi: 10.1111/pbi.14056

Do, P. T., Nguyen, C. X., Bui, H. T., Tran, L., Stacey, G., Gillman, J. D., et al. (2019). Demonstration of highly efficient dual gRNA CRISPR/Cas9 editing of the homeologous *GmFAD2-1A* and *GmFAD2-1B* genes to yield a high oleic, low linoleic and alpha-linolenic acid phenotype in soybean. *BMC Plant Biol.* 19 (1), 311. doi: 10.1186/s12870-019-1906-8

Dong, J., Zielinski, R. E., and Hudson, M. E. (2020). T-SNAREs bind the Rhg1 alpha-SNAP and mediate soybean cyst nematode resistance. *Plant J.* 104, 318–331. doi: 10.1111/tpj.14923

Du, H. P., Fang, C., Li, Y. R., Kong, F. J., and Liu, B. H. (2023). Understandings and future challenges in soybean functional genomics and molecular breeding. *J. Integr. Plant Biol.* 65, 468–495. doi: 10.1111/jipb.13433

Du, Y. T., Zhao, M. J., Wang, C. T., Gao, Y., Wang, Y. X., Liu, Y. W., et al. (2018). Identification and characterization of *GmMYB118* responses to drought and salt stress. *BMC Plant Biol.* 18 (1), 320. doi: 10.1186/s12870-018-1551-7

Duan, N. N., Tang, S. Q., Zeng, B. T., Hu, Z. Q., Hu, Q., Wu, L. Q., et al. (2021). An episomal CRISPR/Cas12a system for mediating efficient gene editing. *Life-Basel* 11 (11), 1262. doi: 10.3390/life11111262

Errum, A., Rehman, N., Uzair, M., Inam, S., Ali, G. M., Khan, M. R., et al. (2023). CRISPR/Cas9 editing of wheat PPD-1 gene homoeologs alters spike architecture and grain morphometric traits. *Funct. Integr. Genomics* 23 (1), 66. doi: 10.1007/s10142-023-00989-2

Fan, Y. L., Liu, J., Lyu, S. H., Wang, Q., Yang, S. M., Zhu, H. Y., et al. (2017). The soybean Rfg1 gene restricts nodulation by sinorhizobium fredii usda193. *Front. Plant Sci.* 8, 1548. doi: 10.3389/fpls.2017.01548

Fan, Y., Wang, X., Li, H., et al. (2020). Anthocyanin, a novel and user-friendly reporter for convenient, non-destructive, low cost, directly visual selection of transgenic hairy roots in the study of rhizobia-legume symbiosis. *Plant Methods.* 16, 94. doi: 10.1186/s13007-020-00638-w

Fan, S. J., Zhang, Z., Song, Y., Zhang, J., and Wang, P. W. (2022). CRISPR/Cas9-mediated targeted mutagenesis of *GmTCP19L* increasing susceptibility to *Phytophthora Sojae* in soybean. *PloS One* 17 (6), e0267502. doi: 10.1371/journal.pone.0267502

Fang, X. L., Sun, Y. Y., Li, J. H., Li, M. A., and Zhang, C. B. (2023). Male sterility and hybrid breeding in soybean. *Mol. Breed.* 43 (6), 47. doi: 10.1007/s11032-023-01390-4

Fang, Y. F., and Tyler, B. M. (2016). Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora Sojae* using CRISPR/Cas9. *Mol. Plant Pathol.* 17, 127–139. doi: 10.1111/mpp.12318

Farooq, R., Hussain, K., Nazir, S., Javed, M. R., and Masood, N. (2018). CRISPR/Cas9; A robust technology for producing genetically engineered plants. *Cell. Mol. Biol.* 64, 31–38. doi: 10.14715/cmb/2018.64.14.6

Feng, C., Gao, H. T., Zhou, Y. G., Jing, Y., Li, S. Q., Yan, Z., et al. (2023). Unfolding molecular switches for salt stress resilience in soybean: recent advances and prospects for salt-tolerant smart plant production. *Front. Plant Sci.* 14, 1162014. doi: 10.3389/fpls.2023.1162014

Fernandez-Marin, B., Milla, R., Martin-Robles, N., Arc, E., Kranner, I., Becerril, J. M., et al. (2014). Side-effects of domestication: cultivated legume seeds contain similar tocopherols and fatty acids but less carotenoids than their wild counterparts. *BMC Plant Biol.* 14, 1599. doi: 10.1186/s12870-014-0385-1

Fields, B., Moffat, E. K., Friman, V. P., and Harrison, E. (2021). The impact of intra-specific diversity in the rhizobia-legume symbiosis. *Microbiology-Sgm* 167 (4), 001051. doi: 10.1099/mic.0.001051

Gan, W. C., and Ling, A. P. K. (2022). CRISPR/Cas9 in plant biotechnology: applications and challenges. *Biotechnologia* 103, 81–93. doi: 10.5114/bta.2022.113919

Gao, Z., Chen, Z. W., Cui, Y. Y., Ke, M. Y., Xu, H. F., Xu, Q. Z., et al. (2021). Gmpin-dependent polar auxin transport is involved in soybean nodule development. *Plant Cell* 33, 2981–3003. doi: 10.1093/plcell/koab183

Gharibzahedi, S., Smith, B., and Altintas, Z. (2022). Bioactive and health-promoting properties of enzymatic hydrolysates of legume proteins: a review. *Crit. Rev. Food Sci. Nutr* 2022, 1–31. doi: 10.1080/10408398.2022.2124399

Gracio, M., Oliveira, S., Lima, A., and Ferreira, R. B. (2023). Rubisco as a protein source for potential food applications: a review. *Food Chem.* 419, 135993. doi: 10.1016/j.foodchem.2023.135993

Gu, B., Shao, G. D., Gao, W. X., Miao, J. Q., Wang, Q. H., Liu, X. L., et al. (2021). Transcriptional variability associated with CRISPR-mediated gene replacements at the *Phytophthora Sojae* avr1b-1 locus. *Front. Microbiol.* 12, 645331. doi: 10.3389/fmicb.2021.645331

Guan, J. N., Xie, Z. M., Rasheed, A., Wang, T. C., Qian, Z., Zhang, Z., et al. (2022). CRISPR/Cas9 applications for improvement of soybeans, current scenarios, and future perspectives. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 50, 12678. doi: 10.15835/nbha50212678

Gupta, S. K., and Manjaya, J. G. (2022). Advances in improvement of soybean seed composition traits using genetic, genomic and biotechnological approaches. *Euphytica* 99 (2022), 218. doi: 10.1007/s10681-022-03046-4

Hawkins, J. P., and Oresnik, I. J. (2022). The rhizobium-legume symbiosis: co-opting successful stress management. *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.796045

Hu, M. X., and Wiatrak, P. (2012). Effect of planting date on soybean growth, yield, and grain quality: review. *Agron. J.* 104, 785–790. doi: 10.2134/agronj2011.0382

Hua, K., Han, P. J., and Zhu, J. K. (2022). Improvement of base editors and prime editors advances precision genome engineering in plants. *Plant Physiol.* 188, 1795–1810. doi: 10.1093/plphys/kiab591

Huang, Q. L., Lin, B. R., Cao, Y. Q., Zhang, Y., Song, H. D., Huang, C. H., et al. (2023). CRISPR/Cas9-mediated mutagenesis of the susceptibility gene *OsHPP04* in rice confers enhanced resistance to rice root-knot nematode. *Front. Plant Sci.* 14, 1134653. doi: 10.3389/fpls.2023.1134653

Hussain, B., Lucas, S. J., and Budak, H. (2018). CRISPR/Cas9 in plants: at play in the genome and at work for crop improvement. *Briefings Funct. Genomics* 17, 319–328. doi: 10.1093/bfgp/ely016

Igiehon, N. O., Babalola, O. O., Cheseto, X., and Torto, B. (2021). Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress. *Microbiol Res.* 242, 126640. doi: 10.1016/j.micres.2020.126640

Impens, L., Jacobs, T. B., Nelissen, H., Inze, D., and Pauwels, L. (2022). Mini-review: transgenerational CRISPR/Cas9 gene editing in plants. *Front. Genome Editing* 4. doi: 10.3389/fgeed.2022.825042

Jia, J. B., Ji, R. H., Li, Z. W., Yu, Y. M., Nakano, M., Long, Y. P., et al. (2020). Soybean dicer-like2 regulates seed coat color via production of primary 22-nucleotide small interfering rnas from long inverted repeats. *Plant Cell* 32, 3662–3673. doi: 10.1105/tpc.20.00562

Jiang, B. J., Chen, L., Yang, C. Y., Wu, T. T., Yuan, S., Wu, C. X., et al. (2021). The cloning and CRISPR/Cas9-mediated mutagenesis of a male sterility gene ms1 of soybean. *Plant Biotechnol. J.* 19, 1098–1100. doi: 10.1111/pbi.13601

Jiang, W. Z., Henry, I. M., Lynagh, P. G., Comai, L., Cahoon, E. B., Weeks, D. P., et al. (2017). Significant enhancement of fatty acid composition in seeds of the allohexaploid, camelina sativa, using CRISPR/Cas9 gene editing. *Plant Biotechnol. J.* 15, 648–657. doi: 10.1111/pbi.12663

Jiang, W., Yang, S. P., Yu, D. Y., and Gai, J. Y. (2011). Cloning and characterization of a novel gene gmmf1 in soybean (*Glycine max* L. Merr.). *Agric. Sci. China* 10, 1834–1841. doi: 10.1016/S1671-2927(11)60183-1

Jimenez-Guerrero, I., Medina, C., Vinardell, J. M., Ollero, F. J., and Lopez-Baena, F. J. (2022). The rhizobial type 3 secretion system: the dr. Jekyll and mr. Hyde in the rhizobium-legume symbiosis. *Int. J. Mol. Sci.* 23 (19), 11089. doi: 10.3390/ijms231911089

Jin, J., Wang, J. F., Li, K. K., Wang, S. W., Qin, J., Zhang, G. H., et al. (2021). Integrated physiological, transcriptomic, and metabolomic analyses revealed molecular mechanism for salt resistance in soybean roots. *Int. J. Mol. Sci.* 22 (23), 12848. doi: 10.3390/ijms222312848

Karmacharya, A., Li, D., Leng, Y., Shi, G. S., Liu, Z., Yang, S. M., et al. (2023). Targeting disease susceptibility genes in wheat through wide hybridization with maize expressing cas9 and guide rna. *Mol. Plant-Microbe Interact. Mpmi*. doi: 10.1094/MPMI-01-23-0004-SC

Khan, M. A. (2018). Achievements and prospects of molecular breeding for drought tolerance in soybean [*Glycine max* (L.) MERR.]. *Genetika-Belgrade* 50, 1095–1109. doi: 10.2298/GENSR1803095K

Khan, M., Khan, S. U., Muhammad, A., Hu, L. M., Yang, Y., Fan, C. C., et al. (2018). Induced mutation and epigenetics modification in plants for crop improvement by targeting CRISPR/Cas9 technology. *J. Cell. Physiol.* 233, 4578–4594. doi: 10.1002/jcp.26299

Kofsky, J., Zhang, H. Y., and Song, B. H. (2018). The untapped genetic reservoir: the past, current, and future applications of the wild soybean (glycine soja). *Front. Plant Sci.* 9. doi: 10.3389/fpls.2018.00949

Kong, K. K., Xu, M. G., Xu, Z. Y., Lv, W. H., Lv, P. Y., Begum, N., et al. (2023). Dysfunction of *GmVPS8a* causes compact plant architecture in soybean. *Plant Sci.* 331, 111677. doi: 10.1016/j.plantsci.2023.111677

Kuzbakova, M., Khassanova, G., Oshergina, I., Ten, E., Jatayev, S., Yerzhebayeva, R., et al. (2022). Height to first pod: a review of genetic and breeding approaches to improve combine harvesting in legume crops. *Front. Plant Sci.* 13, 948099. doi: 10.3389/fpls.2022.948099

L'Hocine, L., and Boye, J. I. (2007). Allergenicity of soybean: new developments in identification of allergenic proteins, cross-reactivities and hypoallergenization technologies. *Crit. Rev. Food Sci. Nutr.* 47, 127–143. doi: 10.1080/10408390600626487

Le, H., Nguyen, N. H., Ta, D. T., Le, T., Bui, T. P., Le, N. T., et al. (2020). CRISPR/Cas9-mediated knockout of galactinol synthase-encoding genes reduces raffinose family oligosaccharide levels in soybean seeds. *Front. Plant Sci.* 11, 612942. doi: 10.3389/fpls.2020.612942

Li, X., Cai, Q., Yu, T., Li, S. J., Li, S. A., Li, Y. L., et al. (2023a). *ZmG6PDH1* in glucose-6-phosphate dehydrogenase family enhances cold stress tolerance in maize. *Front. Plant Sci.* 14, 1116237. doi: 10.3389/fpls.2023.1116237

Li, Z. B., Cheng, Q., Gan, Z. R., Hou, Z. H., Zhang, Y. H., Li, Y. L., et al. (2021). Multiplex CRISPR/Cas9-mediated knockout of soybean LNK2 advances flowering time. *Crop J.* 9, 767–776. doi: 10.1016/j.cj.2020.09.005

Li, J. J., Ding, X. L., Han, S. H., He, T. T., Zhang, H., Yang, L. S., et al. (2016). Differential proteomics analysis to identify proteins and pathways associated with male sterility of soybean using itraq-based strategy. *J. Proteomics* 138, 72–82. doi: 10.1016/j.jprot.2016.02.017

Li, C., Li, Y. H., Li, Y. F., Lu, H. F., Hong, H. L., Tian, Y., et al. (2020). A domestication-associated gene gmprr3b regulates the circadian clock and flowering time in soybean. *Mol. Plant* 13, 745–759. doi: 10.1016/j.molp.2020.01.014

Li, J. J., Nadeem, M., Sun, G. L., Wang, X. B., and Qiu, L. J. (2019b). Male sterility in soybean: occurrence, molecular basis and utilization. *Plant Breed.* 138, 659–676. doi: 10.1111/pbr.12751

Li, C., Nguyen, V., Liu, J., Fu, W., Chen, C., Yu, K. F., et al. (2019a). Mutagenesis of seed storage protein genes in soybean using CRISPR/Cas9. *BMC Res. Notes* 12, 176. doi: 10.1186/s13104-019-4207-2

Li, J. F., Norville, J. E., Aach, J., McCormack, M., Zhang, D. D., Bush, J., et al. (2013). Multiplex and homologous recombination-mediated genome editing in arabidopsis and nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.* 31, 688–691. doi: 10.1038/nbt.2654

Li, X. X., Zhou, H. W., Cheng, L., Ma, N. N., Cui, B. F., Wang, W. F., et al. (2022). Shoot-to-root translocated *GmNN1/FT2a* triggers nodulation and regulates soybean nitrogen nutrition. *PloS Biol.* 20 (8), e3001739. doi: 10.1371/journal.pbio.3001739

Li, H. B., Zhou, R. N., Liu, P. Y., Yang, M. L., Xin, D. W., Liu, C. Y., et al. (2023b). Design of high-monounsaturated fatty acid soybean seed oil using gmpdcts knockout via a CRISPR/Cas9 system. *Plant Biotechnol. J.* 21 (7), 1317–1319. doi: 10.1111/pbi.14060

Liang, D., Liu, Y., Li, C., Wen, Q., Xu, J., Geng, L., et al. (2023). *CRISPR/Lbcas12a-mediated genome editing in soybean*. Eds. B. Yang, W. Harwood and Q. Que. (New York, NY: Springer US), 39–52.

Lin, F., Chhapekar, S. S., Vieira, C. C., Da Silva, M. P., Rojas, A., Lee, D., et al. (2022). Breeding for disease resistance in soybean: a global perspective. *Theor. Appl. Genet.* 135, 3773–3872. doi: 10.1007/s00122-022-04101-3

Lin, X. Y., Liu, B. H., Weller, J. L., Abe, J., and Kong, F. J. (2021). Molecular mechanisms for the photoperiodic regulation of flowering in soybean. *J. Integr. Plant Biol.* 63, 981–994. doi: 10.1111/jipb.13021

Liu, H., Chen, W. D., Li, Y. S., Sun, L., Chai, Y. H., Chen, H. X., et al. (2022). CRISPR/Cas9 technology and its utility for crop improvement. *Int. J. Mol. Sci.* 23 (18), 10442. doi: 10.3390/ijms231810442

Liu, J. Z., Fang, Y., and Pang, H. X. (2016). The current status of the soybean-soybean mosaic virus (SMV) pathosystem. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.01906

Liu, T. F., Ji, J., Cheng, Y. Y., Zhang, S. C., Wang, Z. R., Duan, K. X., et al. (2023). CRISPR/Cas9-mediated editing of *GmTAP1* confers enhanced resistance to *Phytophthora Sojae* in soybean. *J. Integr. Plant Biol.* 65 (7), 1609–1612. doi: 10.1111/jipb.13476

Liu, S. Y., Liu, J. F., Zhang, Y. Z., Jiang, Y. S., Hu, S. W., Shi, A. D., et al. (2022). Cloning of the soybean sHSP26 gene and analysis of its drought resistance. *Phyton-Internat J. Exp. Bot.* 91, 1465–1482. doi: 10.32604/phyton.2022.018836

Liu, D., Yang, H. M., Zhang, Z. H., Chen, Q., Guo, W. L., Rossi, V., et al. (2023). An elite ?-Gliadin allele improves end-use quality in wheat. *New Phytol.* 239, 87–101. doi: 10.1111/nph.18722

Ma, J. J., Yang, S. X., Wang, D. M., Tang, K. Q., Feng, X. X., Feng, X. Z., et al. (2020). Genetic mapping of a light-dependent lesion mimic mutant reveals the function of coproporphyrinogen III oxidase homolog in soybean. *Front. Plant Sci.* 11, 557. doi: 10.3389/fpls.2020.00557

Ma, X. L., Zhu, Q. L., Chen, Y. L., and Liu, Y. G. (2016). CRISPR/Cas9 platforms for genome editing in plants: developments and applications. *Mol. Plant* 9, 961–974. doi: 10.1016/j.molp.2016.04.009

Mammadov, J., Buyyarapu, R., Guttikonda, S. K., Parliament, K., Abdurakhmonov, I. Y., Kumpatla, S. P., et al. (2018). Wild relatives of maize, rice, cotton, and soybean: treasure troves for tolerance to biotic and abiotic stresses. *Front. Plant Sci.* 9, 886. doi: 10.3389/fpls.2018.00886

Mangena, P. (2020). Phytocystatins and their potential application in the development of drought tolerance plants in soybeans (*Glycine max* L.). *Protein Pept. Lett.* 27, 135–144. doi: 10.2174/0929866526666191014125453

Medic, J., Atkinson, C., and Hurburgh, C. R. (2014). Current knowledge in soybean composition. *J. Am. Oil Chem. Soc.* 91, 363–384. doi: 10.1007/s11746-013-2407-9

Meng, L. B., Zhang, A. Y., Wang, F., Han, X. G., Wang, D. J., Li, S. M., et al. (2015). Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* 6, 339. doi: 10.3389/fpls.2015.00339

Molla, K. A., Sretenovic, S., Bansal, K. C., and Qi, Y. P. (2021). Precise plant genome editing using base editors and prime editors. *Nat. Plants* 7, 1166–1187. doi: 10.1038/s41477-021-00991-1

Mu, R. L., Lyu, X., Ji, R. H., Liu, J., Zhao, T., Li, H. Y., et al. (2022). Gmbics modulate low blue light-induced stem elongation in soybean. *Front. Plant Sci.* 13, 803122. doi: 10.3389/fpls.2022.803122

Nakamori, T. (2023). Research on the deliciousness of processed soybean current state and future prospects of soybean breeding ii. *J. Japanese Soc. Food Sci. Technology-Nippon Shokuhin Kagaku Kogaku Kaishi* 70, 43–45. doi: 10.3136/nskkk.NSKKK-D-22-00008

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J., and Kamoun, S. (2013). Targeted mutagenesis in the model plant nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655

Nguyen, C. X., Dohnalkova, A., Hancock, C. N., Kirk, K. R., Stacey, G., Stacey, M. G., et al. (2021). Critical role for uricase and xanthine dehydrogenase in soybean nitrogen fixation and nodule development. *Plant Genome.* 16 (2), e20171. doi: 10.1002/tpg2.20172

Nguyen, C. H., Yan, D. W., and Nambara, E. (2023). Persistence of abscisic acid analogs in plants: chemical control of plant growth and physiology. *Genes* 14 (5), 1078. doi: 10.3390/genes14051078

Niu, F. J., Jiang, Q. Y., Sun, X. J., Hu, Z., Wang, L. X., and Zhang, H. (2021). Large DNA fragment deletion in lncrna77580 regulates neighboring gene expression in soybean (*Glycine max*). *Funct. Plant Biol.* 48, 1139–1147. doi: 10.1071/FP20400

Niu, Q. K., Shi, Z. W., Zhang, P., Su, S., Jiang, B., Liu, X. W., et al. (2023). ZmMS39 encodes a callose synthase essential for male fertility in maize (*Zea* mays l.). *Crop J.* 11, 394–404. doi: 10.1016/j.cj.2022.08.012

Ochola, S., Huang, J., Ali, H., Shu, H. D., Shen, D. Y., Qiu, M., et al. (2020). Editing of an effector gene promoter sequence impacts plant-phytophthora interaction. *J. Integr. Plant Biol.* 62, 378–392. doi: 10.1111/jipb.12883

Osakabe, Y., and Osakabe, K. (2017). Genome Editing to Improve Abiotic Stress Responses in Plants. *Prog Mol Biol Transl Sci* 149 (2017), 99–109. doi: 10.1016/bs.pmbts.2017.03.007

Paul, B., and Montoya, G. (2020). CRISPR/Cas12a: functional overview and applications. *Biomed. J.* 43, 8–17. doi: 10.1016/j.bj.2019.10.005

Phang, T. H., Shao, G. H., and Lam, H. M. (2008). Salt tolerance in soybean. *J. Integr. Plant Biol.* 50, 1196–1212. doi: 10.1111/j.1744-7909.2008.00760.x

Qi, Q. Y., Hu, B. C., Jiang, W. Y., Wang, Y. X., Yan, J. J., Ma, F. W., et al. (2023). Advances in plant epigenome editing research and its application in plants. *Int. J. Mol. Sci.* 24 (4), 3442. doi: 10.3390/ijms24043442

Qian, L. L., Jin, H. X., Yang, Q. H., Zhu, L. M., Yu, X. M., Fu, X. J., et al. (2022). A sequence variation in *GmBADH2* enhances soybean aroma and is a functional marker for improving soybean flavor. *Int. J. Mol. Sci.* 23 (8), 4116. doi: 10.3390/ijms23084116

Qu, S., Jiao, Y. L., Abraham, L., and Wang, P. W. (2021). Correlation analysis of new soybean [*Glycine max* (L.) Merr] gene Gm15g117700 with oleic acid. *Phyton-Internat J. Exp. Bot.* 90, 1177–1192. doi: 10.32604/phyton.2021.015206

Ramesh, S. V., Govindasamy, V., Rajesh, M. K., Sabana, A. A., and Praveen, S. (2019). Stress-responsive mirnaome of *Glycine max* (L.) Merrill: molecular insights and way forward. *Planta* 249, 1267–1284. doi: 10.1007/s00425-019-03114-5

Ramlal, A., Nautiyal, A., Baweja, P., Mahto, R. K., Mehta, S., Mallikarunja, B. P., et al. (2022). Harnessing heterosis and male sterility in soybean [*Glycine max* (L.) Merrill]: a critical revisit. *Front. Plant Sci.* 13, 9817683. doi: 10.3389/fpls.2022.981768

Rangari, S. K., Sudha, M. K., Kaur, H., Uppal, N., Singh, G., Vikal, Y., et al. (2023). DNA-free genome editing for *ZmPLA1* gene via targeting immature embryos in tropical maize. *GM Crops Food-Biotechnol. Agric. Food* 2023, 1–7. doi: 10.1080/21645698.2023.2197303

Rao, Y. C., Yang, X., Pan, C. Y., Wang, C., and Wang, K. J. (2022). Advance of clustered regularly interspaced short palindromic repeats-Cas9 system and its application in crop improvement. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.839001

Rasheed, A., Mahmood, A., Maqbool, R., Albaqami, M., Sher, A., Sattar, A., et al. (2022a). Key insights to develop drought-resilient soybean: a review. *J. King Saud Univ. Sci.* 34, 102089. doi: 10.1016/j.jksus.2022.102089

Rasheed, A., Raza, A., Jie, H. D., Mahmood, A., Ma, Y. S., Zhao, L., et al. (2022b). Molecular tools and their applications in developing salt-tolerant soybean (Glycine max L.) Cultivars. *Bioengineering-Basel* 9 (10), 495. doi: 10.3390/bioengineering9100495

Salvi, P., Varshney, V., and Majee, M. (2022). Raffinose family oligosaccharides (rfos): role in seed vigor and longevity. *Biosci. Rep.* 42 (10), BSR20220198. doi: 10.1042/BSR20220198

Samanta, M. K., Dey, A., and Gayen, S. (2016). CRISPR/Cas9: an advanced tool for editing plant genomes. *Transgenic Res.* 25, 561–573. doi: 10.1007/s11248-016-9953-5

Sarkhel, S., and Roy, A. (2022). Phytic acid and its reduction in pulse matrix: structure-function relationship owing to bioavailability enhancement of micronutrients. *J. Food Process Eng.* 45 (2022). doi: 10.1111/jfpe.14030

Schneider, H. M., Lor, V. S., Zhang, X., Saengwilai, P., Hanlon, M. T., Klein, S. P., et al. (2023). Transcription factor bHLH121 regulates root cortical aerenchyma formation in maize. *Proc. Natl. Acad. Sci. United States America* 120, e2075299176. doi: 10.1073/pnas.2219668120

Shan, Q. W., Wang, Y. P., Li, J., Zhang, Y., Chen, K. L., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR/Cas system. *Nat. Biotechnol.* 31, 686–688. doi: 10.1038/nbt.2650

Song, J. H., Shin, G., Kim, H. J., Lee, S. B., Moon, J. Y., Jeong, J. C., et al. (2022). Mutation of *GmIPK1* gene using CRISPR/Cas9 reduced phytic acid content in soybean seeds. *Int. J. Mol. Sci.* 23 (18), 10583. doi: 10.3390/ijms231810583

Song, H., Taylor, D. C., and Zhang, M. (2023). Bioengineering of soybean oil and its impact on agronomic traits. *Int. J. Mol. Sci.* 24 (3), 2256. doi: 10.3390/ijms24032256

Staniak, M., Szpunar-Krok, E., and Kocira, A. (2023). Responses of soybean to selected abiotic stresses-photoperiod, temperature and water. *Agriculture-Basel* 13 (1), 146. doi: 10.3390/agriculture13010146

Sugano, S., Hirose, A., Kanazashi, Y., Adachi, K., Hibara, M., Itoh, T., et al. (2020). Simultaneous induction of mutant alleles of two allergenic genes in soybean by using site-directed mutagenesis. *BMC Plant Biol.* 20 (1), 513. doi: 10.1186/s12870-020-02708-6

Sun, J., Bie, X. M., Chu, X. L., Wang, N., Zhang, X. S., Gao, X. Q., et al. (2023). Genome-edited *TaTFL1-5* mutation decreases tiller and spikelet numbers in common wheat. *Front. Plant Sci.* 14, 1142779. doi: 10.3389/fpls.2023.1142779

Tian, Y. H., Zhou, Y., Gao, G. J., Zhang, Q. L., Li, Y. H., Lou, G. M., et al. (2023). Creation of two-line fragrant glutinous hybrid rice by editing the wx and *OsBADH2* genes via the CRISPR/Cas9 system. *Int. J. Mol. Sci.* 24 (1), 849. doi: 10.3390/ijms24010849

Tran, L., and Mochida, K. (2010). Functional genomics of soybean for improvement of productivity in adverse conditions. *Funct. Integr. Genomics* 10, 447–462. doi: 10.1007/s10142-010-0178-z

Wada, N., Ueta, R., Osakabe, Y., and Osakabe, K. (2020). Precision genome editing in plants: state-of-the-art in CRISPR/Cas9-based genome engineering. *BMC Plant Biol.* 20 (1), 234. doi: 10.1186/s12870-020-02385-5

Wan, Z., Liu, Y. X., Guo, D. D., Fan, R., Liu, Y., Xu, K., et al. (2022). CRISPR/Cas9-mediated targeted mutation of the E1 decreases photoperiod sensitivity, alters stem growth habits, and decreases branch number in soybean. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.1066820

Wang, J., Kuang, H. Q., Zhang, Z. H., Yang, Y. Q., Yan, L., Zhang, M. C., et al. (2020). Generation of seed lipoxygenase-free soybean using CRISPR/Cas9. *Crop J.* 8, 432–439. doi: 10.1016/j.cj.2019.08.008

Wang, L. W., Sun, S., Wu, T. T., Liu, L. P., Sun, X. G., Cai, Y. P., et al. (2020). Natural variation and CRISPR/Cas9-mediated mutation in *GmPRR37* affect photoperiodic flowering and contribute to regional adaptation of soybean. *Plant Biotechnol. J.* 18, 1869–1881. doi: 10.1111/pbi.13346

Wang, T. Y., Xun, H. W., Wang, W., Ding, X. Y., Tian, H. A., Hussain, S., et al. (2021). Mutation of gmaitr genes by CRISPR/Cas9 genome editing results in enhanced salinity stress tolerance in soybean. *Front. Plant Sci.* 12, 779598. doi: 10.3389/fpls.2021.779598

Weller, J. L., and Ortega, R. (2015). Genetic control of flowering time in legumes. *Front. Plant Sci.* 6. doi: 10.3389/fpls.2015.00207

Widyasari, K., Alazem, M., and Kim, K. H. (2020). Soybean resistance to soybean mosaic virus. *Plants-Basel* 9 (2), 219. doi: 10.3390/plants9020219

Wu, Y., Xiao, N., Cai, Y., Yang, Q., Yu, L., Chen, Z., et al. (2023). CRISPR/Cas9-mediated editing of the oshppd 3' UTR confers enhanced resistance to hppd-inhibiting herbicides in rice. *Plant Commun.* 2023, 100605. doi: 10.1016/j.xplc.2023.100605

Xia, Z. J., Zhai, H., Wu, H. Y., Xu, K., Watanabe, S., and Harada, K. (2021). The synchronized efforts to decipher the molecular basis for soybean maturity loci E1, E2, and E3 that regulate flowering and maturity. *Front. Plant Sci.* 12, 632754. doi: 10.3389/fpls.2021.632754

Xiao, Y. T., Karikari, B., Wang, L., Chang, F. G., and Zhao, T. J. (2021). Structure characterization and potential role of soybean phospholipases a multigene family in response to multiple abiotic stress uncovered by CRISPR/Cas9 technology. *Environ. Exp. Bot.* 188, 104521. doi: 10.1016/j.envexpbot.2021.104521

Xu, R. F., Li, J., Liu, X. S., Shan, T. F., Qin, R. Y., and Wei, P. C. (2020). Development of plant prime-editing systems for precise genome editing. *Plant Commun.* 1 (3), 100043. doi: 10.1016/j.xplc.2020.100043

Xu, H. Y., Li, Y. J., Zhang, K. F., Li, M. J., Fu, S. Y., Tian, Y. Z., et al. (2021). miR169c-NFYA-C-ENOD40 modulates nitrogen inhibitory effects in soybean nodulation. *New Phytol.* 229, 3377–3392. doi: 10.1111/nph.17115

Xu, H., Zhang, L. X., Zhang, K., and Ran, Y. D. (2020). Progresses, challenges, and prospects of genome editing in soybean (Glycine max). *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.571138

Yang, Z. W., Du, H., Xing, X. Z., Li, W. L., Kong, Y. B., Li, X. H., et al. (2022). A small heat shock protein, *GmHSP17.9*, from nodule confers symbiotic nitrogen fixation and seed yield in soybean. *Plant Biotechnol. J.* 20, 103–115. doi: 10.1111/pbi.13698

Yang, C. F., Huang, Y. Z., Lv, P. Y., Antwi-Boasiako, A., Begum, N., Zhao, T. J., et al. (2022). Nac transcription factor GmNAC12 improved drought stress tolerance in soybean. *Int. J. Mol. Sci.* 23 (19), 12029. doi: 10.3390/ijms231912029

Yang, Y., Speth, B. D., Boonyoo, N., Baumert, E., Atkinson, T. R., Palmer, R. G., et al. (2014). Molecular mapping of three male-sterile, female-fertile mutants and generation of a comprehensive map of all known male sterility genes in soybean. *Genome* 57, 155–160. doi: 10.1139/gen-2014-0018

Yang, L., Tang, J., Ma, X. L., Lin, Y., Ma, G. R., Shan, M. H., et al. (2021). Progression and application of CRISPR/Cas genomic editors. *Methods* 194, 65–74. doi: 10.1016/j.ymeth.2021.03.013

Yin, K. Q., Gao, C. X., and Qiu, J. L. (2017). Progress and prospects in plant genome editing. *Nat. Plants* 3, 17107. doi: 10.1038/nplants.2017.107

Yin, J. L., Wang, L. Q., Jin, T. T., Nie, Y., Liu, H., Qiu, Y. L., et al. (2021). A cell wall-localized nlr confers resistance to soybean mosaic virus by recognizing viral- encoded cylindrical inclusion protein. *Mol. Plant* 14, 1881–1900. doi: 10.1016/j.molp.2021.07.013

Yu, T. F., Liu, Y., Fu, J. D., Ma, J., Fang, Z. W., Chen, J., et al. (2021). The nf-y-pyr module integrates the abscisic acid signal pathway to regulate plant stress tolerance. *Plant Biotechnol. J.* 19, 2589–2605. doi: 10.1111/pbi.13684

Yu, H. X., Xiao, A. F., Wu, J. S., Li, H. X., Duan, Y., Chen, Q. S., et al. (2023). *GmNAC039* and *GmNAC018* activate the expression of cysteine protease genes to promote soybean nodule senescence. *Plant Cell*. 35 (8), 2929–2951. doi: 10.1093/plcell/koad129

Yu, G. L., Zou, J. N., Wang, J. H., Zhu, R. S., Qi, Z. M., Jiang, H. W., et al. (2022). A soybean nac homolog contributes to resistance to *Phytophthora Sojae* mediated by dirigent proteins. *Crop J.* 10, 332–341. doi: 10.1016/j.cj.2021.08.009

Zaaboul, F., Zhao, Q. L., Xu, Y. J., and Liu, Y. F. (2022). Soybean oil bodies: a review on composition, properties, food applications, and future research aspects. *Food Hydrocolloids* 124, 107296. doi: 10.1016/j.foodhyd.2021.107296

Zhai, H., Wan, Z., Jiao, S., Zhou, J. W., Xu, K., Nan, H. Y., et al. (2022). Gmmde genes bridge the maturity gene E1 and florigens in photoperiodic regulation of flowering in soybean. *Plant Physiol.* 189, 1021–1036. doi: 10.1093/plphys/kiac092

Zhang, G. Y., Bahn, S. C., Wang, G. L., Zhang, Y. R., Chen, B. B., Zhang, Y. L., et al. (2019). PLDα1-knockdown soybean seeds display higher unsaturated glycerolipid contents and seed vigor in high temperature and humidity environments. *Biotechnol. Biofuels* 12, 9. doi: 10.1186/s13068-018-1340-4

Zhang, Y. F., Blahut-Beatty, L., Zheng, S. Q., Clough, S. J., and Simmonds, D. H. (2023). The role of a soybean 14-3-3 gene (Glyma05g29080) on white mold resistance and nodulation investigations using CRISPR/Cas9 editing and rna silencing. *Mol. Plant-Microbe Interact.* 36, 159–164. doi: 10.1094/MPMI-07-22-0157-R

Zhang, M. H., Cao, J. F., Zhang, T. X., Xu, T., Yang, L. Y., Li, X. Y., et al. (2022). A putative plasma membrane Na^+/H^+ antiporter GmSOS1 is critical for salt stress tolerance in *Glycine* max. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.870695

Zhang, P. P., Du, H. Y., Wang, J., Pu, Y. X., Yang, C. Y., Yan, R. J., et al. (2020). Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus. *Plant Biotechnol. J.* 18, 1384–1395. doi: 10.1111/pbi.13302

Zhang, Y. X., Guo, W., Chen, L. M., Shen, X. J., Yang, H. L., Fang, Y. S., et al. (2022). CRISPR/Cas9-mediated targeted mutagenesis of *GmUGT* enhanced soybean resistance against leaf-chewing insects through flavonoids biosynthesis. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.802716

Zhang, Y. Z., Li, G. L., Hu, S. W., Liu, J. F., Jiang, Y. S., Liu, S. Y., et al. (2022). Cloning and drought resistance analysis of soybean *GmHsps_p23*-like gene. *Phyton-Internat J. Exp. Bot.* 91, 1183–1198. doi: 10.32604/phyton.2022.018853

Zhang, A., Li, Y., Wang, L., Wang, J., Liu, Y., Luan, X., et al. (2023). Analysis of LncRNA44234-associated cerna network reveals oil metabolism in soybean. *J. Agric. Food Chem.* 71 (25), 9815–9825. doi: 10.1021/acs.jafc.3c00993

Zhang, L. X., Liu, W., Tsegaw, M., Xu, X., Qi, Y. P., Sapey, E., et al. (2020). Principles and practices of the photo-thermal adaptability improvement in soybean. *J. Integr. Agric.* 19, 295–310. doi: 10.1016/S2095-3119(19)62850-9

Zhang, M., Liu, S. L., Wang, Z., Yuan, Y. Q., Zhang, Z. F., Liang, Q. J., et al. (2022). Progress in soybean functional genomics over the past decade. *Plant Biotechnol. J.* 20, 256–282. doi: 10.1111/pbi.13682

Zhang, Y. L., Ma, X. L., Xie, X. R., and Liu, Y. G. (2017). CRISPR/Cas9-based genome editing in plants. *Prog. Mol. Biol. Transl. Sci.* 149, 133–150. doi: 10.1016/bs.pmbts.2017.03.008

Zhang, Y., and Showalter, A. M. (2020). CRISPR/Cas9 genome editing technology: a valuable tool for understanding plant cell wall biosynthesis and function. *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.589517

Zhang, Z. H., Wang, W. P., Ali, S., Luo, X., and Xie, L. A. (2022). CRISPR/Cas9-mediated multiple knockouts in abscisic acid receptor genes reduced the sensitivity to aba during soybean seed germination. *Int. J. Mol. Sci.* 23 (24), 16173. doi: 10.3390/ijms232416173

Zhang, Z., Wang, J., Kuang, H., Hou, Z., Gong, P., Bai, M., et al. (2022). Elimination of an unfavorable allele conferring pod shattering in an elite soybean cultivar by CRISPR/Cas9. *Abiotech* 3, 110–114. doi: 10.1007/s42994-022-00071-8

Zhang, M., Wei, H. L., Hao, P. B., Wu, A. M., Ma, Q., Zhang, J. J., et al. (2021). *GhGPAT12/25* are essential for the formation of anther cuticle and pollen exine in cotton (*Gossypium* hirsutum L.). *Front. Plant Sci.* 12, 667739. doi: 10.3389/fpls.2021.667739

Zhang, J., Wu, P., Li, N., Xu, X. L., Wang, S. X., Chang, S. Y., et al. (2023). A male-sterile mutant with necrosis-like dark spots on anthers was generated in cotton. *Front. Plant Sci.* 13, 1102196. doi: 10.3389/fpls.2022.1102196

Zhang, R. Z., Zhang, S. J., Li, J. H., Gao, J., Song, G. Q., Li, W., et al. (2023). CRISPR/Cas9-targeted mutagenesis of *TaDCL4*, *TaDCL5* and *TaRDR6* induces male sterility in common wheat. *Plant Biotechnol. J.* 21, 839–853. doi: 10.1111/pbi.14000

Zhao, R. H., Kang, I. J., and Lee, S. W. (2023). Current status and future prospects in genomic research and breeding for resistance to xanthomonas citri pv. Glycines in soybean. *Agronomy-Basel* 13 (2), 490. doi: 10.3390/agronomy13020490

Zhao, F., Lyu, X., Ji, R. H., Liu, J., Zhao, T., Li, H. Y., et al. (2022). CRISPR/Cas9-engineered mutation to identify the roles of phytochromes in regulating photomorphogenesis and flowering time in soybean. *Crop J.* 10, 1654–1664. doi: 10.1016/j.cj.2022.03.008

Zheng, Y. Z., Li, Q. Q., Ye, M. W., Chen, A., and Wang, H. Y. (2021). Applications of CRISPR/Cas9-based genome editing in the plant biology. *Turkish J. Bot.* 45, 253–268. doi: 10.3906/bot-2103-50

Zhong, X. B., Hong, W., Shu, Y., Li, J. F., Liu, L. L., Chen, X. Y., et al. (2022). CRISPR/Cas9 mediated gene-editing of *GmHDZ4* transcription factor enhances drought tolerance in soybean (*Glycine max* [L.] Merr.). *Front. Plant Sci.* 13, 988505. doi: 10.3389/fpls.2022.988505

Zhou, J. M., Li, Z. Y., Li, Y., Zhao, Q. Z., Luan, X. C., Wang, L. X., et al. (2023a). Effects of different gene editing modes of CRISPR/Cas9 on soybean fatty acid anabolic metabolism based on *GmFAD2* family. *Int. J. Mol. Sci.* 24 (5), 4769. doi: 10.3390/ijms24054769

Zhou, Y. G., Liu, W. C., Li, X. W., Sun, D. Q., Xu, K. H., Feng, C., et al. (2020). Integration of srna, degradome, transcriptome analysis and functional investigation reveals gma-miR398c negatively regulates drought tolerance via *GmCSDs and GmCCS* in transgenic arabidopsis and soybean. *BMC Plant Biol.* 20 (1), 190. doi: 10.1186/s12870-020-02370-y

Zhou, J. M., Luan, X. C., Liu, Y. X., Wang, L. X., Wang, J. X., Yang, S. N., et al. (2023b). Strategies and methods for improving the efficiency of CRISPR/Cas9 gene editing in plant molecular breeding. *Plants-Basel* 12 (7), 1478. doi: 10.3390/plants12071478

Zhu, S. H., Yu, X. L., Li, Y. J., Sun, Y. Q., Zhu, Q. H., and Sun, J. (2018). Highly efficient targeted gene editing in upland cotton using the CRISPR/Cas9 system. *Int. J. Mol. Sci.* 19 (10), 3000. doi: 10.3390/ijms19103000

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 21 September 2023
DOI 10.3389/fpls.2023.1246905

OPEN ACCESS

EDITED BY
Cheng Yuan,
Yunnan Academy of Tobacco Agricultural Sciences, China

REVIEWED BY
Eleonora Cominelli,
National Research Council (CNR), Italy
Per Hofvander,
Swedish University of Agricultural Sciences, Sweden

*CORRESPONDENCE
Pankaj Bhowmik
pankaj.bhowmik@nrc-cnrc.gc.ca
Michel Aliani
michel.aliani@umanitoba.ca

RECEIVED 24 June 2023
ACCEPTED 23 August 2023
PUBLISHED 21 September 2023

CITATION
Bhowmik P, Yan W, Hodgins C, Polley B, Warkentin T, Nickerson M, Ro D-K, Marsolais F, Domoney C, Shariati-Ievari S and Aliani M (2023) CRISPR/Cas9-mediated lipoxygenase gene-editing in yellow pea leads to major changes in fatty acid and flavor profiles. *Front. Plant Sci.* 14:1246905. doi: 10.3389/fpls.2023.1246905

CRISPR/Cas9-mediated lipoxygenase gene-editing in yellow pea leads to major changes in fatty acid and flavor profiles

Pankaj Bhowmik[1*], Wei Yan[1], Connor Hodgins[2], Brittany Polley[1], Tom Warkentin[3], Michael Nickerson[4], Dae-Kyun Ro[2], Frédéric Marsolais[5], Claire Domoney[6], Shiva Shariati-Ievari[7] and Michel Aliani[7,8*]

[1]Aquatic and Crop Resource Development Centre, National Research Council Canada, Saskatoon, SK, Canada, [2]Department of Biological Sciences, University of Calgary, Calgary, AB, Canada, [3]Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK, Canada, [4]Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, SK, Canada, [5]London Research and Development Centre, Agriculture and Agri-Food Canada, London, ON, Canada, [6]Department of Biochemistry and Metabolism, John Innes Centre, Norwich Research Park, Norwich, United Kingdom, [7]Division of Neurodegenerative Diseases (DND), St Boniface Hospital Research Center, Winnipeg, MB, Canada, [8]Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada

Introduction: Although pulses are nutritious foods containing high amounts of protein, fiber and phytochemicals, their consumption and use in the food industry have been limited due to the formation of unappealing flavors/aromas described as beany, green, and grassy. Lipoxygenase (LOX) enzymes are prevalent among pulse seeds, and their activity can lead to the formation of specific volatile organic compounds (VOCs) from certain polyunsaturated fatty acids (PUFAs). As a widespread issue in legumes, including soybean, these VOCs have been linked to certain unappealing taste perception of foods containing processed pulse seeds.

Methods: To address this problem in pea and as proof of principle to promote the wider use of pulses, a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) construct was designed to create null alleles (knockouts) of *PsLOX2* which had been implicated in the generation of VOCs in peas.

Results and discussion: Successful CRISPR/Cas9-mediated LOX gene editing of stable transgenic pea lines (TGP) was confirmed by DNA sequencing of the wild type (WT) and TGP *pslox2* mutant lines. These lines were also assessed for LOX activity, PUFA levels, and VOCs. Compared to WT peas, the TGP lines showed a significant reduction ($p < 0.05$) in LOX activity and in the concentration of key VOCs, including hexanal, 2-hexenal, heptanal, (E)-2-heptenal, (E,E)-2,4-heptadienal, 1-octen-3-ol, octanal, (E)-2-octenal (E,E)-2,4-nonadienal and furan-2-pentyl. The content of two essential PUFAs, linoleic and α-linolenic acids, the known substrates of LOX in plants, was higher in TGP flours, indicating the efficacy of the CRISPR-mediated gene editing in minimizing their oxidation and the further modification of PUFAs and their products. The collection of VOCs

from the headspace of ground pea seeds, using a portable eNose also distinguished the TGP and WT lines. Multiple regression analysis showed that LOX activity correlated with the two VOCs, heptanal and (E,E)-2,4-heptadienal in pea flours. Partial Least Squares Regression (PLS-R) plot for selected PUFAs, VOCs, and sensor responses in WT and TGP lines showed distinct clusters for WT and TGP lines. Together this data demonstrates the utility of CRISPR mediated mutagenesis of *PsLOX2* to quickly improve aroma and fatty acid (FA) profiles of pea seeds of an elite Canadian variety.

KEYWORDS

pea, gene-editing, CRISPR/Cas9, lipoxygenase, fatty acids, aroma, flavor

1 Introduction

Canadian pea production for 2020 was 4.6 million tonnes accounting for nearly one-third (31%) of the total global production, followed by Russia and China (FAOSTAT, 2020). Yellow pea is one of the key pulses grown in Western Canada and elsewhere with considerable interest from the food industry. Although an excellent source of protein, in common with other legumes, yellow pea products face sensory challenges relating to their acceptability and stability over time. A major contributor to these challenges is a group of volatile organic compounds (VOCs) generated from both enzymatic and non-enzymatic chemical reactions during thermal processing which give pulse products unpleasant aromas (e.g., beany, green, grassy) and off-flavors (Ma et al., 2015). The enzymes that generate VOCs in peas and other pulses are lipoxygenases (LOX) – and alcohol oxidoreductases – that convert the polyunsaturated fatty acids (PUFAs), linoleic and α-linolenic acids, to aldehydes and a range of alcohols (Jelen and Wasowicz, 2011).

In recent years, it has been demonstrated that even partial deactivation of LOX activity using heat treatments such as micronization (Shariati-Ievari et al., 2016) and RevTech (RT), a patented heat treatment and pasteurization process (Fahmi et al., 2019), was successful in minimizing the generation of the key VOCs responsible for off-aroma and flavors of cooked legume flours (lentils, chickpeas, red beans, and yellow peas). A significant decrease in LOX activity in split yellow pea flours has also been reported when treated with RevTech at 140°C with 10% steam (RT10%) and bread samples fortified with the treated split yellow pea flour were more acceptable when compared to bread with untreated pea flour or pea flour with RT at 140°C without steam (RT0%) (Fahmi et al., 2019). These results suggested that the total or partial inactivation of LOX enzymes in RT10% pea flours was key to the consumer acceptability results observed. The data generated suggest that the thermostability of the enzyme varied significantly depending on the product type, heat treatment condition, and evaluation assays used.

Although processing studies have indicated the effectiveness of heat in reducing LOX activity in legumes (Smith and Circle, 1972; Zhu et al., 1996; Zilic et al., 2010; Pathiratne et al., 2015) pea germplasm resources and mutant materials with contrasting low and high LOX activity are limited, and no elite materials with low LOX and volatile profiles exist. A natural *LOX2* mutant was identified in a wild pea (*Pisum fulvum*), following a screen of diverse *Pisum* germplasm, and the mutant phenotype was introduced into the *P. sativum* cv. Birte by repeated backcrossing (Forster et al., 1999). However, this cultivar is not generally suitable agronomically for cultivation as a low LOX pea variety. Traditional breeding methods using this mutation entail repeated crossing, genotyping, and phenotyping, which are long and expensive processes to create an elite pea variety with low LOX activity and an improved flavor profile through hybridization.

In contrast, genome editing allows for the regular generation of mutations at preselected genomic loci in plants and could be used to facilitate the improvement of the flavor profile in seeds. In this work, we planned to generate null alleles (knockouts) for the most relevant LOX isoform in pea (Forster et al., 1999) using CRISPR constructs and transformation of elite yellow pea lines. Recent advancements in the deployment of CRISPR/Cas9 for genome editing in legumes was reviewed by Bhowmik et al. (2021). Soybean was the first legume crop targeted for genome editing with CRISPR/Cas technology. Several endogenous soybean genes (*GmLOX1, GmLOX2, GmLOX3*, *GmFT2, GmE1, GmFEI2*, and *GmSHR*) have been edited using the CRISPR/Cas9 method for improving traits such as beany flavor, flowering time, nodulation, and abiotic stress tolerance (Cai et al., 2018; Han et al., 2019; Wang et al., 2020). CRISPR/Cas9-mediated gene editing has also been successfully used in cowpea (Ji et al., 2019) and chickpeas (Badhan et al., 2021) for enhancing symbiotic nitrogen fixation and drought stress tolerance. These examples suggest that application of the CRISPR/Cas9 system for editing LOX gene(s) in elite yellow pea lines could provide improvement of the flavor profile in seeds as an alternative and sustainable approach to thermal treatment.

Therefore, the objectives of this study were two-fold: 1) To generate knockout LOX transgenic peas using a gene editing approach; and 2) To investigate the efficacy of this approach on improving the flavor profile of the transgenic peas.

2 Results

2.1 Generation of *pslox2* mutants and genotyping mutant lines

PsLOX2 was amplified from the pea cultivar CDC Spectrum (Warkentin et al., 2017) and analyzed using the web application CCTop (https://crispr.cos.uni-heidelberg.de; Stemmer et al., 2015) to design gRNAs. The five gRNA sequences with the highest CRISPRater scores (Labuhn et al., 2018) located in the first three exons of *PsLOX2* (Figure 1A) were selected. The cleavage ability of the gRNA candidate sequences was assessed with an *in vitro* Cas9 cleavage assay (Figure 1B). In this assay gRNAs 1 and 5 showed the greatest activity, cleaving almost all of the cleavage template. gRNAs 2, 3, and 4 were also able to cleave the template but to a lesser extent than gRNA 1 or 5. To maximize the probability of an edit we planned to express three gRNAs under the control of a single cauliflower mosaic virus 35S (35S) promoter using a tRNA-gRNA array (Xie et al., 2015). gRNAs 1, 3, and 5 were selected for cloning. However, we were unable to clone a single gRNA expression cassette expressing all three gRNAs. Instead, we were successful in producing a cassette containing only gRNAs 1 and 3. To expedite this project the gRNA expression cassette containing only gRNA1 and 3 was added to a binary vector which expressed Cas9 under the 35S promoter creating the *PsLOX2* CRISPR vector.

It takes 6–8 months to generate stable transgenic pea lines, making it time consuming to test CRISPR vectors in stable transgenic peas. To save substantial amounts of time and resources we assessed the *in planta* efficacy of the *PsLOX2* CRISPR vector using mesophyll protoplasts transfection (Pandey et al., 2022) before initiating stable transformation experiments. Protoplasts were generated from pea leaves and transfected with the *PsLOX2* vector. The *PsLOX2* target locus was PCR amplified from the transfected protoplasts and Sanger sequenced. Mixed signals in the sequencing chromatograms at the double stranded break (DSB) site of gRNA3 were interpreted as evidence of editing in the transfected protoplasts (Figure 1C). Based on this evidence, the *PsLOX2* CRISPR vector was transformed into the *Agrobacterium tumefaciens* strain EHA105 and putative pea transformants were regenerated (Figure 2).

A total of 19 plants were brought out of tissue culture, 17 of which were found to be transgenic. The gRNA1 and gRNA3 sites of *PsLOX2* were PCR amplified and analyzed for evidence of mutation using Sanger sequencing (Table 1; Figure 3). The resulting chromatograms were analyzed using Inference of CRISPR Edits analysis (ICE; https://ice.synthego.com/#/; Conant et al., 2022). Five lines (3, 4, 5, 14, and 17) showed secondary signals in their chromatograms at the DSB site of gRNA3 suggesting the presence of mutant alleles. ICE analysis determined that lines 3, 4, and 5 are heterozygous with a wild-type allele and a mutant allele with a 1 bp deletion at the gRNA3 site. The small secondary peak in the chromatogram of line 14 was not detected as a potential indel in the ICE analysis suggesting that potential mutant cells in this line are in the extreme minority. Interestingly, line 17 produced a secondary peak starting downstream of gRNA3 but did not extend for the entire length of the chromatogram (Figure 3). The ICE analysis of the target locus in line 17 detected a 44 bp deletion in 58% of indels and wild-type sequences in 30% of indels (the remaining 10% and 2% of indels were 14 and 16 bp deletions, respectively). This suggests that line 17 is heterozygous with one allele containing a large deletion of 44 bp at the gRNA3 site which explains why the double peak in the sequencing chromatogram is unable to extend to the full length of the wild-type allele's sequencing chromatogram.

To obtain homozygous mutant lines, three T_1 lines (3, 4, and 17) which displayed strong evidence of mutation were advanced in the greenhouse to produce T_2 plants (Figure 2E). Genomic DNA was extracted from the resulting T_2 plants and analyzed as described for the T_1 lines (Table 2; Figure 4). All T_2 plants analyzed were transgenic. Two homozygous mutant T_2 plants were identified in lines 3 and 4, all of which contained the same T deletion at the gRNA3 DSB site (Figure 4A). It is not uncommon for independent Cas9 cleavage events to result in the same indel mutations in independent transgenic lines (Bertier et al., 2018). Lines 3-4 and 4-3 were determined to be heterozygous containing a wild-type allele and T deletion allele. The identified T deletion causes a frameshift mutation resulting in an early stop codon after 66 amino acids (aa). *PsLOX2* is normally 863 aa and therefore this T deletion was assumed to eliminate *PsLOX2* activity.

Three T_2 plants from line 17 were found to be mutants, one of which was homozygous (Table 2; Figure 4). Line 17-1 was a homozygous mutant that had a GA deletion at the gRNA1 DSB site and a 44 bp deletion that completely removed the gRNA3 site. This GA deletion was not detected in the ICE analysis of the parental T_1 line 17. Lines 17-3 and 17-4 had mixed signals in their chromatograms beginning 26 bp downstream of the gRNA3 DSB site, the same pattern as their T_1 progenitor line. ICE analysis also indicated the predominant indels were 44 bp deletions and wild-type in lines 17-3 and 17-4. The genotypes of line 17's T_2 generation show classic 1:2:1 heterozygous Mendelian inheritance. This is strong evidence that the T_1 progenitor line was heterozygous with one allele being wild-type and the other allele containing the mutations identified in line 17-1. The GA deletion at the gRNA1 site causes a frameshift mutation resulting in an early stop codon which dramatically reduces the size of the mutant protein to 22 aa, likely abolishing the function of *PsLOX2*.

2.2 LOX activity of *pslox2* mutant lines

To test the efficacy of the CRISPR gene edited *PsLOX2*, it was necessary to measure the LOX activity in the pea plants that have been edited and compare it to the LOX activity in unedited plants. Mutant lines 3-3, 3-4 (heterozygous), 3-6, 4-4, 4-5, 17-1, 17-3 (heterozygous), and 17-4 (heterozygous) were re-labelled as TGP-2 to -9, respectively, for LOX activity analysis (Table 3). In addition, the determination of the levels of linoleic acid and α-linolenic acid as the main known substrates of LOX enzymes in pulse flours, in both the edited and unedited peas was warranted. If the CRISPR editing of *PsLOX2* was effective in knocking out the gene, a decrease in LOX activity is expected followed by a significant increase in the levels of these key substrates. Finally, the determination of VOCs in the edited and unedited pea plants is

also critical since these VOCs are directly involved in the generation of the beany aroma in cooked peas. Therefore, effective CRISPR editing of the LOX gene in peas would be expected to result in a significant decrease in beany flavor-related VOCs in the edited plants. Overall, by measuring the LOX activity, substrates, and VOCs in edited and unedited pea plants, the efficacy of the mutant *PsLOX2* lines may be assessed.

The activity of LOX was significantly reduced ($P<0.05$) in all transgenic peas (TGP-2 to -9) including the heterozygous lines (where the WT allele would be expected to be dominant) (Table 3).

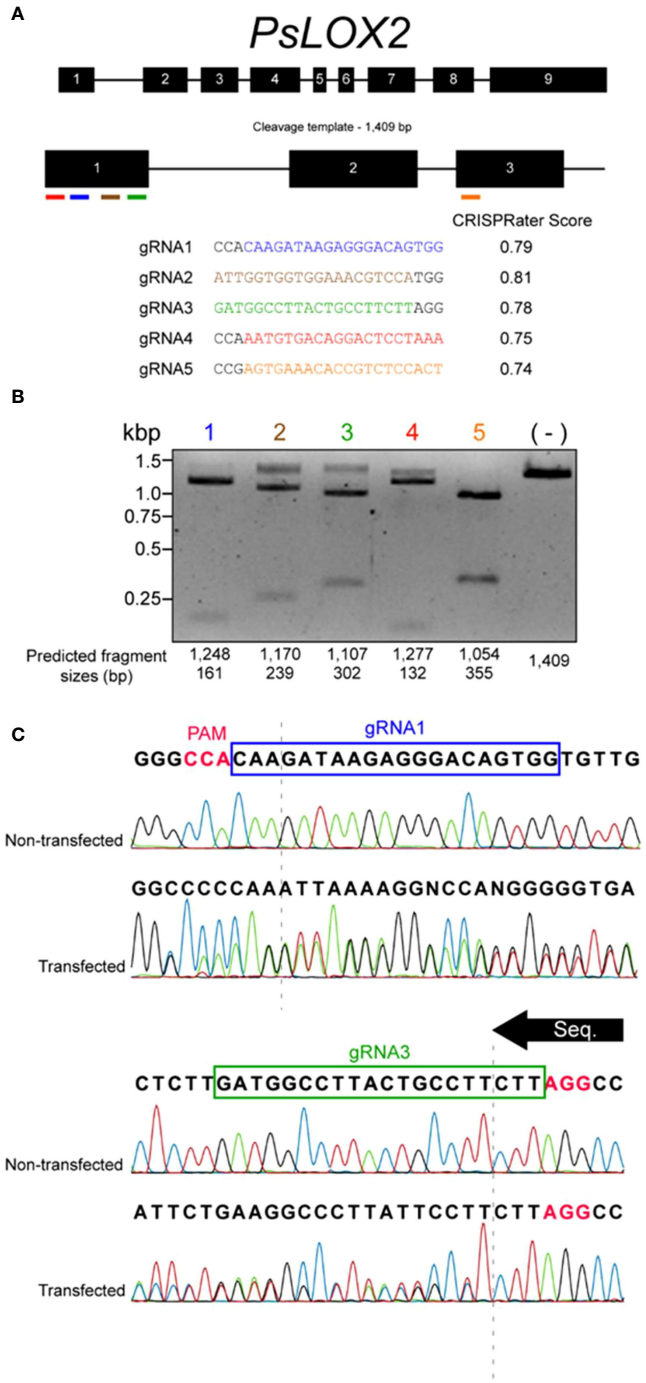


FIGURE 1
gRNA selection and protoplast assessment of *PsLOX2* CRISPR vector. **(A)** *PsLOX2* and location of candidate gRNA sites. Exons are represented by black boxes, introns are represented by black lines, and gRNA candidate sites are represented by colored lines. **(B)** *In vitro* cleavage assay of a PCR generated *PsLOX2* cleavage template incubated with recombinant Cas9 and *in vitro* transcribed gRNAs. (-) represents a reaction with no gRNA added. **(C)** Sanger sequencing chromatograms of the *PsLOX2* locus PCR amplified from protoplasts transfected with the *PsLOX2* CRISPR vector. The sequencing reaction occurred from the gRNA3 site to the gRNA1 site. The dashed lines represent the location of the DSB of the gRNA sites; Protospacer adjacent motif (PAM) sites are indicated in pink font.

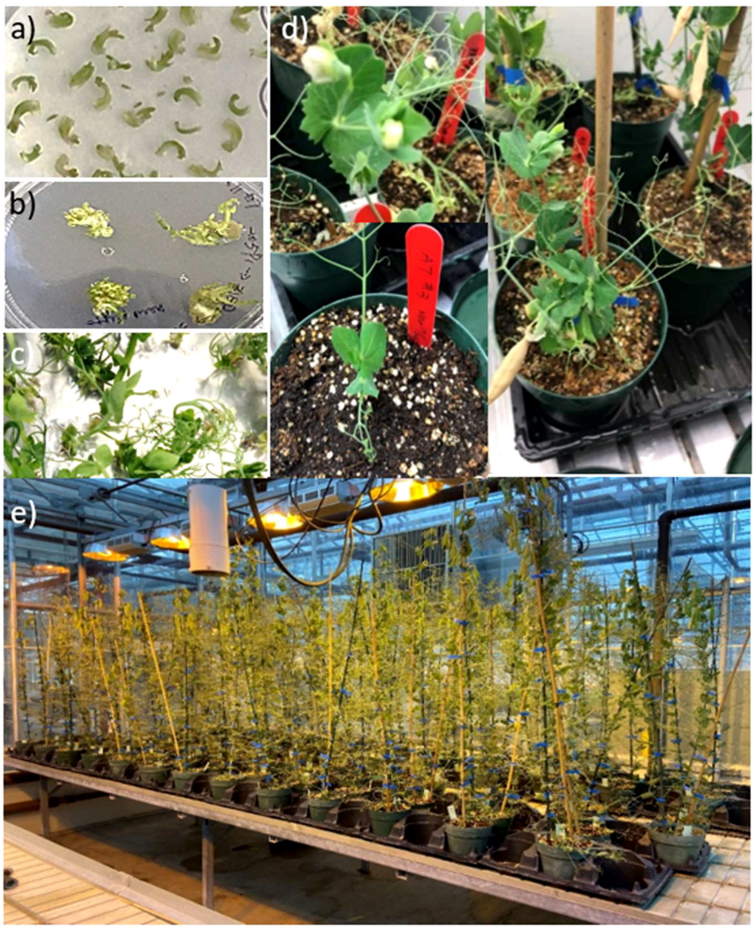


FIGURE 2
Agrobacterium mediated pea transformation and plant regeneration. **(A)** Slices of embryo axes after 4 days of co-cultivation with *Agrobacterium*. **(B)** Callus induction and shoot regeneration. **(C)** Explants at the end of a shoot induction phase. **(D)** Rooted putative transformant in soil. **(E)** Advancing T_2 *PsLOX2* edited pea lines in the green house.

Among the TGP samples, TGP-2 and TGP-7 had the lowest LOX activity (71.8 U/mg) and TGP-6 had the highest (103.2 U/mg). This reflects ~58% and ~40% lower activity of LOX compared to WT samples for TGP-2 and -7, and TGP-6, respectively.

2.3 FA profile of *pslox2* mutant lines

It was also important to investigate the effect of this significant decrease in LOX activity obtained for transgenic pea samples on the levels of FAs since some essential PUFAs have been previously identified as substrates of LOX enzymes in various types of pulses (Murray et al., 1976; Sessa and Rackis, 1977; Lampi et al., 2020). The FA composition of the WT and transgenic pea flours is shown in Table 3. All values are reported as a percentage of total FAs detected using GC-FID. The FA content of the WT peas was composed primarily of unsaturated fatty acids of which 53.54% were PUFA and 28.42% were monounsaturated fatty acids (MUFA). Saturated fatty acids (SFAs) comprised 17.24% of the total lipid content. Several major FAs were present including linoleic acid (C18:2) (45.25%), followed by oleic acid (C18:1, 27.83%), palmitic acid (C16:0, 11.53%), α-linolenic acid (C18:3, 7.74%), and stearic acid (18:0, 4.05%). The SFA levels were not affected by CRISPR-mediated changes of *PsLOX2* while both PUFAs and MUFAs were significantly affected in all transgenic peas but only PUFAs were found at higher percentages in lines TGP-7, -8, and -9 compared to WT peas. Of particular importance, the specific substrates of LOX enzymes, linoleic acid and α-linolenic acid were both found at higher concentrations in TGP -7, -8, and -9. The transgenic peas had a significantly increased range covering from 8.4 to 11.6% in their linoleic acid content and an increased range from 2.8 to 11.2% in their α-linolenic acid content. One of the important indices used for evaluating the nutritional value of dietary foods, the ω-6/ω-3 ratio was also reported in Table 3. The ratio of ω-6/ω-3 in WT pea flours was 5.67 and only significantly increased in TGP–3, -4, and -9 samples by ~11–14%.

10.3389/fpls.2023.1246905

2.4 VOC composition of *pslox2* mutant lines

A list of 11 VOCs collected using SPME extraction is provided in Table 4. Among these, 10 were significantly decreased in transgenic pea flours compared to WT pea flour. Identification of these 11 VOCs was performed by matching the detected mass spectra and LRI values of the VOCs with the values reported by the NIST library (2017, version 2.3). The probable origin and sensory attributes of each compound are also reported for further confirmation for each VOC. The majority of detected compounds in these pea extracts were aldehydes and alcohols.

A significant decrease in the concentration of known key VOCs with unpleasant aromas (i.e., beany, green, grassy) such as hexanal, 2-hexenal, heptanal, (E)-2-heptenal, 1-octen-3-ol, furan-2-pentyl, (E,E)-2,4-heptadienal, (E)-2 octenal, and (E,E) 2,4-nonadienal was observed in at least one of the eight transgenic pea samples presented in our study when compared to WT untreated pea flour. Across eight of the ten significantly reduced VOCs (hexanal, 2-hexanal, (E)-2-heptanal, 1-octen-3-ol, furan-2-pentyl, (E,E)-2,4-heptadienal, (E)-2-octenal, and (E,E)-2,-4-nonadienal) the homozygous mutant lines TGP-2, -4, -5, -6, and -7 seem to have a more severe reduction than the heterozygous mutant lines TGP-3, -8, and -9. The decrease in the aforementioned VOCs in transgenic lines is reasonable given the reduction in LOX activity.

A PCA score plot was generated based on all the VOCs collected from the headspace of WT and transgenic pea samples compared to a blank (air) (Figure 5). The resulting plot shows a clear separation of clusters from the blank sample and a separation of the TGP lines clusters from the WT line cluster. TGP-4 to -9 were further separated from the TGP-2 and -3 lines. Overall, more than 95% of the cluster separation was explained by the first two PC axes with 62.10% and 34.85% for PC1 and PC2 axes, respectively.

Multiple regression analysis showed that two VOCs, heptanal and (E,E)-2,4-heptadienal, were mostly correlated with LOX activity in pea flours. The standardized beta coefficient values that compare the strength of the effect of individual predictors (independent variable) to LOX activity in the investigated model were as follows: 0.946 (p = 0.003) for (E,E)-2,4-heptadienal; 0.055 (p = 0.003) for heptanal. The stepwise model was significant (F1,2 = 3376.572, p = 0.003) with the adjusted R^2 value of 1.00.

TABLE 1 Summary of genotyping data for T_1 pea plants grown in soil.

Plant ID	Transgenic	gRNA 1	gRNA 3
1	Yes	WT	WT
2	Yes	WT	WT
3	Yes	WT	WT and T deletion
4	Yes	WT	WT and T deletion
5	Yes	WT	WT and T deletion
6	Yes	WT	WT
7	No	–	–
8	Yes	WT	WT
9	Yes	WT	WT
10	Yes	WT	WT
11	Yes	WT	WT
12	Yes	WT	WT
13	Yes	WT	WT
14	Yes	WT	WT
15	Yes	WT	WT
16	No	–	–
17	Yes	WT	WT and 44 bp deletion
18	Yes	WT	WT
19	Yes	WT	WT
WT	No	WT	WT

Genotyping assays were conducted using PCR from the T_1 plants genomic DNA. Successful PCR using primers specific to *neomycin phosphotransferase II*, present on the T-DNA, was used to determine if plants were transgenic. Sanger sequencing of *PsLOX2* PCR products containing gRNA1 and gRNA3 sites was used to detect mutations. A single sequencing reaction reading from the gRNA3 to gRNA1 site was used. ICE analysis was used to determine the alleles present in mutated samples.
"–" indicates that no sequencing reaction was performed as the T_1 plant was not transgenic and therefore could not be mutated.

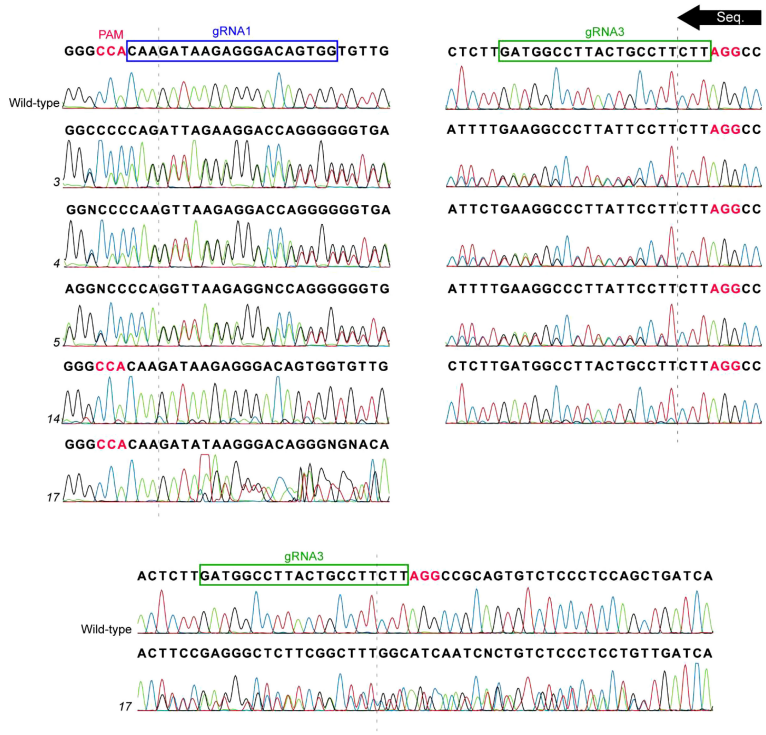


FIGURE 3
Sequencing data of T_1 lines which showed evidence of mutation. The *PsLOX2* locus was PCR amplified and Sanger sequenced. The sequencing reaction occurred from the gRNA3 site to the gRNA1 site. The dashed lines represent the location of the DSB of the gRNA sites; Protospacer adjacent motif (PAM) sites are indicated in pink font.

3 Discussion

LOX activity in yellow peas causes the production of VOCs from PUFAs which are associated with unpleasant aromas in pea flour. While other approaches like micronization and RevTech heat treatment have been demonstrated to diminish the unpleasant aromas of various pulse flours by totally or partially deactivating LOX activity (Shariati-Ievari et al., 2016; Fahmi et al., 2021), these methods lack selectivity and can impact other enzymes in pulse flours. The utilization of CRISPR/Cas9 technology presents an excellent option to target *PsLOX* genes precisely for mutation thereby reducing LOX activity in pea flour and reducing the production of undesirable VOCs.

The application of CRISPR/Cas9 as a tool for rapid and efficient genome modification and accelerated crop improvement has been successful for soybean genome editing (Cai et al., 2015; Jacobs et al., 2015; Li et al., 2015; Michno et al., 2015; Sun et al., 2015; Du et al., 2016; Curtin et al., 2018; Kanazashi et al., 2018). The recently available sequence of the pea genome (Kreplak et al., 2019) has provided a valuable tool for reverse genetics approaches such as CRISPR/Cas9 to realize the agricultural productivity and nutritional value of this crop. Li et al. (2023) demonstrated CRISPR/Cas9 mutation of *phytoene desaturase* in peas. However, to the best of our knowledge, this is the first report of the use of CRISPR/Cas9 to generate mutant pea lines with improved flavor traits.

Soybean lines with mutations of all three *GmLOX* isogenes (*GmLOX1/2/3*) have been identified (Hildebrand and Hymowitz, 1982; Kitamura et al., 1983; Davies et al., 1987). Lines with null mutations of *GmLOX2* were particularly valuable as they were determined to have improved flavor in human sensory tests whereas lines with mutations in *GmLOX1/3* did not have improvments over the wild-type (Davies et al., 1987). Importantly, disease resistance and seed yield were not negatively impacted by the *GmLOX2* null mutation (Pfeiffer et al., 1992). Similarly, mutants of *pslox2*, one of the two seed *PsLOX* isogenes, had no decrease in seed weight or field yield despite having reduced VOC levels (Forster et al., 1999). VOCs have been reported to have roles in a diverse set of plant metabolic pathways (Vancanneyt et al., 2001). However, the unique environmental conditions experienced by soybean and pea *LOX* mutants in agricultural fields can potentially explain their ability to combat this deficiency. The introgression of the identified *pslox2 i*nto elite cultivars is possible but will be a time intensive endeavor. In contrast recreating this trait in an elite cultivar is an obvious application for CRISPR/Cas9 mutagenesis as a simple single gene knockout can dramatically improve the flavor of the pea seeds.

To this end, we implemented a previously successful CRISPR/Cas9 editing system in the elite yellow pea cultivar CDC Spectrum and successfully generated eight transgenic *pslox2* mutant lines. Confirming significantly decreased LOX activity in pea flours generated from each of the eight transgenic lines was crucial. Although all transgenic lines had significantly ($p<0.05$) lower LOX activity, TGP-7 had the lowest LOX activity. Previous studies have demonstrated that heat treatments such as RevTech

10.3389/fpls.2023.1246905

TABLE 2 Summary of genotyping data for T_2 pea plants.

T_1 Line	Plant ID	Transgenic	gRNA1	gRNA3
3	3-1	Yes	WT	WT
	3-2	Yes	WT	WT
	3-3	Yes	WT	T deletion
	3-4	Yes	WT	WT and T deletion
	3-5	Yes	WT	WT
	3-6	Yes	WT	T deletion
	3-7	Yes	WT	WT
4	4-1	Yes	WT	WT
	4-2	Yes	WT	WT
	4-3	Yes	WT	WT and T deletion
	4-4	Yes	WT	T deletion
	4-5	Yes	WT	T deletion
	4-6	Yes	WT	WT
17	17-1	Yes	GA deletion	44 bp deletion
	17-2	Yes	WT	WT
	17-3	Yes	WT and GA deletion	WT and 44 bp deletion
	17-4	Yes	WT and GA deletion	WT and 44 bp deletion
WT		No	WT	WT

Genotyping assays were conducted as described in Table 2 for T_1 plants.

at 140°C with 0% and/or 10% moisture were effective means to decrease LOX activity in yellow peas by 43% and 89%, respectively (Fahmi et al., 2021). The *pslox2* mutants described here had similar reductions in LOX activity (39.4% to 57.8%) as the RevTech treatment but required no heating or further processing.

The FA compositions presented in Table 3 are consistent with those previously reported in different varieties of peas, suggesting that the major FAs in pea flours are linoleic, oleic, palmitic, and α-linolenic acid, in decreasing order (Murcia and Rincón, 1992; Grela and Günter, 1995; Ryan et al., 2007; Villalobos Solis et al., 2013; Caprioli et al., 2016; Padhi et al., 2017; Khrisanapant et al., 2019; Fahmi et al., 2021). The overall FA profiles were not affected in transgenic pea samples. However, some essential PUFAs, linoleic and α-linolenic acid, were found at higher concentrations in some transgenic peas (TGP-7, -8, and -9). This is expected as LOX activity was reduced in the transgenic pea lines due to mutations in *PsLOX2* (Table 3). If this significant increase in the proportion of linoleic and α-linolenic acid is due to reduced LOX activity, a decrease in the key known VOCs generated from these PUFAs is to be expected. In line with this expectation, there were significantly lower concentrations of 10 out of 11 VOCs in the transgenic peas (Table 4). The difference among the TGPs tested here might relate to other interfering co-factors required for LOX activity.

The *ω*-6/*ω*-3 ratios reported here are consistent with previously reported values ranging from 4.4 to 6.3 (Grela and Günter, 1995; Ryan et al., 2007; Caprioli et al., 2016; Khrisanapant et al., 2019).

Identification and semi-quantification of VOCs associated with unpleasant aromas was achieved based on the comparison of mass spectra and LRI values of VOCs detected in WT and *pslox2* mutant pea flours with those reported in the NIST library (2017, version 2.3) (Table 4). For additional clarity, the likely fatty acid source and odor description of each VOC can be found in Table 4. Most VOCs identified in the pea seed extracts were aldehydes or alcohols. A significant decrease in all TGP lines was only seen for VOCs (E)-2-heptenal, octanal, (E,E)-2,-4-heptadienal, and (E)-2-octenal.

The standardized beta coefficient values [the measure of the contribution of the variables (all significant VOCs and FAs) to the model)] indicated the strength of the effect of each model predictor variable used on LOX activity. Overall, the LOX activity of pea samples was highly influenced by (E,E)-2,4-heptadienal and heptanal. Beta values were 0.946 and a p-value of 0.003 ((E,E)-2,4-heptadienal) and 0.055 and a p-value of 0.003 (heptanal). These findings suggest that (E,E)-2,4-heptadienal and heptanal are important VOC markers of LOX activity in pea flours, with (E,E)-2,4-heptadienal having a much stronger effect compared to heptanal. The stepwise model was significant with an F1,2 value of 3376.572 and a p-value of 0.003, indicating that the model is a good fit for the data. The adjusted R2 value of 1.00 suggests that the model explains 100% of the variance in LOX activity, which is a very strong result. These VOCs could help food developers to create suitable screening models to select pea flours and/or products made with them to predict unpleasant aroma.

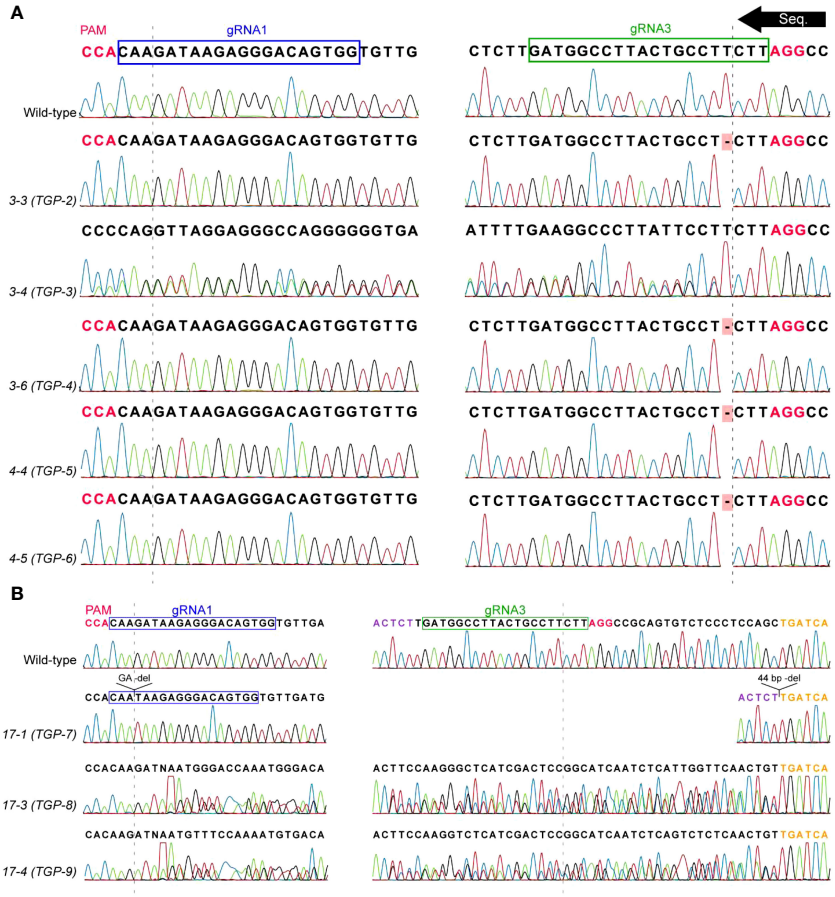


FIGURE 4
Sequencing data from T_2 mutants used in LOX activity experiments. The *PsLOX2* locus was PCR amplified and Sanger sequenced. **(A)** T_2 progeny of lines 3 and 4. **(B)** T_2 progeny of line 17. Sequencing reactions occurred from the gRNA3 to gRNA1 site. The dashed lines represent the DSB site of the gRNA sites; Protospacer adjacent motif (PAM) sites are indicated in pink font; Purple and orange font indicate the flanking regions around the 44 bp deletion at the gRNA3 site of line 7-1.

This means that as the LOX activity increases the concentration of (E,E)-2,4-heptadienal increases. LOX activity also had a significant effect on heptanal, but its effect was much weaker, with a standardized beta coefficient of 0.055 and a p-value of 0.003. This suggests that as the LOX activity increases, the concentration of heptanal also increases, but the effect is much smaller compared to (E,E)-2,4-heptadienal.

Collecting volatiles using sensors typically involves using specialized devices or instruments that are designed to detect and measure the concentration or presence of volatile compounds in the air or other environments. These sensors can be based on various principles such as chemical reactions, physical properties, or electrical signals, depending on the type of volatile compound being targeted. The SENSIGENT MSEM 160 Portable Odor and Chemical Monitor System used in our study uses an array of 32 sensors to mimic the sense of smell for detecting volatiles from a wide range of chemical classes which makes them useful to screen food samples. These results show the overall VOC profile of the pea flours and not the individual VOCs that were identified using the GC-MS analysis outlined above. The score plot in principal component analysis (PCA) for WT and eight transgenic yellow pea flours (TGP-2 to -9) presented in Figure 5 clearly shows the ability of this device to distinguish between CRISPR-mediated and nonmediated pea flours with more than 96% of the separation explained by the first two PCA axes.

The homozygous mutant lines TGP-2, -4, -5, -6, and -7 tended to have a more severe phenotype than the heterozygous mutant lines TGP-3, -8, and -9 across the parameters examined, LOX activity, FA composition, and VOC composition. The data suggest that *PsLOX2* shows semi-dominant inheritance as the heterozygous mutants have an intermediate phenotype between the homozygous mutant and WT lines. Although unexpected, a similar semi-dominant inheritance has been observed in tomato (*Solanum lycopersicum*) mutants of *suppressor of prosystemin-mediated responses8* (*spr8*) which is also a lipoxygenase (Yan et al., 2013).

To visually examine the linear correlation between LOX activity and all significant measurements presented in Tables 3 and 4, a correlogram is provided in Figure 6. This graph presents a positive linear correlation between LOX and detected VOCs with the sensor responses. A negative correlation is obtained for total PUFAs, selective PUFAs (linoleic and α-linolenic acid), and also for the ω-6/ω-3 ratios.

10.3389/fpls.2023.1246905

TABLE 3 Lipoxygenase specific activity (U/mg) and fatty acid composition (% of total fatty acid) in wild type (WT) and transgenic yellow pea (TGP) flours.

Enzyme Activity	F value (8, 18) †	Yellow Pea Samples								
		WT	TGP-2	TGP-3	TGP-4	TGP-5	TGP-6	TGP-7	TGP-8	TGP-9
Specific LOX activity (U/mg)	29.135 ***	170.18^{a} (14.64)	71.85^{c} (6.7)	94.75^{bc} (8.29)	89.04^{bc} (8.37)	93.86^{bc} (14.09)	103.2^{b} (6.39)	71.82^{c} (6.7)	100.6^{b} (5.58)	86.54^{bc} (8.90)
Fatty Acid Composition	**F value (8, 18)**	**WT**	**TGP-2**	**TGP-3**	**TGP-4**	**TGP-5**	**TGP-6**	**TGP-7**	**TGP-8**	**TGP-9**
Myristic acid (C14:0)	1.101 NS	0.23 (0.13)	0.35 (0.2)	0.23 (0.02)	0.17 (0.01)	0.17 (0.05)	0.18 (0.02)	0.18 (0.02)	0.18 (0.00)	0.35 (0.16)
Pentadecanoic acid (C:15)	11.770 ***	0.11^{c} (0.01)	0.14^{ab} (0.005)	0.15^{a} (0.005)	0.15^{a} (0.011)	0.14^{ab} (0.005)	0.15^{a} (0.007)	0.13^{b} (0.02)	0.14^{ab} (0.04)	0.15^{ab} (0.02)
Palmitic acid (16:0)	1.571 NS	11.53 (1.20)	11.39 (0.64)	11.42 (0.44)	11.62 (0.50)	10.88 (0.48)	11.47 (0.59)	10.42 (0.16)	10.81 (0.40)	11.59 (0.20)
Palmitoleic acid (C16:1)	4.556 *	0.070^{a} (0.006)	0.063^{ab} (0.010)	0.063^{ab} (0.005)	0.060^{abc} (0.003)	0.062^{abc} (0.003)	0.062^{abc} (0.002)	0.058^{abc} (0.008)	0.056^{bc} (0.001)	0.054^{c} (0.002)
Margaric acid (C17:0)	2.104 NS	0.17 (0.02)	0.19 (0.01)	0.19 (0.01)	0.19 (0.01)	0.18 (0.01)	0.18 (0.01)	0.18 (0.00)	0.19 (0.00)	0.20 (0.00)
Stearic acid (C 18:0)	6.700 ***	4.05^{a} (0.37)	3.55^{b} (0.10)	3.72^{ab} (0.14)	3.56^{b} (0.06)	3.39^{b} (0.05)	3.47^{b} (0.05)	3.42^{b} (0.03)	3.61^{b} (0.01)	3.36^{b} (0.06)
Oleic acid (C18:1) n-9	143.257 ***	27.83^{e} (0.88)	28.40^{de} (0.50)	32.89^{a} (0.13)	30.63^{b} (0.29)	30.26^{bc} (0.33)	29.39^{cd} (0.40)	25.94^{f} (0.13)	25.46^{f} (0.41)	23.44^{g} (0.20)
Linoleic acid (C18:2)	195.731 ***	45.25^{c} (0.39)	45.66^{c} (0.70)	42.27^{e} (0.26)	44.47^{d} (0.2)	45.35^{cd} (0.08)	45.4^{cd} (0.01)	49.05^{b} (0.05)	49.10^{b} (0.24)	50.50^{a} (0.38)
α-Linolenic acid (C18:3) n-3	27.181 ***	7.74^{c} (0.26)	7.38^{cd} (0.2)	6.48^{e} (0.4)	7.08^{de} (0.13)	7.56^{cd} (0.10)	7.67^{cd} (0.14)	8.61^{a} (0.03)	8.42^{ab} (0.17)	7.96^{bc} (0.07)
Arachidic acid (C20:0)	5.232 **	0.38^{a} (0.01)	0.366^{ab} (0.013)	0.370^{ab} (0.004)	0.351^{b} (0.003)	0.345^{b} (0.005)	0.371^{ab} (0.009)	0.36^{ab} (0.01)	0.37^{ab} (0.01)	0.34^{b} (0.01)
Behenic acid (C22:0)	26.637 ***	0.23^{a} (0.01)	0.156^{b} (0.002)	0.156^{b} (0.003)	0.146^{b} (0.003)	0.138^{b} (0.003)	0.147^{b} (0.003)	0.14^{b} (0.01)	0.13^{b} (0.02)	0.14^{b} (0.01)
Erucic acid (C22:1)	1.253 NS	0.05 (0.01)	0.044 (0.01)	0.061 (0.01)	0.062 (0.01)	0.053 (0.01)	0.060 (0.01)	0.05 (0.02)	0.04 (0.01)	0.05 (0.01)
Lignoceric acid (C24:0)	1.803 NS	0.38 (0.02)	0.35 (0.07)	0.35 (0.02)	033 (0.02)	0.32 (0.02)	0.32 (0.03)	0.32 (0.01)	0.32 (0.04)	0.31 (0.01)
Others ♣	4.203 **	1.98^{a} (0.26)	1.90^{ab} (0.70)	1.90^{ab} (0.70)	1.59^{ab} (0.20)	1.13^{ab} (0.04)	1.10^{b} (0.02)	1.08^{ab} (0.01)	1.13^{ab} (0.01)	1.06^{ab} (0.08)
∑ SFA	1.983 NS	17.25 (1.68)	17.03 (1.36)	16.80 (0.69)	16.57 (0.56)	15.60 (0.55)	16.33 (0.68)	15.20 (0.18)	15.78 (0.48)	16.94 (0.66)
∑ MUFA	132.557 ***	28.42^{d} (0.91)	28.96^{cd} (0.53)	33.51^{a} (0.11)	31.17^{b} (0.29)	30.82 ^{b} (0.35)	29.92 ^{bc} (0.45)	26.48^{e} (0.13)	25.98^{e} (0.42)	23.95^{f} (0.23)
∑ PUFA	119.466 ***	53.54^{b} (0.75)	53.36^{b} (0.91)	49.07^{d} (0.68)	51.64^{c} (0.24)	52.96^{bc} (0.19)	53.13^{b} (0.25)	57.70^{b} (0.06)	57.64^{b} (0.07)	58.56^{b} (0.45)
PUFA/SFA	6.174 ***	3.13^{bc} (0.36)	3.14^{bc} (0.30)	2.92^{c} (0.15)	3.11^{bc} (0.12)	3.39^{ab} (0.13)	3.25^{ab} (0.14)	3.80^{a} (0.05)	3.65^{ab} (0.11)	3.46^{ab} (0.16)
ω-6/ω-3	7.626 ***	5.67^{c} (0.22)	5.97^{bc} (0.14)	6.51^{a} (0.42)	6.25^{ab} (0.09)	5.98^{bc} (0.06)	5.90^{bc} (0.09)	5.68^{c} (0.02)	5.82^{bc} (0.15)	6.32^{ab} (0.04)

• Mean values (followed in brackets by the standard deviation) within the same row with the same letter are not significantly different when a probability level of 0.05 is applied. All analyses were conducted in three replicates (n = 3).
• MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids.
• Wild Type (WT), Transgenic 2 to 9; (TGP-2 to TGP-9).
• NS, not significant $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
• †Numbers in the brackets reflect the degrees of freedom: F-value (dfbetween, dfwithin).
• ♣ Includes unidentified acids.

TABLE 4 Concentrations of selected volatile compounds (μg/100g of flour) in wild type (WT) and transgenic yellow pea (TGP) flours.

Volatile Organic Compound (VOC)	Probable origin	Ref	Odor description	LRI♠	F Value (8, 33)†	WT	TGP-2	TGP-3	TGP-4	TGP-5	TGP-6	TGP-7	TGP-8	TGP-9
Hexanal	Linoleic acid	(Eriksson, 1975) (Murray et al., 1976) (Jelen and Wasowicz, 2011) (Murat et al., 2013) (Mandal et al., 2014) (Lampi et al., 2020) (Belitz et al., 2009)	Green, Grassy	881	10.574 ***	25.321^{a} (9.213)	9.546^{bc} (3.83)	19.860^{ab} (5.90)	7.360^{bc} (1.93)	7.090^{bc} (1.16)	7.150^{bc} (0.96)	5.736^{bc} (0.440)	13.384^{bc} (3.550)	11.634^{bc} (2.956)
2-Hexenal	Linoleic acid Linolenic acid	(Murray et al., 1976) (Sessa & Rackis, 1977) (Mandal et al., 2014) (Jelen and Wasowicz, 2011) (Lampi et al., 2020)	Green, Leafy	958	19.613 ***	8.910^{a} (3.621)	0.648^{b} (0.24)	6.462^{a} (1.35)	0.672^{b} (0.25)	0.476^{b} (0.21)	0.522^{b} (0.13)	0.353^{b} (0.033)	1.757^{b} (0.764)	2.543^{b} (0.792)
1-Hexanol	Linoleic acid	(Murray et al., 1976) (Jelen and Wasowicz, 2011) (Matoba et al., 1989)	Green, Herbacious	978	0.680 NS	2.672^{a} (1.689)	1.866^{a} (0.60)	2.032^{a} (0.43)	1.686^{a} (0.46)	2.126^{a} (0.16)	2.061^{a} (0.09)	2.053^{a} (0.208)	2.102^{a} (0.215)	1.851^{a} (0.748)
Heptanal	Linoleic and oleic acids Linolenic acid	(Murray et al., 1976) (Belitz et al., 2009) (Lampi et al., 2020) (Sessa & Rackis, 1977)	Green, Fatty, Pungent	984	4.155 **	2.216^{a} (1.179)	1.255^{ab} (0.47)	1.376^{ab} (0.26)	0.769^{b} (0.11)	0.819^{b} (0.13)	0.927^{b} (0.10)	0.731^{b} (0.043)	1.210^{ab} (0.497)	0.977^{b} (0.173)
(E)-2-Heptenal	Linoleic acid α-Linolenic acid	(Hoffmann, 1962) (Eriksson, 1975) (Sessa and Rackis, 1977) (Belitz et al., 2009) (Lampi et al., 2020) (Murray et al., 1976) (Belitz et al., 2009)	Beany, Pungent, Green	1064	8.583 ***	7.785^{a} (3.309)	2.637^{b} (0.55)	3.429^{b} (0.32)	1.913^{b} (0.50)	1.767^{b} (0.41)	2.069^{b} (0.26)	1.855^{b} (0.106)	2.918^{b} (1.601)	3.195^{b} (1.298)
1-Octen-3-ol	Linoleic acid α-Linolenic and Linoleic acids	(Lampi et al., 2020) (Hoffmann, 1962) (Khrisanapant et al., 2019) (Murray et al., 1976) (Zhang et al., 2020) (Lumen et al., 1978) (Mandal et al., 2014)	Earthy, Mushroom, Vegetable	1082	5.590 **	7.779^{a} (3.80)	3.114^{b} (1.02)	3.768^{b} (0.52)	2.694^{b} (0.48)	2.717^{b} (0.30)	3.085^{b} (0.74)	2.717^{b} (0.39)	3.646^{ab} (0.74)	3.140^{b} (0.59)

(Continued)

10.3389/fpls.2023.1246905

TABLE 4 Continued

Volatile Organic Compound (VOC)	Probable origin	Ref	Odor description	LRI♠	F Value (8, 33)†	WT	TGP-2	TGP-3	TGP-4	TGP-5	TGP-6	TGP-7	TGP-8	TGP-9
Furan-2-Pentyl	Linoleic acid	(Min and Boff, 2002)	Green	990	4.921 *	42.657^{a} (21.406)	16.205^{b} (5.70)	18.353^{ab} (5.54)	9.672^{b} (2.32)	11.844^{b} (1.51)	13.021^{b} (2.74)	11.367^{b} (2.697)	22.204^{ab} (12.743)	24.419^{ab} (12.283)
Octanal	Oleic acid Linoleic acid	(Murray et al., 1976) (Belitz et al., 2009) (Belitz et al., 2009)	Green	1088	2.796 *	4.423^{a} (3.015)	1.781^{b} (0.85)	1.343^{b} (0.34)	0.784^{b} (0.39)	0.923^{b} (0.17)	1.348^{b} (0.26)	1.109^{b} (0.247)	1.477^{b} (0.622)	1.289^{b} (0.544)
(E,E)-2,4-Heptadienal	α-Linolenic acid	(Murray et al., 1976) (Lampi et al., 2020)	Rancid hazelnut, Brown beany	1138	8.192 **	3.897^{a} (1.804)	1.130^{b} (0.18)	1.479^{b} (0.05)	0.810^{b} (0.20)	0.616^{b} (0.28)	0.877^{b} (0.10)	0.786^{b} (0.106)	1.640^{b} (0.643)	1.739^{b} (0.853)
(E)-2-Octenal	Linoleic acid	(Murray et al., 1976) (Sessa & Rackis, 1977) (Belitz et al., 2009) (Murat et al., 2013) (Lampi et al., 2020)	Green, Leafy, Brown, Pea, Vegetable	1168	7.885 ***	3.678^{a} (1.595)	1.139^{b} (0.36)	1.547^{b} (0.25)	0.754^{b} (0.26)	0.685^{b} (0.22)	0.900^{b} (0.15)	0.739^{b} (0.153)	1.667^{b} (0.798)	1.446^{b} (0.909)
(E,E)-2,4-Nonadienal	Linoleic acid α-Linolenic acid	(Murray et al., 1976) (Sessa & Rackis, 1977) (Jelen and Wasowicz, 2011)	Green, Grassy, Sweat	1348	6.033 **	1.723^{a} (0.908)	0.636^{b} (0.20)	0.737^{ab} (0.04)	0.352^{b} (0.09)	0.289^{b} (0.11)	0.403^{b} (0.08)	0.324^{b} (0.086)	0.887^{ab} (0.452)	0.685^{b} (0.390)

- Mean values (followed in brackets by the standard deviation) within the same row with the same letter are not significantly different when a probability level of 0.05 is applied. All analyses were conducted in triplicate (n=3) except for Wild Type (n=7).
- Wild Type (WT), Transgenic 2 to 9; (TGP-2 to TGP-9).
- NS, not significant $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
- ♠ Linear Retention Index (LRI): calculated using the retention time obtained for a series of n-alkane (C8-C20, 40 µg/ml hexane) as described previously (Aliani and Farmer, 2005).
- †Numbers in the brackets reflect the degrees of freedom: F-value (dfbetween, dfwithin).

10.3389/fpls.2023.1246905

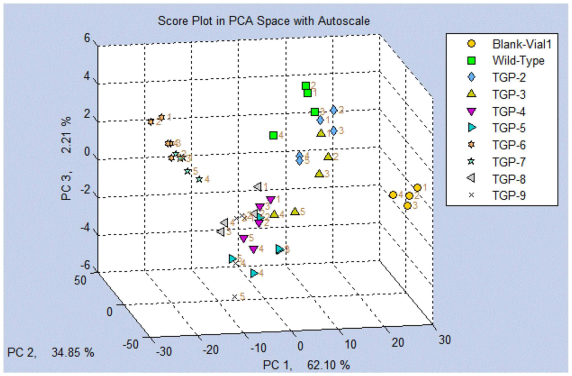


FIGURE 5
Score plot using principal component analysis (PCA) for Wild Type (WT) and all transgenic yellow pea flours (TGP-2 to -9) by SENSIGENT MSEM 160 Portable Odor and Chemical Monitor System.

In addition, the overall correlations between selected VOCs, FAs, sensor responses, and LOX activity in WT and TGP lines are presented in the PLS-R plot presented in Figure 7. The eight TGP lines were clustered separately in different areas on the plot opposite to WT lines which were located in the quadrant close to the LOX activity, sensor responses, and also with the majority of VOCs responsible for unpleasant aromas. Overall, the plot depicts a positive association between the LOX activity and VOC formation and is a useful visual model to examine the significant

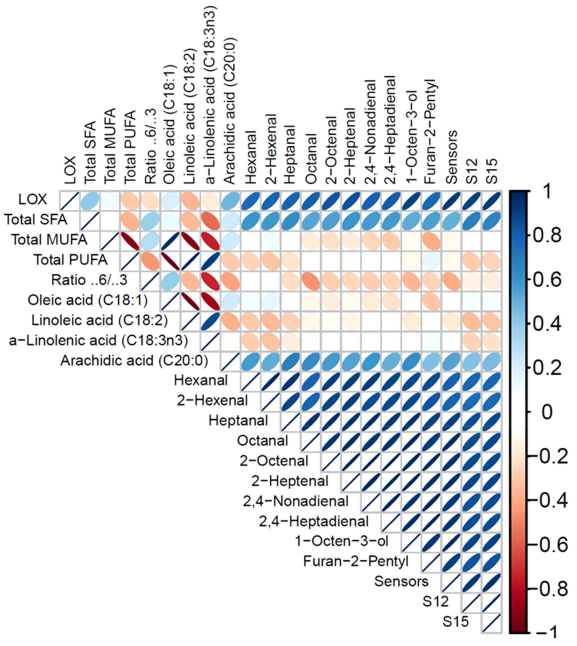


FIGURE 6
R Correlogram for LOX activity, selected fatty acids (FAs), and concentration of volatile organic compounds (VOCs) in Wild Type and all Transgenic Yellow Pea Flours (TGP-2 to -9). The correlogram is color-coded with the correlation scale indicated. Blue color shows a positive correlation and red color a negative correlation, as indicated by opposite directions of ellipses within each square. The width of the ellipse represents the strength of the correlation, perfect linear is just a line.

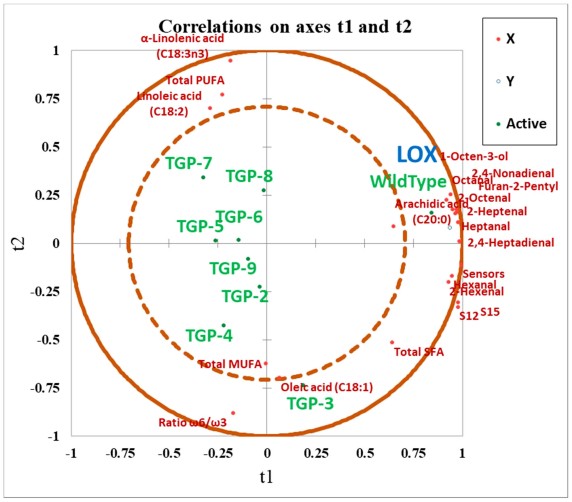


FIGURE 7
Partial least squares regression (PLS-R) plot for wild type and all transgenic yellow pea flours (TGP-2 to -9). Observations showing the correlation between *X* (t1) and *Y* (t2) variables where: *X* variables • = volatile organic compounds (VOCs) responsible for beany off-flavor, selected fatty acids, and E-nose sensor response; *Y* variable • = LOX activity observations; ○ = Wild Type, and transgenic yellow pea flours.

results as a whole rather than discussing them one by one. It is also clear that as expected for a *LOX* mutant, the SFAs and MUFAs were not affected by CRISPR/Cas9 gene editing of the *PsLOX2* gene and these FAs were all found to be associated with WT lines.

4 Conclusions

The results presented in this study demonstrated a successful CRISPR-Cas9 mediated editing of the pea *PsLOX2* gene. The results obtained for LOX activity, the known substrates, and products of this enzyme confirmed that this mutation led to lower activity of LOX in all mutant pea lines with a significant decrease in several VOCs generated from some PUFAs. Among all the detected VOCs, (E,E)-2,4-heptadienal may be of particular interest for monitoring the success of CRISPR-Cas9 mediated editing of the pea *PsLOX2* gene.

The use of sensors to monitor the headspace of the pea flours in less than 3 minutes provided a rapid and sensitive tool for screening the VOCs profile of any transgenic crop which can accelerate breeding and gene discovery in pea and other important legume crop plants.

5 Materials and methods

5.1 Pea growth conditions

Yellow pea seeds of the elite genotype CDC Spectrum were obtained from the Crop Development Centre, University of Saskatchewan (Dr. Tom Warkentin's pea breeding program).

Plants were grown in a controlled environment chamber at 24°C with a 16 h photoperiod at 200 µmol quanta m^{-2} s^{-1}.

5.2 Amplification of *PsLOX2* from cv. CDC Spectrum, identification and designing of gRNAs, and CRISPR/Cas9 vector construction

Primers (all primers are listed in Table 5) were designed based on the accession X78580 (GenBank) and were used to PCR amplify the first three exons of *PsLOX2* from the pea cv. CDC Spectrum (Warkentin et al., 2017). The resulting PCR product was cloned into the *EcoRV* site of pBluescript using Gibson assembly and sequenced using primers 3/4. The web-application CCTop (https://crispr.cos.uni-heidelberg.de; Stemmer et al., 2015) was then used to identify gRNA sites in this sequence. The five gRNA sites located in the first three exons with the highest CRISPRater scores (Labuhn et al., 2018) were selected for *in vitro* testing. CRISPRater is an algorithm which predicts the efficiency of a gRNA based on its sequence. *In vitro* tests were performed as described by Kwon et al. (2023). Briefly, DNA templates for *in vitro* transcription of gRNA candidates were produced using PCR with primers 5–9 and a universal reverse primer (10) from a tracrRNA template used in a previous study (Kwon et al., 2023; Invitrogen, Waltham, MA, USA). gRNAs were prepared using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, Ipswich, MA, USA). The cleavage template was prepared from the pBluescript clones using primers 3/4. Recombinant Cas9 was prepared as described by Kwon et al. (2023). The gRNA, cleavage template, and recombinant Cas9 were incubated at 37°C for 1 h and the resulting DNA fragments were analyzed using gel electrophoresis.

TABLE 5 List of primers used in this work.

No.	Sequence
1	GCTGCAGGAATTCGAT ATGTTTCCAAATGTGACAGGACTCC
2	CGGTATCGATAAGCTTGAT CTTGTATCTAACCTTTCCTCGTTGG
3	CACACAGGAAACAGCTATGA
4	CGTTGTAAAACGACGGCCAG
5	GAAATTAATACGACTCACTATAGGCCACTGTCCCTCTTATCTTG GTTTTAGAGCTAGAAA
6	GAAATTAATACGACTCACTATAGGATTGGTGGTGGAAACGTCCA GTTTTAGAGCTAGAAA
7	GAAATTAATACGACTCACTATAGGGATGGCCTTACTGCCTTCTT GTTTTAGAGCTAGAAA
8	GAAATTAATACGACTCACTATAGGTTTAGGAGTCCTGTCACATT GTTTTAGAGCTAGAAA
9	GAAATTAATACGACTCACTATAGGAGTGGAGACGGTGTTTCACT GTTTTAGAGCTAGAAA
10	AAAAAAGCACCGACTCGGTGC
11	AGGCTACCGCATAAGTCC
12	CCACTGTCCCTCTTATCTTG GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG
13	AAGAAGGCAGTAAGGCCATC TGCACCAGCCGGGAATC
14	GATGGCCTTACTGCCTTCTT GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG
15	ACTGGTGATTTTTGCGGACT TGCACCAGCCGGGAATC
16	TTCGATTCCCGGCTGGTGCA CCACTGTCCCTCTTATCTTG
17	AAGAAGGCAGTAAGGCCATC
18	TGAGAATTAAGGGAGTCACGTTATGACC
19	CGGCCATTTTCCACCATGATATT

Underlined sections are overhangs.

The *PsLOX2* CRISPR vector was prepared as described by Kwon et al. (2023). Briefly, gRNAs 1 and 3 were selected for cloning into the CRISPR vector based on the *in vitro* cleavage assay. To express both gRNAs simultaneously, a self-processing tRNA-gRNA array was implemented (Xie et al., 2015). The tRNA-gRNA arrays were constructed using Gibson assembly. To generate this unique gRNA, overhang sequences were added to tracrRNA-tRNA templates from a previous study (Kwon et al., 2023; Invitrogen, Waltham, MA, USA) using primers 12/13 and 14/15 and PCR amplification to produce two gRNA fragments. The first gRNA fragment underwent an additional PCR step using primers 16/17 to add an additional tRNA overhang to allow for Gibson assembly. The gRNA fragments were added to the *BasI* site of a pENTR/D-TOPO (Thermo Fisher Scientific, Waltham, MA, USA) vector containing a synthetic gRNA expression cassette driven by the cauliflower mosaic virus 35S (35S) promoter using Gibson assembly. The completed gRNA expression cassette was then added to a binary destination vector containing a 35S driven Cas9 expression cassette using Gateway cloning, resulting in the CRISPR vector targeting *PsLOX2* used in this work.

5.3 Validation of gRNAs using mesophyll protoplasts

Tissue for preparation of mesophyll protoplasts was prepared from pea plants grown *in vitro*. First pea seeds were surface sterilized with 75% ethanol for 1 min in a 15 mL centrifuge tube. The ethanol was removed and then 10 mL of bleach solution was added. The seeds were incubated in the bleach solution for 15 min on a shaker. Seeds were then washed five time in sterilie water and placed on germination media (PhytoTech Labs; Product ID A 1375) in Magenta boxes and placed in a phytochamber. Leaf tissue from three week old plants was surface sterilized with 70% ethanol and cut into 0.5–1 mm strips using a fresh scalpel blade. The strips were then quickly transferred to a dish containing 10 mL of 0.6 M mannitol and incubated for 10 min on a shaker at 50 rpm in the dark. The mannitol solution was then removed and the leaf tissue was incubated in 10 mL of warm enzyme (37°C) solution consisting of 0.6 M mannitol, 10 mM MES, 1.5% Cellulase R-10, 0.75% Macerozyme R-10, 10 mM $CaCl_2$, and 0.1% BSA. The dish was placed back onto the shaker at 50 rpm for 4 h in the dark. Protoplasts freed from the leaf tissue were isolated with a 40 µm filter, and washed with W5 consisting of 5 mM KCl, 154 mM NaCl,

125 mM $CaCl_2$, and 2 mM MES pH 5.7, and added to the top of 5 mL of 0.55 M sucrose in a round bottom culture tube (Fisher Scientific, Hampton, NH). Protoplasts were further purified by centrifugation for 10 min at 100 g and 4°C in a swinging bucket rotor without using brakes. A green intermediate layer formed and was carefully transferred to a sterile round bottom culture tube. Then 7 mL of W5 solution was carefully added to the protoplasts followed by another centrifugation for 5 min at 100 g with brakes on. The supernatant was discarded and 7 mL of W5 solution was added to the purified protoplasts followed by a 2 min centrifugation at 100 g with brakes on. Lastly, the supernatant was removed and 1 mL of W5 solution was added to the purified protoplasts. A hemocytometer was used to estimate the number of protoplasts isolated.

MMG (0.5M mannitol, 15mM $MgCl_2$ and 4mM MES; pH 5.7) solution was used to dilute the protoplasts to a concentration of 2×10^6 protoplasts mL^{-1}. Thirty µg of the *PsLOX2* CRISPR vector was added to a 2 mL microcentrifuge tube followed by 200 µL of the protoplast solution. The vector and protoplasts were then gently mixed. 230 µL of PEG solution was then added to the protoplasts and fully mixed with gentle tapping and inverting the tube several times. The mixture was then incubated for 30 min at room temperature in the dark. Two volumes (900 µL) of W5 solution was then added to the mixture and fully mixed by inverting the tube several times. This halted the transfection process. Finally, the transfected protoplasts were centrifuged for 5 min at 100 rpm.

Genomic DNA was extracted 72 h after transfection from the protoplasts and used to detect CRISPR-mediated mutations. *PsLOX2* was then amplified from the extracted protoplast genomic DNA as described above. The resulting PCR products were sequenced using primer 11. PCR products were determined to be mutated if their sequencing chromatograms contained mixed signals occurring near the double stranded break (DSB) site of either gRNA site.

5.4 Pea transformation and genotype analysis

Mutant *pslox2* pea lines were produced by *Agrobacterium*-mediated transformation of thin slices from developing embryo axes following Polowick et al. (2000) with slight modification. Colonies of *Agrobacterium tumefaciens* strain EHA105 harboring the CRISPR vector targeting *PsLOX2* were grown for 16–18 h at 28°C and 200 rpm in LB broth supplemented with 50 mg/L spectinomycin and 50 mg/L rifampicin. The overnight culture was pelleted by centrifugation at 3,000 rpm for 25 min. The pelleted cells were used to prepare infection media by resuspending them in MS media supplemented with 100 µM acetosyringone up to an OD_{600} of 0.2. Pea seeds cv. CDC Spectrum were surface sterilized, as described for protoplast isolation, and imbibed for 20 h in sterile water. Cotyledons were removed from the embryonic axes and explants were prepared by removing the top and bottom of the axes and slicing them four to five times perpendicular to their long axis. To prevent bacterial overgrowth, sterile filter paper was placed onto cocultivation media consiting of 1× B5 media, 3.0% sucrose, 0.8% phytoagar, 3 g/L KNO_3, 770 mg/L $CaCl_2$, 800 mg/L L-glutamine, 500 mg/L $MgSO_4{\cdot}7H_2O$, 100 mg/L L-serine, 10 mg/L glutathione, 1 mg/L adenine, 1 mg/L 2,4-D, 0.2 mg/L kinetin, and pH 5.7. The explants were placed onto the filter paper and then covered with 5 µL of infection media and incubated for 4 days. Following co-cultivation, the explants were transferred to callus-inducing media consisting of 1× MS macro- and micronutrients, 1× B5 vitamins, 3.0% sucrose, 0.8% phytoagar, 2 mg/L BAP, 2 mg/L NAA, 150 mg/L Timentin©, 40 mg/L kanamycin, and pH 5.7. The explants were incubated for 2 weeks on callus-inducing media, after which the calli that formed were transferred to shoot-inducing media consisting of 1× MS macro- and micro-nutrients, 1× B5 vitamins, 3.0% sucrose, 0.8% phytoagar, 4.5 mg/L BAP, 0.02 mg/L NAA, 150 mg/L Timentin©, 50 mg/L kanamycin, and pH 5.7. Incubation for 6–9 weeks was required to induce shoot growth, with fresh media being supplied every 2 weeks. Shoots that were 0.5–1 cm in length were transferred to root-inducing media consisting of 0.5× MS, 3.0% sucrose, 0.8% phytoagar, 0.185 mg/L NAA, 150 mg/L Timentin©, and pH 5.7. When the shoots were 5–6 cm in length, with prolific root growth, the plantlets were transferred to soil. The plantlets in soil were transferred to resealable plastic bags to allow them to slowly harden by gradually opening the plastic bags over 2 weeks.

Genomic DNA was extracted from 20–50 mg of leaf tissue of plants brought out of tissue culture and T_2 plants derived from them using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Plants were determined to be transgenic using primers 18/19 which are specific to the *neomycin phosphotransferase II* of the CRISPR vector. The *PsLOX2* target locus of transgenic plants was analyzed for mutations in the same way described for protoplasts.

5.5 Lipoxygenase assay

LOX assays were conducted in triplicates by preparing enzyme extracts from 100 mg of ground seeds (WT, TGP-2 to -9) with 1 mL of 50 mM sodium phosphate buffer pH 6.8 containing 4 mM sodium sulfite and 2 mM sodium ascorbate at 4°C for 4 h. Samples were centrifuged and subjected to fractionation to obtain a crude LOX fraction as described previously (Domoney et al., 1990). In brief, samples were brought to 25% saturation with ammonium sulfate, incubated on ice for 10 minutes, and centrifuged. Recovered supernatants were then brought to 60% saturation with ammonium sulfate and again incubated and centrifuged. The resulting pellets were resuspended in 500 µL of 50 mM sodium phosphate pH 6.8 giving the crude LOX fraction from the samples. This preparation was subjected to protein quantification by direct measurement of absorbance at 280 nm and used in the LOX assays described below.

LOX substrate was prepared by dissolving 4.5 µL linoleic acid (30 mM final concentration) in 500 µL of 50 mM sodium phosphate pH 6.8 containing 2% v/v Tween-20. Reactions were performed in triplicate and assembled by combining 200 µL of aerated 50 mM sodium phosphate pH 6.8, 5 µL of the crude LOX fraction, and 2 µL of 30 mM linoleic acid substrate solution. Reactions were vortexed and the absorbance at 234 nm was measured immediately

and again after five minutes on a Nanodrop One UV-Visual spectrophotometer. LOX activity was determined by subtraction of the initial from the final absorbance values divided by the exact time between measurements, with one unit of activity defined as an increase of 0.001 in absorbance at 234 nm with a path length of 1 cm. Data were combined and reported as the average with the standard error. Specific activity was calculated as unit activity per milligram protein (U/mg) in the partially purified LOX fraction with appropriate error propagation.

5.6 Fatty acid extraction and analysis by GC-FID

Lipids were extracted from pea flour samples (WT, TGP-2 to -9) based on Folch et al. (1957) with some modifications (Shariati-Ievari et al., 2016). The extracted methylated fatty acids were analyzed using a gas chromatograph coupled with a flame ionization detector (GC-FID). The GC-FID column was held at 100°C for 2 min before increasing to 175°C at 25°C/min for 30 min. The column temperature was then increased to 220˚C at 15°C/min and held for another 10 min. The final temperature reached 240°C at 20°C/min and was held for 11 min. Each sample was run for a total of 60 min with a split ratio of 10:1 and a flow rate of 1.8 mL/min. Hydrogen was used as a carrier gas. A set of high-purity fatty acid standards (Nu-check; Funakoshi Co. Ltd.) was used for the quantification of fatty acids in each sample (Shariati-Ievari et al., 2016). Lipid extractions and fatty acid analysis were conducted in four replicates for each flour sample.

5.7 Extraction of volatile organic compounds (VOCs) using SPME fibers

Volatile extractions were performed using solid-phase microextraction (SPME) fibers (75µm Carboxen. PDMS, Supleco), with 5 g of each of the pea flour samples (WT, TGP-2 to -9) that were mixed with 10 g of NaCl and 90 mL of Milli-Q water in a 250 mL PYREX^{TM} bottle. An internal standard, 1,2-dichlorobenzene (50 µL of 1 ng/mL in methanol), was added to the mixture before the SPME extraction. The Pyrex bottle was placed in a water bath held at 90°C (using CORNING PC-420D heater/magnetic stirrer) and the mixture was stirred carefully with a magnetic stirrer to prevent clump formation. A hole was made in the lid through which the SPME needle was introduced to the headspace above the mixture. The extraction was performed for 60 min.

5.8 GC-MS analysis of VOCs

Collected volatiles on SPME fibers were immediately analyzed using a 7890B GC with a 7693 Auto-Sampler connected to a 7000 GC/Triple Quadrupole mass spectrometry (MS) detector (Agilent Ltd, USA). The column was a HP-5MS (5%-phenyl)-methylpolysiloxane (Agilent Technologies; Santa Clara, CA, USA; 30 m × 250 µm × 0.25 µm film thickness). The carrier gas was helium (99.999% purity, Air Liquide Canada Inc., Montreal, QC, Canada) maintained at a constant flow (1 mL/min) with nitrogen (99.999% purity, Air Liquide Canada Inc.) as the make-up gas. The run was performed with the following temperature gradient, the initial 40°C temperature was held for 1 min, followed by a temperature increase of 4°C/min up to 200°C, a hold for 1 min, and a second increase of 10°C/min to 270°C which was held for 1 min for a total run of 54 min. The injector, at a temperature of 250°C, was operated in splitless mode with an injection volume of 1 µL. The Agilent Ltd. Triple Quadrupole MS was operated in electron impact (EI) mode with an ionization energy of 70 eV. The GC-MS transfer line and ion source temperatures were set at 250°C and 230°C, respectively. All analyses were performed in full scan mode with a scanning range of 29 to 500 m/z. A 3.7 min solvent delay was used to eliminate possible interfering solvent peaks from the chromatogram.

The semi-quantification of each volatile was calculated from the ratio of the base ion peak area for each VOC to the internal standard's (m/z 146) base ion peak. The identity of each peak was determined by matching their mass spectra with the mass spectra of authentic compounds analyzed and reported in the National Institute of Standards and Technology (NIST version 2.3, 2017) library. The relative linear retention indices (LRIs) of each of the compounds were also calculated using the retention time obtained for a series of n-alkanes (C8–C20, 40 µg/mL hexane) as described previously (Aliani and Farmer, 2005).

5.9 E-nose sensor analysis

The WT and transgenic yellow pea flours (2g) were placed in a 20 mL glass tube covered with GC-MS vial septa lids where a hose equipped with a needle was placed for VOCs and chemical collection using a Sensigent MSEM 160. The collection was done over 60 sec with a pre-collection and purging time of 60 and 60 sec, respectively. Air was used as a blank and all analyses were performed using 5 replicates.

5.10 Statistical analysis

The differences in LOX activity, FAs, and VOCs in the different pea flours were determined using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The relationship between LOX activity (Y variable) and the other measured parameters (X variables including the concentration of FAs, E-nose sensor response, and selected VOCs) was calculated using partial least squares regressions (PLS-R) using mean values for all variables (XLSTAT, version 19.4; Addinsoft, Paris, France). To visualize linear correlations between LOX activity and the concentration of the different VOCs and FAs in different pea flours, a correlogram was generated using R statistical package (version 4.02). The collected data was transferred into CDAnalysis software (Sensigent, Version 11.2), and processed using the specific parameters as follows: sensors ΔR/R as the data source, the baseline correction using the 'Adv min max' algorithm followed by

normalization 'Norm1' and generation of the principal component analysis (PCA). A stepwise multiple regression analysis was performed using SPSS with all detected FAs, VOCs, and sensor responses to determine variables that can be used to predict the transgenic effect of reduced LOX activity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PB conceptualized the study and all authors contributed to the writing and critical review of the manuscript. MA conceptualized the study, a co-applicant for the NRC funding, analytical and statistical analysis, interpretation, writing, and critical review of the manuscript. SS-I provided technical support for extraction and analysis of fatty acids and volatile compounds. BP performed eNose sampling and LOX assay and WY conducted the pea transformation and plant regeneration CH and D-KR designed and cloned the CRISPR/Cas9 vector. All authors contributed to the article and approved the submitted version.

Funding

We thank the Aquatic and Crop Resource Development Research Centre (ACRD) of the National Research Council Canada (NRC) under the Sustainable Protein Production (SPP) program for providing in-kind research support and the publication fee and also acknowledge Saskatchewan Ministry of Agriculture (Agriculture Development Fund, ADF Project # 20180240) for providing research grant to PB. We thank the funding support from Alberta Innovates (grant number: 2018F160R) to DKR. CD also acknowledges support from the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/PBBS/E/J/000PR9799) and the John Innes Foundation.

Acknowledgments

The authors acknowledge the technical support from Dr. Patricia Polowick for the pea transformation protocol.

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.

Publisher's note

References

Aliani, M., and Farmer, L. J. (2005). Precursors of chicken flavor. II. Identification of key flavor precursors using sensory methods. *J. Agric. Food Chem.* 53, 6455–6462. doi: 10.1021/jf050087d

Badhan, S., Ball, A. S., and Mantri, N. (2021). First report of CRISPR/cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts. *Int. J. Mol. Sci.* 22, 1–15. doi: 10.3390/ijms22010396

Belitz, H.-D., Grosch, W., and Schieberle, P. (2009). *Food chemistry* (© Springer-Verlag Berlin Heidelberg).

Bertier, L. D., Ron, M., Huo, H., Bradford, K. J., Britt, A. B., and Michelmore, R. W. (2018). High-resolution analysis of the efficiency, heritability, and editing outcomes of CRISPR/Cas9-induced modifications of NCED4 in lettuce (Lactuca sativa). *G3 (Bethesda)* 8, 1513–1521. doi: 10.1534/g3.117.300396

Bhowmik, P., Konkin, D., Polowick, P., Hodgins, C. L., Subedi, M., Xiang, D., et al. (2021). CRISPR/Cas9 gene editing in legume crops: opportunities and challenges. *Legume Sci.* 3 (3), 1–16. doi: 10.1002/leg3.96

Cai, Y., Chen, L., Liu, X., Guo, C., Sun, S., Wu, C., et al. (2018). CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. *Plant Biotechnol. J.* 16, 176–185. doi: 10.1111/pbi.12758

Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., et al. (2015). CRISPR/Cas9-mediated genome editing in soybean hairy roots. *PLoS One* 10, e0136064. doi: 10.1371/journal.pone.0136064

Caprioli, G., Giusti, F., Ballini, R., Sagratini, G., Vila-Donat, P., Vittori, S., et al. (2016). Lipid nutritional value of legumes: Evaluation of different extraction methods and determination of fatty acid composition. *Food Chem.* 192, 965–971. doi: 10.1016/j.foodchem.2015.07.102

Conant, D., Hsiau, T., Rossi, N., Oki, J., Maures, T., Waite, K., et al. (2022). Inference of CRISPR edits from sanger trace data. *CRISPR J.* 5 (1), 123–130. doi: 10.1089/crispr.2021.0113

Curtin, S. J., Xiong, Y., Michno, J. M., Campbell, B. W., Stec, A. O., Cermak, T., et al. (2018). CRISPR/Cas9 and TALENs generate heritable mutations for genes involved in small RNA processing of Glycine max and Medicago truncatula. *Plant Biotechnol. J.* 16, 1125–1137. doi: 10.1111/pbi.12857

Davies, C., Nielsen, S., and Nielsen, N. (1987). Flavor improvement of soybean preparations by genetic removal of lipoxygenase-2. *J. Am. Oil Chemists' Soc.* 64, 1428–1433. doi: 10.1007/BF02636994

Domoney, C., Firmin, J. L., Sidebottom, C., Ealing, P. M., Slabas, A., and Casey, R. (1990). Lipoxygenase heterogeneity in Pisum sativum. *Planta* 181, 35–43. doi: 10.1007/BF00202322

Du, H., Zeng, X., Zhao, M., Cui, X., Wang, Q., Yang, H., et al. (2016). Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *J. Biotechnol.* 217, 90–97. doi: 10.1016/j.jbiotec.2015.11.005

Eriksson, C. (1975). Aroma compounds derived from oxidized lipids. Biochemical and analytical aspects. *J. Agric. Food Chem.* 23, 126–128. doi: 10.1021/jf60198a011

Fahmi, R., Ryland, D., Sopiwnyk, E., and Aliani, M. (2019). Sensory and physical characteristics of pan bread fortified with thermally treated split yellow pea (Pisum sativum L.) flour. *J. Food Sci.* 84 doi: 10.1111/1750-3841.14908

Fahmi, R., Ryland, D., Sopiwnyk, E., Malcolmson, L. J., Ievari-Shariati, S., McElrea, A., et al. (2021). Effect of revtech thermal processing on volatile organic compounds and chemical characteristics of split yellow pea (Pisum sativum L.) flour. *J. Food Sci.* 84, 1–24. doi: 10.1111/1750-3841.15913

FAOSTAT (2020) *FAO (Food and Agriculture Organisation of the United Nation)*. Available at: https://www.fao.org/faostat/en/#data/QCL.

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509. doi: 10.1016/S0021-9258(18)64849-5

Forster, C., North, H., Afzal, N., Domoney, C., Hornostaj, A., Robinson, D. S., et al. (1999). Molecular analysis of a null mutant for pea (Pisum sativum L.) seed lipoxygenase-2. *Plant Mol. Biol.* 39, 1209–1220. doi: 10.1023/A:1006173313548

Grela, E. R., and Günter, K. D. (1995). Fatty acid composition and tocopherol content of some legume seeds. *Anim. Feed Sci. Technol.* 52, 325–331. doi: 10.1016/0377-8401(94)00733-P

Han, J., Guo, B., Guo, Y., Zhang, B., Wang, X., and Qiu, L. J. (2019). Creation of early flowering germplasm of soybean by CRISPR/Cas9 technology. *Front. Plant Sci.* 10, 1446. doi: 10.3389/fpls.2019.01446

Hildebrand, D., and Hymowitz, T. (1982). Inheritance of lipoxygenase-1 activity in soybean seeds1. *Crop Sci.* 22, 851–853. doi: 10.2135/cropsci1982.0011183X002200040036x

Hoffmann, G. (1962). 1-Octen-3-ol and its relation to other oxidative cleavage products from esters of linoleic acid. *J. Am. Oil Chemists' Soc.* 39, 439–444. doi: 10.1007/BF02637660

Jacobs, T. B., LaFayette, P. R., Schmitz, R. J., and Parrott, W. A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol.* 15, 16. doi: 10.1186/s12896-015-0131-2

Jelen, H., and Wasowicz, E. (2011). "Lipid-derived flavor," in *Food Flavors: Chemical, Sensory, and Technological Properties*. Ed. H. Jelen (Boca Raton, FL: CRC Press), 65–93.

Ji, J., Zhang, C., Sun, Z., Wang, L., Duanmu, D., and Fan, Q. (2019). Genome editing in cowpea vigna unguiculata using CRISPR-Cas9. *Int. J. Mol. Sci.* 20, 1–13. doi: 10.3390/ijms20102471

Kanazashi, Y., Hirose, A., Takahashi, I., Mikami, M., Endo, M., Hirose, S., et al. (2018). Simultaneous site-directed mutagenesis of duplicated loci in soybean using a single guide RNA. *Plant Cell Rep.* 37, 553–563. doi: 10.1007/s00299-018-2251-3

Khrisanapant, P., Kebede, B., Leong, S. Y., and Oey, I. (2019). A comprehensive characterisation of volatile and fatty acid profiles of legume seeds. *Foods* 8, 1–19. doi: 10.3390/foods8120651

Kitamura, K., Davies, C., Kaizuma, N., and Nielsen, N. (1983). Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. *Crop Sci.* 23, 924–927. doi: 10.2135/cropsci1983.0011183X002300050026x

Kreplak, J., Madoui, M. A., Capal, P., Novak, P., Labadie, K., Aubert, G., et al. (2019). A reference genome for pea provides insight into legume genome evolution. *Nat. Genet.* 51, 1411–1422. doi: 10.1038/s41588-019-0480-1

Kwon, M., Hodgins, C. L., Salama, E. M., Dias, K. R., Parikh, A., Mackey, A. V., et al. (2023). New insights into natural rubber biosynthesis from rubber- deficient lettuce mutants expressing goldenrod or guayule *cis*-prenyltransferase. *New Phytol.* 239, 1098–1111. doi: 10.1111/nph.18994

Labuhn, M., Adams, F. F., Ng, M., Knoess, S., Schambach, A., Charpentier, E. M., et al. (2018). Refined sgRNA efficacy prediction improces large- and small-scale CRISPR-Cas9 applications. *Nucleic Acid Res.* 46 (3), 1375–1385. doi: 10.1093/nar/gkx1268

Lampi, A. M., Yang, Z., Mustonen, O., and Piironen, V. (2020). Potential of faba bean lipase and lipoxygenase to promote formation of volatile lipid oxidation products in food models. *Food Chem.* 311, 125982. doi: 10.1016/j.foodchem.2019.125982

Li, Z., Liu, Z. B., Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L., et al. (2015). Cas9-guide RNA directed genome editing in soybean. *Plant Physiol.* 169, 960–970. doi: 10.1104/pp.15.00783

Li, G., Liu, R., Xu, R., Varshney, R. K., Ding, H., Li, M., et al. (2023). Development of an Agrobacterium-mediated CRISPR/Cas9 system in pea (*Pisum sativum* L.). *Crop J.* 11, 132–139. doi: 10.1016/j.cj.2022.04.011

Lumen, B. O., Stone, E. J., Kazeniac, S. J., and Forsythe, R. H. (1978). Formation of volatile flavor compounds in green beans from linoleic and linolenic acids. *J. Food Sci.* 43, 698–702. doi: 10.1111/j.1365-2621.1978.tb02396.x

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al. (2015). A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant* 8, 1274–1284. doi: 10.1016/j.molp.2015.04.007

Mandal, S., Dahuja, A., Kar, A., and Santha, I. M. (2014). *In vitro* kinetics of soybean lipoxygenase with combinatorial fatty substrates and its functional significance in off flavour development. *Food Chem.* 146, 394–403. doi: 10.1016/j.foodchem.2013.08.100

Matoba, T., Sakurai, A., Taninoki, N., Saitoh, T., Kariya, F., Kuwahata, M., et al. (1989). n-Hexanol formation from n-hexanal by enzyme action in soybean extracts. *Journal of Food Science* 54, 1607–1610.

Michno, J. M., Wang, X., Liu, J., Curtin, S. J., Kono, T. J., and Stupar, R. M. (2015). CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. *GM Crops Food* 6, 243–252. doi: 10.1080/21645698.2015.1106063

Min, D. B., and Boff, J. M. (2002). "Lipid oxidation of edible oil," in *Food lipids: chemistry, nutrition, and biotechnology*, vol. 2 . Eds. C. C. Akoh and D. B. Min (Boca Raton: CRC Press).

Murat, C., Bard, M.-H., Dhalleine, C., and Cayot, N. (2013). Characterisation of odour active compounds along extraction process from pea flour to pea protein extract. *Food Res. Int.* 53, 31–41. doi: 10.1016/j.foodres.2013.03.049

Murcia, M. A., and Rincón, F. (1992). Size as source of variance in lipid composition of pea. *Food Chem.* 44, 29–35. doi: 10.1016/0308-8146(92)90253-X

Murray, K. E., Shipton, J., Whitfield, F. B., and Last, J. H. (1976). The volatiles of off-flavoured unblanched green peas (Pisum sativum). *J. Sci. Food Agric.* 27, 1093–1107. doi: 10.1002/jsfa.2740271204

Padhi, E. M. T., Liu, R., Hernandez, M., Tsao, R., and Ramdath, D. D. (2017). Total polyphenol content, carotenoid, tocopherol and fatty acid composition of commonly consumed Canadian pulses and their contribution to antioxidant activity. *J. Funct. Foods* 38, 602–611. doi: 10.1016/j.jff.2016.11.006

Pandey, P. K., Bhowmik, P., and Kagale, S. (2022). Optimized methods for random and targeted mutagenesis in field pea (Pisum sativum L.). *Front. Plant Sci.* 13, 995542. doi: 10.3389/fpls.2022.995542

Pathiratne, S. M., Shand, P. J., Pickard, M., and Wanasundara, J. P. D. (2015). Generating functional property variation in lentil (Lens culinaris) flour by seed micronization: Effects of seed moisture level and surface temperature. *Food Res. Int.* 76 (P1), 122–131. doi: 10.1016/j.foodres.2015.1003.1026

Pfeiffer, T., Hildebrand, D., and TeKrony, D. (1992). Agronomic performance of soybean lipoxygenase isolines. *Crop Sci.* 32, 357–362. doi: 10.2135/cropsci1992.0011183X003200020016x

Polowick, P. L., Quandt, J., and Mahon, J. D. (2000). The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. *Plant Sci.* 153 (2), 161–170. doi: 10.1016/s0168-9452(99)00267-8

Ryan, E., Galvin, K., O'Connor, T. P., Maguire, A. R., and O'Brien, N. M. (2007). Phytosterol, squalene, tocopherol content and fatty acid profile of selected seeds, grains, and legumes. *Plant Foods Hum. Nutr.* 62, 85–91. doi: 10.1007/s11130-007-0046-8

Sessa, D. J., and Rackis, J. J. (1977). Lipid-derived flavors of legume protein products. *J. Am. Oil Chemists' Soc.* 54, 468–473. doi: 10.1007/BF02671039

Shariati-Ievari, S., Ryland, D., Edel, A., Nicholson, T., Suh, M., and Aliani, M. (2016). Sensory and physicochemical studies of thermally micronized chickpea (Cicer arietinum) and green lentil (Lens culinaris) flours as binders in low-fat beef burgers. *J. Food Sci.* 81, S1230–S1242. doi: 10.1111/1750-3841.13273

Smith, A. K., and Circle, S. (1972). "Soybeans: chemistry and technology," in *Soybeans: chemistry, technology and utilization*. Avi Publishing, Westport.

Stemmer, M., Thumberger, T., del Sol Keyer, M., and Mateo, J. L. (2015). CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PloS One* 10 (4), e0124633. doi: 10.1371/journal.pone.0124633

Sun, X., Hu, Z., Chen, R., Jiang, Q., Song, G., Zhang, H., et al. (2015). Targeted mutagenesis in soybean using the CRISPR-Cas9 system. *Sci. Rep.* 5, 10342. doi: 10.1038/srep10342

Vancanneyt, G., Sanz, C., Farmaki, T., Paneque, M., Ortego, F., Castanera, P., et al. (2001). Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8139–8144. doi: 10.1073/pnas.141079498

Villalobos Solis, M. I., Patel, A., Orsat, V., Singh, J., and Lefsrud, M. (2013). Fatty acid profiling of the seed oils of some varieties of field peas (*Pisum sativum*) by RP-LC/ESI-MS/MS: Towards the development of an oilseed pea. *Food Chem.* 139, 986–993. doi: 10.1016/j.foodchem.2012.12.052

Wang, J., Kuang, H., Zhang, Z., Yang, Y., Yan, L., Zhang, M., et al. (2020). Generation of seed lipoxygenase-free soybean using CRISPR-Cas9. *Crop J.* 8 (3), 432–439. doi: 10.1016/j.cj.2019.08.008

Warkentin, T., Tar'an, B., Banniza, S., Vandenberg, A., Bett, K., Arganosa, G., et al. (2017). CDC Spectrum yellow field pea. *Can. J. Plant Science.* 98 (1), 230–232. doi: 10.1139/cjps-2017-0143

Xie, K., Minkenberg, B., and Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capacity with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci.* 112, 3570–3575. doi: 10.1073/pnas.1420294112

Yan, L., Zhai, Q., Wei, J., Li, S., Wang, B., Huang, T., et al. (2013). Role of tomato lipoxygenase D in wound-induced jasmonate biosynthesis and plant immunity to insect herbivores. *PLos Genet.* 9 (12), e1003964. doi: 10.1371/journal.pgen.1003964

Zhang, C., Hua, Y., Li, X., Kong, X., and Chen, Y. (2020). Key volatile off-flavor compounds in peas (*Pisum sativum* L.) and their relations with the endogenous precursors and enzymes using soybean (*Glycine max)* as a reference. *Food Chem.* 333, 127469. doi: 10.1016/j.foodchem.2020.127469

Zhu, S., Riaz, M. N., and Lusas, E. W. (1996). Effect of different extrusion temperatures and moisture content on lipoxygenase inactivation and protein solubility in soybeans. *J. Agric. Food Chem.* 44, 3315–3318. doi: 10.1021/jf960065e

Zilic, S., Sobajic, S., Mladenovic-Drinic, S., Kresovic, B., and Vasic, M. (2010). Effects of heat processing on soya bean fatty acids content and the lipoxygenase activity. *J. Agric. Sci.* 55, 55–64. doi: 10.2298/JAS1001055Z

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 29 September 2023
DOI 10.3389/fpls.2023.1255819

OPEN ACCESS

EDITED BY
Tong Zhang,
South China Agricultural University, China

REVIEWED BY
Phetole Mangena,
University of Limpopo, South Africa
Aftab Ahmad,
University of Agriculture, Faisalabad, Pakistan

*CORRESPONDENCE
Sarah Zanon Agapito-Tenfen
saag@norceresearch.no

RECEIVED 09 July 2023
ACCEPTED 08 September 2023
PUBLISHED 29 September 2023

CITATION
Subburaj S and Agapito-Tenfen SZ (2023) Establishment of targeted mutagenesis in soybean protoplasts using CRISPR/Cas9 RNP delivery via electro–transfection. *Front. Plant Sci.* 14:1255819. doi: 10.3389/fpls.2023.1255819

Establishment of targeted mutagenesis in soybean protoplasts using CRISPR/Cas9 RNP delivery via electro–transfection

Saminathan Subburaj and Sarah Zanon Agapito-Tenfen*

NORCE Norwegian Research Centre AS, Climate & Environment Department, Siva Innovasjonssenter, Tromsø, Norway

The soybean (*Glycine max* L.) is an important crop with high agronomic value. The improvement of agronomic traits through gene editing techniques has broad application prospects in soybean. The polyethylene glycol (PEG)-mediated cell transfection has been successfully used to deliver the CRISPR/Cas9-based ribonucleoprotein (RNP) into soybean protoplasts. However, several downstream analyses or further cell regeneration protocols might be hampered by PEG contamination within the samples. Here in this study, we attempted to transfect CRISPR/Cas9 RNPs into trifoliate leaf-derived soybean protoplasts using Neon electroporation to overcome the need for PEG transfection for the first time. We investigated different electroporation parameters including pulsing voltage (V), strength and duration of pulses regarding protoplast morphology, viability, and delivery of CRISPR/Cas9. Electroporation at various pulsing voltages with 3 pulses and 10 ms per pulse was found optimal for protoplast electro-transfection. Following electro-transfection at various pulsing voltages (500 V, 700 V, 1,000 V, and 1,300 V), intact protoplasts were observed at all treatments. However, the relative frequency of cell viability and initial cell divisions decreased with increasing voltages. Confocal laser scanning microscopy (CLSM) confirmed that the green fluorescent protein (GFP)-tagged Cas9 was successfully internalized into the protoplasts. Targeted deep sequencing results revealed that on-target insertion/deletion (InDel) frequencies were increased with increasing voltages in protoplasts electro-transfected with CRISPR/Cas9 RNPs targeting *constitutive pathogen response 5* (*CPR5*). InDel patterns ranged from +1 bp to –6 bp at three different target sites in *CPR5* locus with frequencies ranging from 3.8% to 8.1% following electro-transfection at 1,300 V and 2.1% to 3.8% for 700 V and 1,000 V, respectively. Taken together, our results demonstrate that the CRISPR/Cas9 RNP system can be delivered into soybean protoplasts by the Neon electroporation system for efficient and effective gene editing. The electro-transfection system developed in this study would also further facilitate and serve as an alternative delivery method for DNA-free genome editing of soybean and other related species for genetic screens and potential trait improvement.

KEYWORDS

CRISPR/Cas9 RNPs, gene-editing, neon electroporation system, soybean, protoplasts, target-deep sequencing, trait improvement

Introduction

The CRISPR/Cas9 system has become a versatile technology in plant breeding and functional genomics due to its design simplicity and high efficiency in genome editing (Jiang and Doudna, 2017; Liu et al., 2022). Genome editing via CRISPR/Cas9 system uses single guide RNA (sgRNA) and Cas9 protein to make mutation events including insertions and deletions (InDels) at desired locations in the host genome through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways (Jinek et al., 2013). The CRISPR/Cas9 system enables targeted modifications in the genome by gene knock-in/out, base editing, prime editing, and guided transcriptional regulation (Feng et al., 2013; Zong et al., 2017; Mao et al., 2018; Lin et al., 2020; Rezazade Bazaz and Dehghani, 2022). Therefore, an optimized CRISPR/Cas9 cell system would facilitate efficient genetic screens for target genes and off-target activity in the plant genomes, thereby accelerating breeding and biosafety research.

The DNA expression cassettes of the CRISPR/Cas9 system can be transformed into plant cells using the most common delivery methods including *Agrobacterium*-mediated (Zhang et al., 2019), virus-mediated (Lei et al., 2022), particle bombardment (Hamada et al., 2018), and polyethylene glycol (PEG)-mediated transfection (Wu et al., 2020). Despite the fact that *Agrobacterium* and biolistic methods have been widely used in many organisms to deliver components for gene editing (Sandhya et al., 2020; Ghogare et al., 2021), their inadvertent incorporation of transposable elements (T-DNA) into the host genomes results in continued expression of CRISPR machinery, often resulting in unintended off-target mutation and genomic rearrangement (Zhang et al., 2018; Jupe et al., 2019; Chu and Agapito-Tenfen, 2022). Although virus-induced gene editing systems have many advantages including high editing efficiency and without integration of exogenous DNA into the host genome, their capacity to deliver the entire CRISPR/Cas9 system into plant cells is lower, which limits their application as a delivery method (Liu and Zhang, 2020; Zhang et al., 2022). Developing gene-edited lines without CRISPR T-DNA remnants in the host genome is gaining importance in the global genetically modified (GM) regulatory landscapes. Therefore, the DNA-free Cas9 ribonucleoprotein (RNP) (gRNA precomplexed to Cas9 nuclease) delivery into protoplasts has been adopted as a versatile method for genome editing of a diverse range of plants.

The direct delivery of RNPs into plant cells can be achieved by PEG-mediated as well as electroporation-mediated transfection or electro-transfection. PEG-mediated transfection has been widely used to deliver RNPs into plant protoplasts including *Arabidopsis*, tobacco and rice (Woo et al., 2015), petunia (Subburaj et al., 2016), apple and grape (Malnoy et al., 2016), wheat (Liang et al., 2017), cabbage (Murovec et al., 2018), pepper (Kim et al., 2020), maize (Sant'Ana et al., 2020), tomato (Nicolia et al., 2021), and soybean (Subburaj et al., 2022). PEG might exhibit incompatibility to several downstream analyses (e.g., proteomic analysis) and may cause some degree of cell cytotoxicity, with toxic effects occasionally seen in protoplasts when transfection with PEG is performed (Tyagi et al., 1989; Masani et al., 2014). Electro-transfection is another direct delivery method for the efficient transfection of the CRISPR/Cas9 system to living cells, and it has been widely used in the transformation of human and mouse primary T cells (Rupp et al., 2017; Seki and Rutz, 2018). Electro-transfection of RNPs has been demonstrated successfully in the microalga model *Chlamydomonas reinhardtii* (Baek et al., 2016; Shin et al., 2016). In addition, electroporation was also used for the delivery of RNPs (cabbage protoplast) and plasmid DNA containing CRISPR reagents (oil palm protoplast and wheat microspores) to plant cells (Bhowmik et al., 2018; Lee et al., 2020; Yeap W. et al., 2021). In soybean protoplasts, the transient expression of electroporated DNA has been reported by several studies (Dhir et al., 1991; Christou et al., 1987; Lin et al., 1987), but these available methods have not been adopted and or updated for targeted genome editing through transient introduction of RNA-guided endonuclease (RGEN) RNPs into soybean protoplasts.

Soybean is an economically important agronomic crop with high protein and oil, and several genetic engineering approaches have been made to improve the soybean traits (Rahman et al., 2023). Very recently, an efficient DNA-free genome editing platform for soybean protoplast systems using direct delivery of Cas9-RNP through PEG-mediated transfection was established by our research group (Subburaj et al., 2022). To date, no standardized protocols exist to transfect RNPs to soybean protoplasts through electroporation with reasonable mutation efficiency, which would greatly facilitate the CRISPR/Cas9 system to soybean protoplasts of different genetic backgrounds and further downstream analysis such as the impact of CRISPR exposure to soybean proteome.

In the present study, we report the establishment of a CRISPR/Cas9 RNP delivery system that facilitates efficient RNA-based genome editing in soybean protoplasts using electro–transfection. Using the Neon electroporation system (Thermo Fisher Scientific, Waltham, MA, USA), we investigated the important electrical factors including voltage strength and pulse duration on protoplasts, and we analyzed protoplasts of post-electroporation. With the established electrical parameters, we successfully demonstrated the delivery of exogenous green fluorescent protein (GFP)-Cas9 into the nucleus of soybean protoplasts using electro-transfection. By targeting the *Glycine max CPR5* locus (*GmCPR5*) that is associated with soybean trichome growth, we validated the mutations at three different sgRNA targeted sites and determined the mutagenesis efficiency of CRISPR/Cas9 in soybean protoplasts by targeted deep sequencing.

Abbreviations: 9M CPW, 9% mannitol with CPW salts; CLSM, confocal laser scanning microscope; CPR5, constitutive pathogen response 5; CPW, cell and protoplast washing; ESID, electronically switchable illumination and detection module; FDA, fluorescein diacetate; GFP, green fluorescent protein; HDR, homology-directed repair; InDels, insertions/deletions; NCBI, National Center for Biotechnology Information; NHEJ, non-homologous end-joining; PEG, polyethylene glycol; RGEN, RNA-guided endonuclease; RNP, ribonucleoprotein; sgRNA, single guide RNA; SRA, Sequence Read Archive; T7E1, T7 endonuclease I; V, voltage.

Materials and methods

Plant material and protoplast extraction

The soybean (*G. max*) seeds cv. OAC Bayfield was planted and grown in soil pots under a photoperiod of 8-h light and 16-h dark at

25°C in a growth chamber (Enviro Plant®) for 3 weeks. The newly expanded trifoliate leaves from 14–16-day- old soybean seedlings were used for protoplast isolation. The extraction of protoplasts was carried out according to our previous study (Subburaj et al., 2022) with minor modifications. Briefly, 12–18 trifoliate leaves were sliced into 0.2–0.4- mM strips and were agitated in 20 ml of VCP (Viscozyme® L, Celluclast® 1.5L, and Pectinex® Mltra SPL) (Sigma-Aldrich, Darmstadt, Germany) cell wall digesting enzyme solution for 4–5 h at 25°C in the dark to isolate protoplasts. After digestion, the solution was filtered through 0.75- µm nylon mesh, and the filtrate was pelleted at 600 rpm for 5 min. Harvested protoplasts were rinsed thrice with 10 ml of 9M cell and protoplast washing (CPW) solution, followed by resuspending and centrifugation. The washed and purified protoplasts were kept in ice for 1 h prior to further use.

Target gene and guide RNA selection

In this study, we chose *GmCPR5* as the target gene, as it was already attempted to make site-directed mutations in their coding region using CRISPR/Cas9 RNP through the PEG-mediated delivery method; to accomplish this, we designed five candidate gRNAs (denoted as T1–T5) against *CPR5* coding region in our previous study (Subburaj et al., 2022). In the present study, we selected and used best gRNAs of T1 (5′-AGGCTGCGGCGTTCAAACGACGG-3′), T3 (5′-GTCTCCCAGTCATCTTTCGATGG-3′), and T5 (5′-AGCTTTAGTAATCCGCTCGTAGG-3′) due to their higher mutation frequency at the *CPR5* locus (Subburaj et al., 2022). We carried out the *in vitro* transcript synthesis and purification of these gRNAs as reported previously (Subburaj et al., 2022).

Electroporation-mediated soybean protoplast transformation

The purified protoplasts were centrifuged and resuspended in 9M CPW solution and then counted using a hemocytometer. CPW solution with a volume of 100 µl containing approximately 4×10^5 protoplasts was transferred to a 1.5-ml Eppendorf tube as needed for each transfection. After brief centrifugation, CPW solution was removed, and protoplasts were resuspended in 80 µl of transfection buffer, which included R buffer (Neon suspension buffer), MMG solution (4 mM of MES, 0.4 M of mannitol, and 15 mM of MgCl), and HEPES electroporation buffer (10 mM of Hepes (pH 7.2), 0.2 M of mannitol, 5 mm of $CaCl_2$, and 150 mM of NaCl). Next, 20 µl of RNP complex was added to 80 µl of protoplast resuspension to bring a final volume of 100 µl. Then, the electroporation was carried out using the Neon™ Transfection System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions in 100-µl volumes. Protoplasts were electroporated with various voltages (500 V, 700 V, 1,000 V, and 1,300 V), pulse (1 and 3), and width (10 ms, 20 ms, and 30 ms) to optimize and obtain efficient transfection conditions as described in the Results section. With the optimized electro-transfection conditions, 20 µl of RNP complexes (1:3 molar ratio) consisted of 10 µg of ready-to-use GFP-tagged Cas9 from Sigma-Aldrich (CAS9GFPPRO), and 30 µg of sgRNA was electrophoretically introduced into 80 µl of resuspended protoplasts. Control and electroporated cells were immediately transferred in a 12-well poly-D-lysine-coated cell plate with 0.5 ml of precooled KP8 liquid medium (Kao, 1977) (supplemented with 9% mannitol and 3% sucrose). Then, the plates were kept on ice for 1–2 h and shifted to 25°C in darkness for 16–24 h prior to DNA extraction. Control and transfected cells were further cultured in 1 ml of KP8 liquid medium for 4 weeks at 25°C in the dark.

Microscopic observation of protoplasts

The bright-field images of isolated and transfected cells were analyzed using Motic AE2000 inverted microscope and captured with Moticam (Motic Hong Kong Limited, Hong Kong). The viability of control and electro-transfected cells was assessed by 0.5% fluorescein diacetate (FDA) staining, observed under reflected light with Axio Vert.A1 (FL-LED Stand) inverted light microscope, and captured via Axiocam 202 mono (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany). The fluorescence images of GFP-tagged Cas9 were acquired under bright field and electronically switchable illumination and detection module (ESID) using confocal laser scanning microscopy (CLSM) (LSM 800; Carl Zeiss) using a diode laser (488-nm line) with a 40× objective lens. The viability and transfection efficiency were calculated as the number of fluorescent protoplasts divided by the total number of protoplasts in one representative microscope field (Adedeji et al., 2020; Subburaj et al., 2022).

T7E1 validation and targeted deep sequencing

The genomic DNA from control and electro-transfected samples was prepared using Plant DNAzol™ Reagent (Invitrogen Co., Carlsbad, CA, USA). For each sgRNA (T1, T3, and T5), the target-specific nested PCR primers were designed in *GmCPR5* loci (Supplementary Table 1), and the extracted DNA was PCR amplified using designed primers. Targeted mutagenesis in PCR products of both control and electro-transfected protoplasts was detected by T7 endonuclease I (T7E1) assay. Further, the InDels at the targeted locations were also analyzed by targeted deep sequencing using the Illumina NovaSeq™ 6000 platform at Novogene Europe (Cambridge Science Park, UK) as described previously (Subburaj et al., 2022). CRISPR/Cas9 RNP induced InDels at *GmCPR5* loci for each sgRNA were determined using Cas-Analyzer from the CRISPR RGEN tools (http://www.rgenome.net/cas-analyzer/) (Subburaj et al., 2016; Subburaj et al., 2022). Briefly, the raw data FASTQ files along with basic information about query sequences were submitted in the Cas-Analyzer software with the following analysis parameters: comparison range of 40 and minimum frequency of 1. Following submission, the total number of reads, the number of reads with InDels, and the calculated InDel frequency (defined as the

percentage by dividing the count of reads that contained InDels at the target site by the total number of reads) were obtained from the output of a summarized result table.

Results

Isolation of protoplasts from trifoliate leaves

The initial electroporation experiments showed that trifoliate protoplasts are the most suitable for electro-transfection compared to unifoliate cells, as they were severely damaged after electroporation. In this study, we efficiently isolated the protoplast from 15- day-old trifoliate leaves with a duration of 5–6 h of enzyme digestion (Figures 1A, B). The yield of protoplasts was approximately 2×10^6 cells per gram fresh weight. Isolated protoplasts were 10 µm to 50 µm in size, and most were spherical in shape (Figure 1C). Following FDA staining, it was determined that 70% ± 2.1% of the protoplasts were alive (Figures 1D, E). The isolated protoplasts were cultured in KM medium, and first cell divisions were noted after 4–6 days of culture initiation (Figure 1F), indicating that the trifoliate leaf-derived protoplasts could be suitable for the electroporation-mediated transformation.

Electro-transfection of protoplasts using Neon electroporation system

To establish an electroporation-mediated soybean protoplast transformation using the Neon system, the electro-transfection method was optimized based on cabbage and wheat protocols where transfection efficiency reaches 3.4% (1 pulse of 1,000 V, 20 seconds each) and 2.2% (3 pulses of 500 V, 30 seconds each), respectively (Bhowmik et al., 2018; Lee et al., 2020). Following cabbage and wheat electroporation conditions, initial soybean testing failed, as we could not detect or could only detect a few GFP fluorescent spots in electroporated cells. Subsequently, the various pulse strengths (1–3) along with voltages (500–1,300 V) and time duration (10–30 ms) were optimized. It was found that 3 pulses of 500 V and 10 ms per pulse were sufficient to transport the exogenous GFP-Cas9 with lower cell death rates. The effects of various ranges of pulsing voltages were further evaluated with optimized 3 pluses and 10 ms per pulse in the protoplasts (100 µl of 4×10^5 cells) for 500 V, 700 V, 1,000 V, and 1,300 V. As shown in Figure 2, the protoplasts' morphology, viability, and cell division efficiency were examined after electroporation. It was noted that intact protoplasts with a large and round shape were observed at all the applied voltages. However, at 1,000 and 1,300 V, a certain proportion of broken and debris of dead cells was also noted.

The calculated relative frequency of cell viability was 86.5% ± 2.4% under 0 V compared to the non-treatment control of 100%. Under various voltage treatments, the viability of cells was decreased with the increase in pulsing voltage. The observed relative frequency of viable rates for treatments under 500 V, 700 V, 1,000 V, and 1,300 V were 76.7% ± 2.8%, 66% ± 1.4%, 59.1% ± 1.2%, and 57.6% ± 1.1%, respectively (Figure 2). Upon culturing of electroporated protoplasts, the primary cell divisions were noted in all treatments. However, a higher proportion of cell divisions was only observed at 0 V and 500 V when compared to the higher voltages of 700 V, 1,000 V, and 1,300 V (Figure 2).

To further optimize and validate the delivery of Cas9 protein, a fixed amount of 10 µg of GFP-conjugated Cas9 was electro-

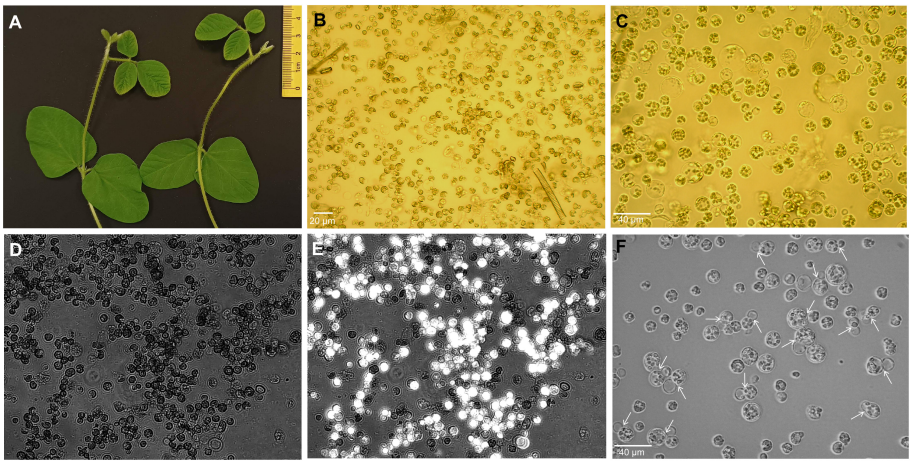


FIGURE 1
Isolation of protoplasts from trifoliate leaves of soybean plantlets. **(A)** Fifteen-day-old plants showing trifoliate leaves of suitable size. **(B, C)** Protoplasts of freshly extracted **(B)** and purified cells **(C)** under the Motic AE2000 inverted microscope with × 20 and × 40 objectives, respectively. Black scale bar, 30 µm. **(D, E)** The protoplast viability was assessed by FDA staining and observed under both bright field **(D)** and fluorescence channel, and simultaneously merged images are depicted **(E)** using Axio Vert.A1 inverted microscope with a × 20 objective. **(F)** Division of protoplasts (shown by white arrows) at 4 days after isolation in culture medium. FDA, fluorescein diacetate.

transfected into protoplasts with the abovementioned pulsing voltages. After 24 h of electroporation, the internalization of GFP-tagged Cas9 inside the protoplasts was confirmed under the CLSM microscope (Figure 3). Regardless of voltage, the GFP-Cas9 was successfully localized inside of cells. However, the calculated internalization efficiency of GFP-Cas9 with an unsupervised eye was $\gtrsim$ 40% under 1,300 V compared to other treatments such as 500 V, 700 V, and 1,000 V where there was only ~ 20%–23% noted. These results suggest that the established soybean protoplast transfection using the Neon electroporation system can be suitable for the delivery of RNPs into protoplasts.

RNP-based targeted mutagenesis of *CPR5* in soybean via electro-transfection

To demonstrate whether the established electro-transfection system can be employed for RGEN RNP-mediated genome editing in soybean, three sgRNAs were chosen and prepared based on our previous study (Subburaj et al., 2022), namely, T1, T3, and T5 to target exons 1, 2, and 4 of *CPR5* gene, respectively (Figure 4A). RNP complex consisting of a 1:3 molar ratio of Cas9 (10 µg) and synthesized sgRNAs (30 µg) were electro-transfected into protoplasts using the Neon system at different pulsing voltages

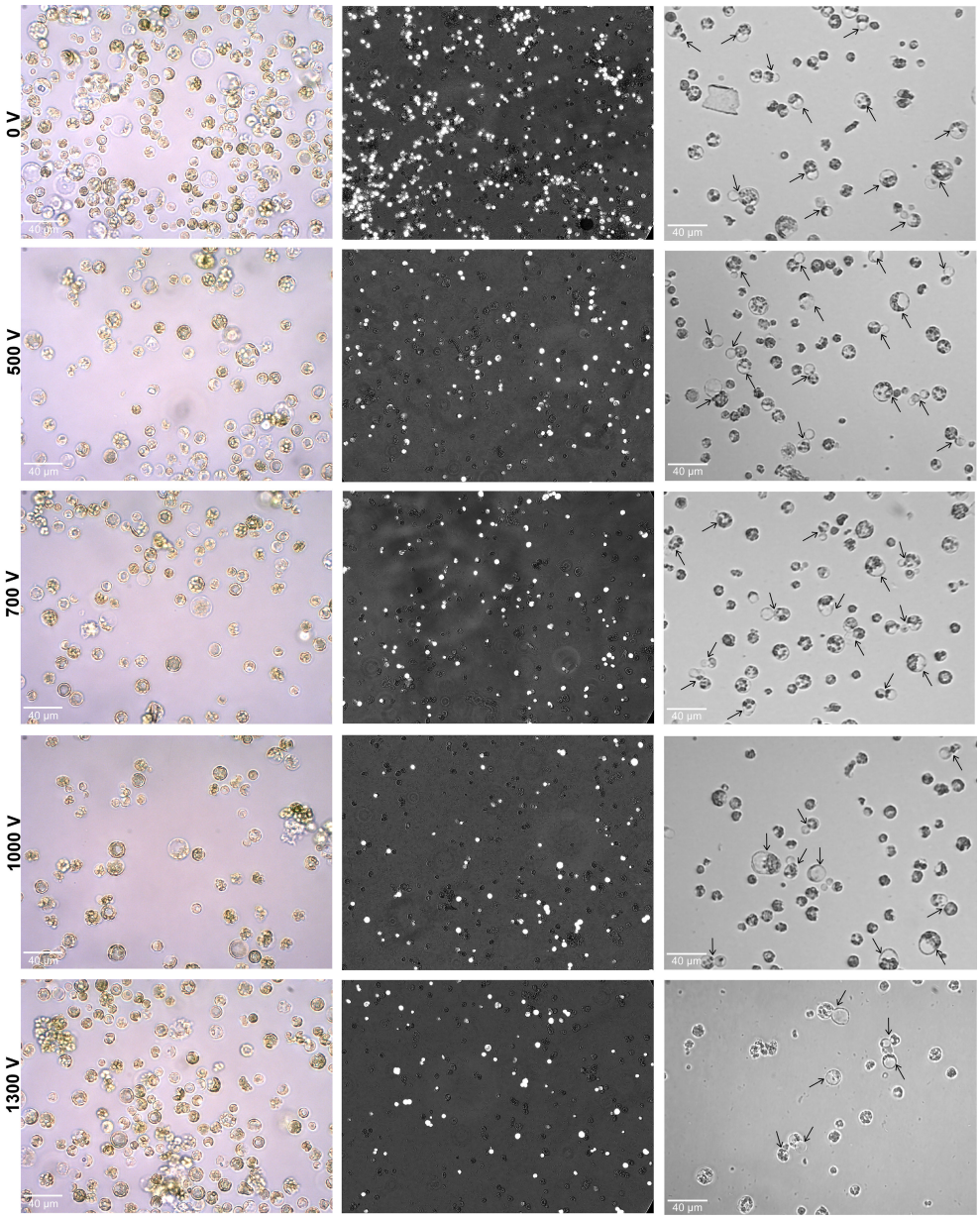


FIGURE 2
Effect of various pulsing voltage on the protoplasts morphology, viability, and cell division efficiency following electro-transfection. Left panel: representative images of electroporated protoplasts after 24 h are shown. Middle panel: the merged fluorescence images (under bright and fluorescence field using Axio Vert.A1 inverted microscope with a × 20 objective) showing the FDA-stained viable cells after 24 h of electroporation. Right panel: images showing primary divisions (shown by black arrows) of electroporated protoplasts at 4 days after culture initiation. FDA, fluorescein diacetate.

as mentioned earlier. Following transfection, the genomic DNA was extracted from control and transfected samples after 24 h of incubation. The targeted sites were PCR amplified using designed nested primers (Supplementary Table 1), and a T7E1 assay was carried out for preliminary detection of RNP-induced mutations for electro-transfected samples under different pulsing voltages. Upon agarose gel electrophoresis of T7E1-digested PCR products, cleaved PCR fragments at expected sizes were noted for all the RNP electro-transfected samples under 1,000 V and 1,300 V (Figures 4B–D). In addition, there were no cleaved PCR products observed for all the target sites under 500 V and or 700 V, except for T1 under 700 V; the same was observed for the wild type and the Cas9 alone transfected samples. This suggests that RNPs successfully induced site-specific double-strand breaks followed by DNA repair mechanisms within the *GmCPR5* locus in soybean.

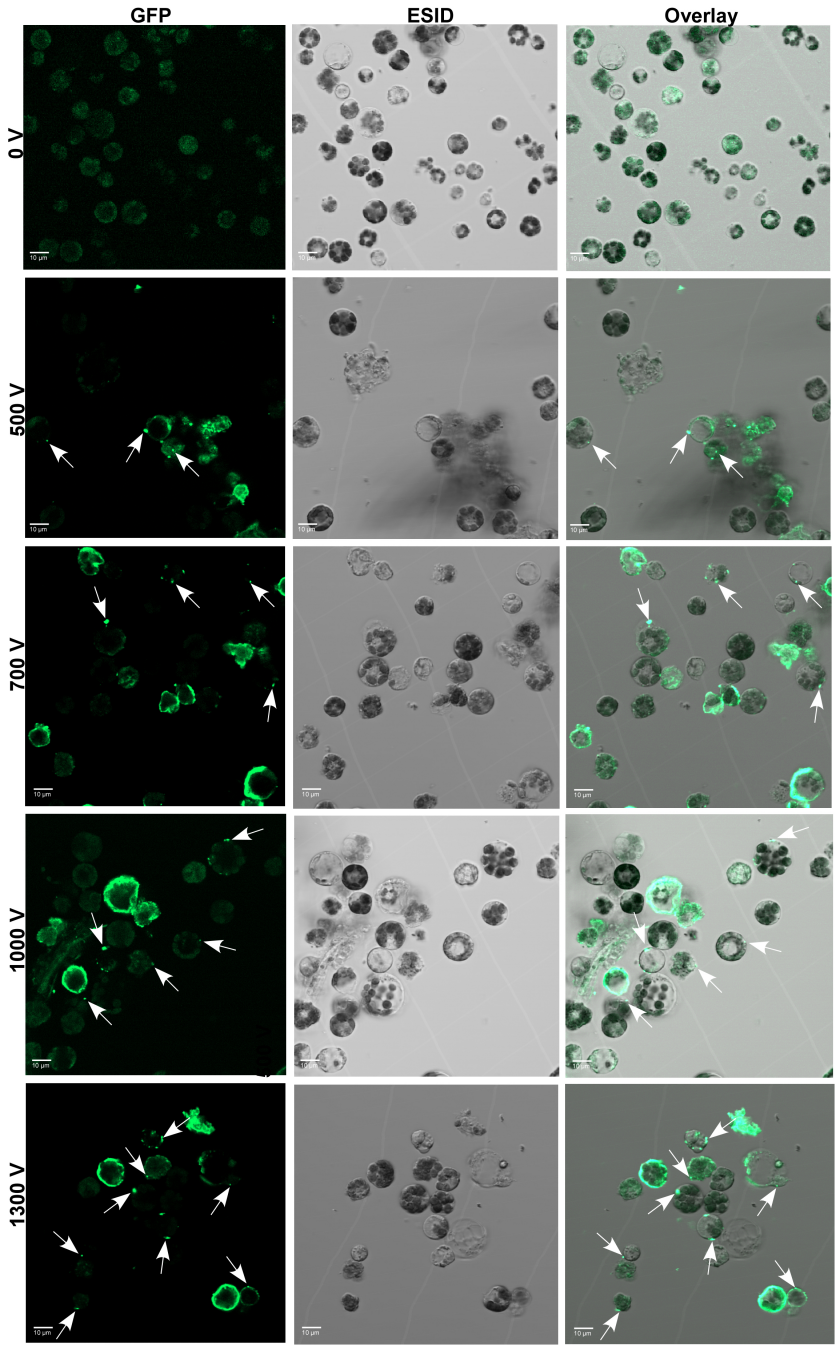


FIGURE 3
Delivery and cellular localization of Cas9-GFP to soybean protoplasts through electro-transfection. GFP-Cas9 in electro-transfected (0 V to 1,300 V) protoplasts at 24 h after electroporation was seen using a laser scanning confocal microscope under GFP (left panel) and bright field of ESID channel (middle panel). Right panel: representative overlay images of GFP and bright field are shown. White arrows show the location of internalized GFP-Cas9. GFP, green fluorescent protein; ESID, electronically switchable illumination and detection module.

To estimate InDel frequency and characterization of mutation patterns, targeted deep sequencing was performed on the DNA samples with positive results from the T7E1 assay. The raw data from targeted deep sequencing were submitted to the National Center for Biotechnology Information (NCBI) Bioproject (http://www.ncbi.nlm.nih.gov/bioproject/) under the accession number PRJNA983990. Targeted deep sequencing results confirmed that the InDels present at all three target sites including T1, T3, and T5 under different pulsing voltages, similar to that in the T7E1 cleavage assay. As shown in Table 1, the percentage of InDel (mutation) frequency of each target site was estimated by dividing the number of mutated InDel sequences by the number of total sequences in the *CPR5* target sequences using the Cas-Analyzer tool. The target sites of T1, T3, and T5 have InDel frequencies that ranged from a minimum of 2.1% to a maximum of 8.1% with an average frequency of 4.21% ± 0.73% in the *GmCPR5* locus. It was found that the estimated InDel frequencies were increased with the increase in pulsing voltage. For the T1 target site, InDel frequency following electro-transfection at 700 V, 1,000 V, and 1,300 V were 2.1%, 3.7%, and 8.1%, respectively. Similarly, in T3 and T5, the frequency was increased from 2.1% and 3.8% (at 1,000 V) to 3.8% and 5.7% at 1,300 V, respectively (Table 1).

The mutation pattern for each target site was further characterized. The distribution of the five most frequent alleles observed around the cleavage site in *GmCPR5* loci after electro-transfection under different pulsing voltages is presented in Supplementary Figure 1 (700 V and 1,000 V) and Figure 4 (1,300 V). gRNAs produced InDels at corresponding target sites, which ranged from +1 to −6 nucleotide (nt) in length, and all induced mutations were observed prevalently at 4th nt upstream of the PAM site except for target sites with few alleles, which were the T1 (1,000 V and 1,300 V), T3 (1,300 V), and T5 (1,000 V) (Supplementary Figures 1B, C; Figures 4B–D). As shown in Table 1, the highest mutation rate was observed only in protoplast samples under 1,300 V, in which the five most frequent alleles were responsible for 6%, 3.14%, and 4% of the total mutation rates for T1 (8.1%), T3 (3.8%), and T5 (5.7%) (Figures 4B–D). Further, +1 nt insertion of adenine or thiamine was found prevalent among the observed InDels in the frequent alleles for all the target sites (Supplementary Figure 1; Figure 4). Apart from this, some of the target sites possessed +1 nt insertion of guanine (T3 under 1,000 V and 1,300 V) and cytosine (T5 under 1,300 V) (Supplementary Figure 1C; Figures 4C, D). A +2 nt insertion (thiamine and adenine) was also noted for T1 under

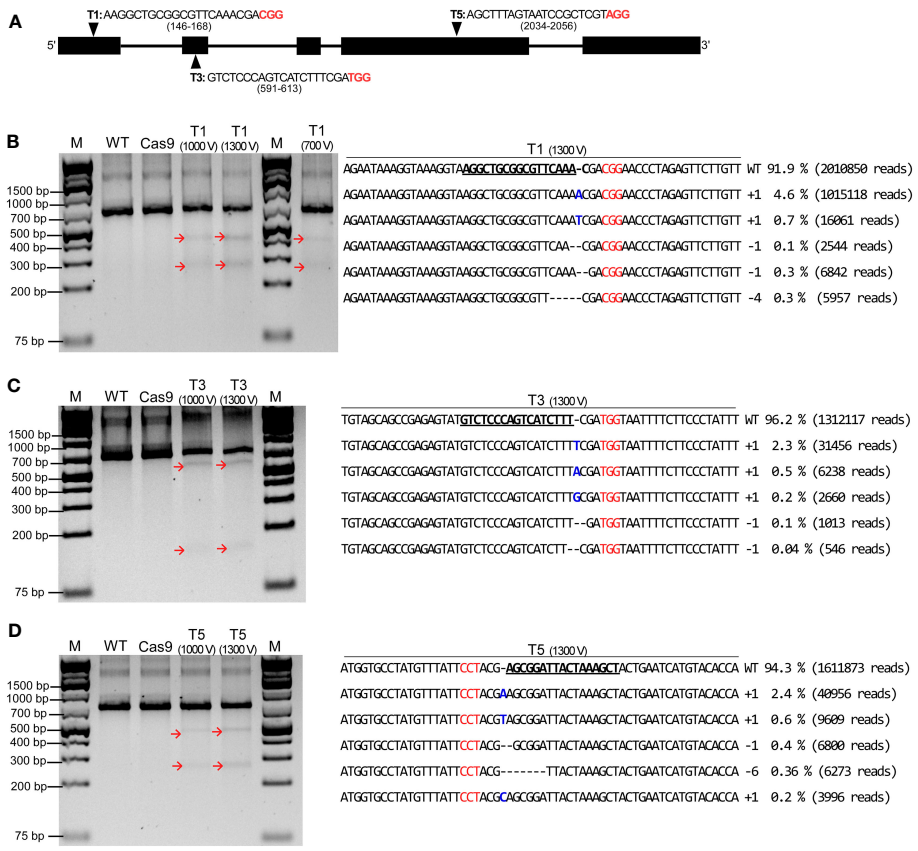


FIGURE 4
CRISPR/Cas9-mediated editing of the exogenous *GmCPR5* gene in soybean protoplasts using electro-transfection. **(A)** *GmCPR5* locus, location of target sites (T1, T3, and T5), and their gRNA sequences. **(B–D)** Results of T7E1 endonuclease assay for target sites T1, T3 and T5. Lane M: a DNA ladder. Lane WT: non- transfected wild type (control). Lane Cas9: transfected with SpCas9 only. Lanes T1–T5: electro-transfected with RNPs at 700 V, 1,000 V, and 1,300 V. Red arrows indicate the T7E1-mediated cleaved bands. The mutation patterns observed by targeted deep sequencing for the corresponding target sites of T1–T5 at *GmCPR5* loci by electro-transfection at 1,300 V are shown on the right panel. Wild-type (WT) nuclease target sequences are in bold and underlined. PAM sites are denoted in red. RNPs, ribonucleoproteins; gRNA, guide RNA.

TABLE 1 Estimation of mutation rates at *GmCPR5* locus following electro-transfection of Soybean protoplasts.

Protoplast samples	Wild-type negative control				
	Total	InDel	InDel frequency (%)	Insertion[a]	Deletion[b]
T1 (0 V)	1,713,502	164	0.009	5	159
T3 (0 V)	1,207,415	82	0.006	3	79
T5 (0 V)	1,285,534	239	0.018	0	239
Average[c]	1,402,150 ± 128,435.4	161 ± 37.01	0.011 ± 0.003	2.66 ± 1.18	159 ± 37.71
Cas9 RNP transformants					
T1 (700 V)	2,233,489	47,431	2.1	47,238	193
T1 (1,000 V)	1,964,762	72,550	3.7	39,940	32,610
T1 (1,300 V)	2,186,990	176,140	8.1	142,852	33,288
T3 (1,000 V)	736,342	16,884	2.3	14,417	2,467
T3 (1,300 V)	1,363,363	51,246	3.8	47,636	3,610
T5 (1,000 V)	1,812,143	69,095	3.8	38,366	30,729
T5 (1,300 V)	1,709,290	97,417	5.7	60,815	36,602
Average[c]	1,715,197 ± 183,218	75,823 ± 17,768	4.21 ± 0.72	55,894 ± 14,293	19,928 ± 5,880

RNP, ribonucleoprotein.
[a]Numbers of insertions analyzed.
[b]Numbers of deletions analyzed.
[c]Values of average and standard deviation error.

700 V (Supplementary Figure 1A). A maximum of −5- and −6-bp deletions were observed for T1 (1,000 V) and T5 (1,000 and 1,300 V) following treatments, respectively (Supplementary Figures 1B, D; Figure 4D). In summary, all the mutant alleles have frameshift mutations, which would result in a complete loss of CPR5 protein function compared to the wild-type alleles of *GmCPR5*. The result analysis from targeted deep sequencing demonstrated that the electro-transfection of RGEN RNPs using the Neon system can be used for site-directed mutagenesis in soybean protoplasts.

Discussion

The CRISPR/Cas9 technology has emerged as a powerful tool for genome editing and crop improvement. Transient gene expression in leaf mesophyll-derived protoplasts is an excellent resource for genetic manipulation and genome editing using CRISPR/Cas9, which enables the high-throughput analysis of gene functions (Zhu et al., 2016; Xu et al., 2020; Yu et al., 2021). The efficiency of genome editing highly relies on the transfection system and delivery methods including PEG-mediated, electroporation, and microinjection, which have been utilized to introduce DNA, RNA, or protein into plant cells (Masani et al., 2014; Subburaj et al., 2016; Lee et al., 2020; Yeap W. et al., 2021). In addition to electroporation and microinjection, PEG-mediated transient expression technology has been predominantly applied in both model and non-model plants due to their high transfection efficiency as reviewed by Zhang et al. (2021). To date, there have been no reports of CRISPR/Cas9-mediated genome editing via electroporation in soybean species, although several studies have utilized it for transfecting plasmid DNA into soybean protoplasts (Christou et al., 1987; Lin et al., 1987; Dhir et al., 1991). In this study, we standardized the protocols for the delivery of CRISPR/Cas9 RNPs into soybean protoplasts using the Neon electroporation system for the first time. The detection of site-directed mutations in endogenous targeted genes in this work would provide an additional and alternative methodology to the PEG-mediated transient expression technology-based genome editing of the soybean and their related species.

A stable protoplast isolation method and choice of protoplast source with high-quality cells are required for efficient transient gene expression studies (Huang et al., 2013; Li et al., 2018; Lee et al., 2020). Recently, we developed a successful protoplast isolation method for unifoliate leaves of soybean (Subburaj et al., 2022). Following this method, we isolated the protoplasts from trifoliate leaves and obtained a high quantity (2×10^6 cells) and viability of protoplasts (70% ± 2.1%) in this study. While ensuring high transfection efficiency in protoplast cells, and at the same time retaining viability and ability to differentiate (cell division), we have tested the protoplasts of both unifoliate and trifoliate to the different pulsing voltages of 500 V to 1,000 V in a pilot study. It was found that trifoliate cells endure and survive under electrophoretic conditions compared to the unifoliate cells, as they were greatly damaged after electroporation, even at 500 V. In addition, the isolated trifoliate cells had exhibited first protoplast divisions at 5 days after culture initiation as noted for the unifoliate cells in our previous study (Subburaj et al., 2022). Cotyledon and zygotic embryo-derived protoplasts were used in earlier soybean studies for the electroporation-mediated transient expression of introduced DNA molecules (Christou et al., 1987; Lin et al., 1987; Dhir et al., 1991).

In this study, we suggest that trifoliate leaf-derived protoplasts of Neon electro-transfection would facilitate DNA-free gene editing using the CRISPR/Cas9 system because of its easy isolation and manipulation.

Electroporation experiments often need an appropriate buffer to provide conductivity as well as cell survival. Therefore, we have investigated the impact of electroporation buffers on protoplasts survival by electroporating them with three different buffers including MMG solution, Neon R, and HEPES buffer as those reported in earlier studies (Dhir et al., 1992; Bhowmik et al., 2018; Lee et al., 2020). Soybean protoplasts did not survive Neon R and HEPES buffers but only survived in MMG solution following electro-transfection, similar to the observed results in cabbage protoplasts (Lee et al., 2020). Upon electroporation with optimized protocol, our results revealed that cell survival and division efficiency decreased with increased pulse voltage. At the same time, the transfection efficiency was increased when the protoplasts were subjected to increasing pulse voltage. This difference might be due to the high electrical pulse applied to protoplast membranes, but more likely, the cellular damage could be induced by increasing voltage, which results in cell death. Many transient pores could also be created on the protoplast membranes under high voltage, which allows the uptake of exogenous GFP-Cas9, thereby increasing the efficiency of transfection. The relative frequency of cell viability was the highest at 500 V (76%) and the lowest at 1,300 V (57%), suggesting that high voltages could decrease the survival rate of cells. Similarly, Bhowmik et al. (2018) also reported that the microspore survival increased with decreasing voltage, with the highest microspore survival of 50% noted at 500 V compared to 1,000 V. Moreover, the efficiency of cell division following 0 V and 500 V was higher than that of other treatments such as 700 V, 1,000 V, and 1,300 V, indicating that the pulsing voltages did not affect the process of mitotic divisions of protoplasts, as they have undergone primary cell divisions at 5 days after culture initiation. In wheat microspores, the transfection efficiency has been noted to increase with decreasing voltage (Bhowmik et al., 2018). In contrast, in cabbage protoplasts, InDel frequencies increased with increasing voltage (Lee et al., 2020). GFP-tagged Cas9 was electro-transfected into soybean protoplasts, the GFP-Cas9 signal was successfully detected in intracellular compartments of electroporated protoplasts, and the highest transfection efficiency was noted under 1,300 V (~ 40%) compared to other treatments (~ 20%–23%), indicating that electro-transfection efficiency increased with increasing pulse voltage in soybean protoplasts. This has also corresponded well with the observed InDel frequency rates from targeted deep sequencing results. The InDel frequency of *GmCPR5* following sgRNA (T1, T3, and T5) transfection was increased with increasing pulse voltage from 700 V or 1,000 V to 1,300 V. In this study, the calculated InDel frequencies of T1 (700 V) and T5 (1,000 V) were slightly higher than the observed InDel frequencies of 1.2% (750 V) and 3.4% (1,000 V) for *PDS1* gene in cabbage protoplasts following electro-transfection (Lee et al., 2020). By PEG-mediated transfection of RNPs, a very low editing efficiency has been achieved in protoplasts of several plant species including cabbage (0.09%–2.25%) (Murovec et al., 2018), Cavendish banana (0.19%– 0.92%) (Wu et al., 2020), wild tobacco (0.01–0.9%) (Kim et al., 2017), and grapevine (0.1%) (Malnoy et al., 2016). Further, the targeted deep sequencing results showed that three sgRNAs (T1, T3, and T5) were induced with various mutation pattern sizes ranging from +1 to −6 nt in length at targeted sites, which were found similar to our previous mutagenic CRISPR/Cas9 study in soybean using PEG-mediated delivery method (Subburaj et al., 2022). Taken together, in this study, by electro-transfection, we achieved much higher mutation rates (2.1%–8.1%), suggesting that electro-transfection may potentially be helpful and applicable to the abovementioned plant species to improve editing efficiency compared with the PEG-mediated method.

The high pulse voltage of 1,400 V or 1,250 V has been reported to be lethal to wheat microspores and cabbage protoplasts (Bhowmik et al., 2018; Lee et al., 2020). In this study, we attempted to electro-transfect the soybean protoplasts at 1,300 V and found that trifoliate leaf-derived protoplasts could survive well in post-electroporation, as it had shown 57% viability and ability to divide in culture. However, this study expects further improvements to increase transfection efficiency with a progressive decrease in pulsing voltage within 1,000 V. The regeneration of protoplasts is highly necessary for DNA-free genome editing systems. In this study, after 12 days in culture, dividing protoplasts could not form colonies regardless of treatment of pulsing voltages. This is probably due to the constraining factors including the recalcitrant nature of soybean, genotype-dependent response to culture conditions, type of explant, and age (Wei and Xu, 1988; Cutler et al., 1991; Dhir et al., 1992; Eeckhaut et al., 2013). As demonstrated in previous studies (Wei and Xu, 1988; Dhir et al., 1991), well-established protocols exist for the regeneration of soybean protoplasts. Perhaps a genotype of the perfect donor species or cultivar of soybean is advisable to improve the chances of regeneration of gene-edited protoplasts. In addition, the reduction of non-transfected cells by enriching transfected cells through the application of a fluorescence-activated cell sorting (FACS) system could also have a positive effect on the regeneration of successfully edited cells. Altogether, the protoplast-based gene editing through the Neon electro-transfection system described in this study provides an alternative gene editing platform to the PEG-mediated system for evaluating the efficacy of CRISPR systems as well as gene functional validations in soybean and other related species.

Conclusion

Our study demonstrated that the CRISPR/Cas9 DNA-free genome editing is effective and efficient in editing soybean genes using the Neon electroporation system. In this study, we demonstrated a time- and cost-efficient *in vitro* electro-transfection assay that provides a rapid assessment and evaluation of gRNA efficiency in soybean protoplasts. The balance between higher voltages and higher targeted mutagenesis will be the challenge for future applications of this method. Nevertheless, a

DNA-free transformation system in soybeans to generate non-transgenic gene-edited mutants is highly desired to reduce the occurrence of vector backbone spurious introgressions. This enabling platform for genome editing may accelerate the exploration of gene function for trait improvement in soybean lines. In addition, our study offers new insights into other related species, such as the pinto bean and other *Phaseolus* species, that share similar limitations in genetic transformation and inefficient tissue culture propagation and regeneration processes using other non-transgenic approaches.

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 below: NCBI Bioproject accession PRJNA983990.

Author contributions

SS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SA-T: Conceptualization, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Funding

This project was funded by The Research Council of Norway (Project Number: 301911 - FOODPRINT: Traceability and labelling of gene-edited products in the food chain).

Acknowledgments

We would like to thank Jennifer A. L. Nunn for helping to take the confocal microscopy images.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1255819/full#supplementary-material

SUPPLEMENTARY FIGURE 1
CRSIPR/Cas9-mediated editing of the exogenous *GmCPR5* gene in Soybean protoplasts using electro-transfection and characterization of five most frequent mutation patterns. **(A)** Target sites of T1 at *GmCPR5* loci by electro-transfection at 700 V. **(B)** T1 at *GmCPR5* loci by electro-transfection at 1000 V. **(C)** T3 at *GmCPR5* loci by electro-transfection at 1000 V. **(D)** T5 at *GmCPR5* loci by electro-transfection at 1000 V. Wild type (WT) nuclease target sequences were in bold and underlined. PAM sites denoted by red.

References

Adedeji, O. S., Naing, A. H., and Kim, C. K. (2020). Protoplast isolation and shoot regeneration from protoplast-derived calli of Chrysanthemum cv. White ND. *Plant Cell Tiss. Organ. Cult.* 141, 571–581. doi: 10.1007/s11240-020-01816-3

Baek, K., Kim, D. H., Jeong, J., Sim, S. J., Melis, A., Kim, J. S., et al. (2016). DNA-free two-gene knockout in *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. *Sci. Rep.* 6, 30620. doi: 10.1038/srep30620

Bhowmik, P., Ellison, E., Polley, B., Bollina, V., Kulkarni, M., Ghanbarnia, K., et al. (2018). Targeted mutagenesis in wheat microspores using CRISPR/Cas9. *Sci. Rep.* 8, 6502. doi: 10.1038/s41598-018-24690-8

Christou, P., Murphy, J. E., and Swain, W. F. (1987). Stable transformation of soybean by electroporation and root formation from transformed callus. *Proc. Natl. Acad. Sci. U.S.A.* 84, 3962–3966. doi: 10.1073/pnas.84.12.3962

Chu, P., and Agapito-Tenfen, S. Z. (2022). Unintended genomic outcomes in current and next generation GM techniques: A systematic review. *Plants (Basel).* 11, 2997. doi: 10.3390/plants11212997

Cutler, A. J., Saleem, M., and Wang., H. (1991). Cereal protoplast recalcitrance. *In Vitro Cell. Dev. Biol. Plant* 27, 104–111. doi: 10.1007/BF02632192

Dhir, S. K., Dhir, S., and Widholm, J. M. (1991). Plantlet regeneration from immature cotyledon protoplasts of soybean (*Glycine max* L.). *Plant Cell Rep.* 10, 39–43. doi: 10.1007/BF00233030

Dhir, S. K., Dhir, S., and Widholm, J. M. (1992). Regeneration of fertile plants from protoplasts of soybean (*Glycine max* L. Merr.): genotypic differences in culture response. *Plant Cell Rep.* 11, 285–289. doi: 10.1007/BF00235083

Eeckhaut, T., Lakshmanan, P. S., Deryckere, D., Van Bockstaele, E., and Van Huylenbroeck, J. (2013). Progress in plant protoplast research. *Planta.* 238, 991–1003. doi: 10.1007/s00425-013-1936-7

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D., Wei, P., et al. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23, 1229–1232. doi: 10.1038/cr.2013.114

Ghogare, R., Ludwig, Y., Bueno, G. M., Slamet-Loedin, I. H., and Dhingra, A. (2021). Genome editing reagent delivery in plants. *Transgenic Res.* 30, 321–335. doi: 10.1007/S11248-021-00239-W

Hamada, H., Liu, Y., Nagira, Y., Miki, R., Taoka, N., and Imai, R. (2018). Biolistic-delivery-based transient CRISPR/Cas9 expression enables in planta genome editing in wheat. *Sci. Rep.* 8, 14422. doi: 10.1038/s41598-018-32714-6

Huang, H., Wang, Z., Cheng, J., Zhao, W., Li, W., Wang, H., et al. (2013). An efficient cucumber (*Cucumis sativus* L.) protoplast isolation and transient expression system. *Sci. Hortic. Amst.* 150, 206–212. doi: 10.1016/j.scienta.2012.11.011

Jiang, F., and Doudna, J. A. (2017). CRISPR-cas9 structures and mechanisms. *Annu. Rev. Biophys.* 46, 505–529. doi: 10.1146/annurev-biophys-062215-010822

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., and Doudna, J. (2013). RNA programmed genome editing in human cells. *eLife* 2, e00471. doi: 10.7554/eLife

Jupe, F., Rivkin, A. C., Michael, T. P., Zander, M., Motley, S. T., Sandoval, J. P., et al. (2019). The complex architecture and epigenomic impact of plant T-DNA insertions. *PloS Genet.* 15, e1007819. doi: 10.1371/journal.pgen.1007819

Kao, K. N. (1977). Chromosomal behaviour in somatic hybrids of soybean-*Nicotiana glauca*. *Molec. Gen. Genet.* 150, 225–230. doi: 10.1007/BF00268120

Kim, H., Choi, J., and Won, K. H. (2020). A stable DNA-free screening system for CRISPR/RNPs-mediated gene editing in hot and sweet cultivars of *Capsicum annuum*. *BMC Plant Biol.* 20, 449. doi: 10.1186/s12870-020-02665-0

Kim, H., Kim, S. T., Ryu, J., Kang, B. C., Kim, J. S., and Kim, S. G. (2017). CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nat. Commun.* 8, 14406. doi: 10.1038/ncomms14406

Lee, M. H., Lee, J., Choi, S. A., Kim, Y.-S., Koo, O., Choi, S. H., et al. (2020). Efficient genome editing using CRISPR-Cas9 RNP delivery into cabbage protoplasts via electrotransfection. *Plant Biotechnol. Rep.* 14, 695–702. doi: 10.1007/s11816-020-00645-2

Lei, J., Li, Y., Dai, P., Liu, C., Zhao, Y., You, Y., et al. (2022). Efficient virus-mediated genome editing in cotton using the CRISPR/Cas9 system. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.1032799

Li, J., Liao, X., Zhou, S., Liu, S., Jiang, L., and Wang, G. (2018). Efficient protoplast isolation and transient gene expression system for Phalaenopsis hybrid cultivar 'Ruili Beauty'. *In Vitro Cell. Dev. Biol. Plant* 54, 87–93. doi: 10.1007/s11627-017-9872-z

Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., et al. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8, 14261. doi: 10.1038/ncomms14261

Lin, W., Odell, J. T., and Schreiner, R. M. (1987). Soybean protoplast culture and direct gene uptake and expression by cultured soybean protoplasts. *Plant Physiol.* 84, 856–861. doi: 10.1104/pp.84.3.856

Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., et al. (2020). Prime genome editing in rice and wheat. *Nat. Biotechnol.* 38, 582–585. doi: 10.1038/s41587-020-0455-x

Liu, H., Chen, W., Li, Y., Sun, L., Chai, Y., Chen, H., et al. (2022). CRISPR/cas9 technology and its utility for crop improvement. *Int. J. Mol. Sci.* 23, 10442. doi: 10.3390/ijms231810442

Liu, H., and Zhang, B. (2020). Virus-based CRISPR/cas9 genome editing in plants. *Trends Genet.* 36, 810–813. doi: 10.1016/j.tig.2020.08.002

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., et al. (2016). DNAFree genetically edited grapevine and apple protoplast using CRISPR/cas9 ribonucleoproteins. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01904

Mao, Y., Yang, X., Zhou, Y., Zhang, Z., Botella, J. R., and Zhu, J.-K. (2018). Manipulating plant RNA-silencing pathways to improve the gene editing efficiency of CRISPR/Cas9 systems. *Genome Biol.* 19, 1–15. doi: 10.1186/s13059-018-1529-7

Masani, M. Y., Noll, G. A., Parveez, G. K., Sambanthamurthi, R., and Prüfer, D. (2014). Efficient transformation of oil palm protoplasts by PEG-mediated transfection and DNA microinjection. *PloS One* 9, e96831. doi: 10.1371/journal.pone.0096831

Murovec, J., Gu˘cek, K., Bohanec, B., Avbelj, M., and Jerala, R. (2018). DNA-Free Genome Editing of *Brassica oleracea* and *B. rapa* Protoplasts Using CRISPRCas9 Ribonucleoprotein Complexes. *Front. Plant Sci.* 9. doi: 10.3389/fpls.2018.01594

Nicolia, A., Andersson, M., Hofvander, P., Festa, G., and Cardi, T. (2021). Tomato protoplasts as cell target for ribonucleoprotein (RNP)- mediated multiplexed genome editing. *Plant Cell Tiss. Organ. Cult.* 144, 463–467. doi: 10.1007/s11240-020-01954-8

Rahman, S. U., McCoy, E., Raza, G., Ali, Z., Mansoor, S., and Amin, I. (2023). Improvement of soybean; A way forward transition from genetic engineering to new plant breeding technologies. *Mol. Biotechnol.* 65, 162–180. doi: 10.1007/s12033-022-00456-6

Rezazade Bazaz, M., and Dehghani, H. (2022). From DNA break repair pathways to CRISPR/Cas-mediated gene knock-in methods. *Life Sci.* 295, 120409. doi: 10.1016/j.lfs.2022.120409

Rupp, L. J., Schumann, K., Roybal, K. T., Gate, R. E., Ye, C. J., Lim, W. A., et al. (2017). CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Sci. Rep.* 7, 737. doi: 10.1038/s41598-017-00462-8

Sandhya, D., Jogam, P., Allini, V. R., Abbagani, S., and Alok, A. (2020). The present and potential future methods for delivering CRISPR/cas9 components in plants. *J. Genet. Eng. Biotechnol.* 18, 25. doi: 10.1186/S43141-020-00036-8

Sant'Ana, R., Caprestano, C. A., Nodari, R. O., and Agapito-Tenfen, S. Z. (2020). PEG-delivered CRISPR-cas9 ribonucleoproteins system for gene-editing screening of maize protoplasts. *Genes.* 11, 1029. doi: 10.3390/genes11091029

Seki, A., and Rutz, S. (2018). Optimized RNP transfection for highly efficient CRISPR/Cas9-mediated gene knockout in primary T cells. *J. Exp. Med.* 215, 985–997. doi: 10.1084/jem.20171626

Shin, S. E., Lim, J. M., Koh, H. G., Kim, E. K., Kang, N. K., Jeon, S., et al. (2016). CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. *Sci. Rep.* 6, 27810. doi: 10.1038/srep27810

Subburaj, S., Chung, S. J., Lee, C., Ryu, S. M., Kim, D. H., Kim, J. S., et al. (2016). Site-directed mutagenesis in *Petunia* × *hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep.* 35, 1535–1544. doi: 10.1007/s00299-016-1937-7

Subburaj, S., Zanatta, C. B., Nunn, J. A. L., Hoepers, A. M., Nodari, R. O., and Agapito-Tenfen, S. Z. (2022). A DNA-free editing platform for genetic screens in soybean via CRISPR/cas9 ribonucleoprotein delivery. *Front. Plant Sci.* 13. doi: 10.3389/fpls.2022.939997

Tyagi, S., Spörlein, B., Tyagi, A. K., Herrmann, R. G., and Koop, H. U. (1989). PEG- and electroporation-induced transformation in *Nicotiana tabacum*: influence of genotype on transformation frequencies. *Theor. Appl. Genet.* 78, 287–292. doi: 10.1007/BF00288813

Wei, Z. M., and Xu, Z. H. (1988). Plant regeneration from protoplasts of soybean (*Glycine max* L.). *Plant Cell Rep.* 7, 348–351. doi: 10.1007/BF00269935

Woo, J. W., Kim, J., Kwon, S. I., Corvalán, C., Cho, S. W., Kim, H., et al. (2015). DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33, 1162–1164. doi: 10.1038/nbt.3389

Wu, S., Zhu, H., Liu, J., Yang, Q., Shao, X., Bi, F., et al. (2020). Establishment of a PEG-mediated protoplast transformation system based on DNA and CRISPR/Cas9 ribonucleoprotein complexes for banana. *BMC Plant Biol.* 20, 425. doi: 10.1186/s12870-020-02609-8

Xu, J., Kang, B. C., Naing, A. H., Bae, S. J., Kim, J. S., Kim, H., et al. (2020). CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances Petunia flower longevity. *Plant Biotechnol. J.* 18, 287–297. doi: 10.1111/pbi.13197

Yeap W., -C., Norkhairunnisa, C. M. K., Norfadzilah, J., Muhammad Rashdan, M., Appleton, D. R., and Harikrishna, K. (2021). An efficient clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 mutagenesis system for oil palm (*Elaeis guineensis*). *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.773656

Yu, J., Tu, L., Subburaj, S., Bae, S., and Lee, G. J. (2021). Simultaneous targeting of duplicated genes in Petunia protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Rep.* 40, 1037–1045. doi: 10.1007/s00299-020-02593-1

Zhang, Z., Hua, L., Gupta, A., Tricoli, D., Edwards, K. J., Yang, B., et al. (2019). Development of an Agrobacterium-delivered CRISPR/Cas9 system for wheat genome editing. *Plant Biotechnol. J.* 17, 1623–1635. doi: 10.1111/pbi.13088

Zhang, Y., Iaffaldano, B., and Qi., Y. (2021). CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant Commun.* 2, 100168. doi: 10.1016/j.xplc.2021.100168

Zhang, C., Liu, S., Li, X., Zhang, R., and Li, J. (2022). Virus-induced gene editing and its applications in plants. *Int. J. Mol. Sci.* 23, 10202. doi: 10.3390/ijms231810202

Zhang, Q., Xing, H. L., Wang, Z. P., Zhang, H. Y., Yang, F., Wang, X. C., et al. (2018). Potential high-frequency off-target mutagenesis induced by CRISPR/Cas9 in Arabidopsis and its prevention. *Plant Mol. Biol.* 96, 445–456. doi: 10.1007/s11103-018-0709-x

Zhu, J., Song, N., Sun, S., Yang, W., Zhao, H., Song, W., et al. (2016). Efficiency and inheritance of targeted mutagenesis in maize using CRISPR-cas9. *J. Genet. Genomics* 43, 25–36. doi: 10.1016/j.jgg.2015.10.006

Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., et al. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35, 438–440. doi: 10.1038/nbt.3811

frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 16 October 2023
DOI 10.3389/fpls.2023.1271368

OPEN ACCESS

EDITED BY
Tong Zhang,
South China Agricultural University, China

REVIEWED BY
Changjun Huang,
Yunnan Academy of Tobacco Agricultural Sciences, China
U. M. Aruna Kumara,
University of Colombo, Sri Lanka

*CORRESPONDENCE
Kyun Oh Lee
leeko@gnu.ac.kr
Jae-Yean Kim
kimjy@gnu.ac.kr

†These authors have contributed equally to this work

RECEIVED 02 August 2023
ACCEPTED 02 October 2023
PUBLISHED 16 October 2023

CITATION
Vu BN, Vu TV, Yoo JY, Nguyen NT, Ko KS, Kim J-Y and Lee KO (2023) CRISPR-Cas-mediated unfolded protein response control for enhancing plant stress resistance.
Front. Plant Sci. 14:1271368.
doi: 10.3389/fpls.2023.1271368

CRISPR-Cas-mediated unfolded protein response control for enhancing plant stress resistance

Bich Ngoc Vu[1,2†], Tien Van Vu[1†], Jae Yong Yoo[1], Ngan Thi Nguyen[1,2], Ki Seong Ko[1], Jae-Yean Kim[1,2,3*] and Kyun Oh Lee[1,2*]

[1]Plant Molecular Biology and Biotechnology Research Center (PMBBRC), Gyeongsang National University, Jinju, Republic of Korea, [2]Division of Applied Life Science (BK21 Four), Gyeongsang National University, Jinju, Republic of Korea, [3]Nulla Bio Inc., Jinju, Republic of Korea

Plants consistently encounter environmental stresses that negatively affect their growth and development. To mitigate these challenges, plants have developed a range of adaptive strategies, including the unfolded protein response (UPR), which enables them to manage endoplasmic reticulum (ER) stress resulting from various adverse conditions. The CRISPR-Cas system has emerged as a powerful tool for plant biotechnology, with the potential to improve plant tolerance and resistance to biotic and abiotic stresses, as well as enhance crop productivity and quality by targeting specific genes, including those related to the UPR. This review highlights recent advancements in UPR signaling pathways and CRISPR-Cas technology, with a particular focus on the use of CRISPR-Cas in studying plant UPR. We also explore prospective applications of CRISPR-Cas in engineering UPR-related genes for crop improvement. The integration of CRISPR-Cas technology into plant biotechnology holds the promise to revolutionize agriculture by producing crops with enhanced resistance to environmental stresses, increased productivity, and improved quality traits.

KEYWORDS

endoplasmic reticulum (ER) stress, unfolded protein response (UPR), genome editing, CRISPR-Cas, crop improvement

Introduction

The endoplasmic reticulum (ER) is a crucial organelle responsible for protein folding and modifications in eukaryotic cells (Schuldiner and Schwappach, 2013). Proper protein folding is essential for the proper function of secretory and membrane proteins, which account for approximately 30% of the total proteome (Wallin and Von Heijne, 1998; Schubert et al., 2000). Various post-translational modifications, such as N-linked glycosylation, disulfide bond formation, and chaperone-assisted folding, occur in the ER lumen to ensure proper protein folding (Duwi Fanata et al., 2013). However, protein folding, occurring within the ER, can be disrupted not only by internal factors, such as

genetic mutations and hereditary metabolic defects, but also by external factors, such as biotic and abiotic stresses (Park and Park, 2019). When proteins fail to fold properly or become misfolded due to these intrinsic or extrinsic factors, their accumulation within the ER leads to an aberrant cellular condition known as ER stress (Howell, 2013). To address ER stress, eukaryotic cells activate a series of compensatory adaptive mechanisms, collectively called the unfolded protein response (UPR) (Kozutsumi et al., 1988; Harding et al., 2002). The UPR activates a process that increases the expression of ER chaperone genes, enhancing the protein folding capacity of the ER, while also inhibiting protein synthesis and promoting ER-associated protein degradation (ERAD) to alleviate the burden of misfolded proteins (Duwi Fanata et al., 2013). When the UPR is not able to mitigate ER stress, it can lead to apoptosis or cell death, which may contribute to the development of prominent stress-related phenotypes, such as inhibited growth or developmental abnormalities (Hetz, 2012; Angelos et al., 2017).

Numerous studies have reported on the evolutionarily conserved UPR mechanism in eukaryotes, from yeast to animals and plants (Chakraborty et al., 2016; Howell, 2021). The UPR has been extensively characterized in mammals, where it consists of three ER signaling pathways: activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1)-mediated splicing activation of X-box binding protein 1 (XBP1) mRNA, and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) (Chakrabarti et al., 2011). Aberrant UPR has been implicated in a wide range of disease states, including diabetes, immune and inflammatory disorders, and cancers (Marciniak, 2019). Thus, signaling pathways of the UPR have emerged as a potential therapeutic axis for treating various diseases (Marciniak, 2019). While UPR mechanisms in mammals have been a subject of extensive research, exploration into the molecular mechanisms of ER stress responses in plants began more recently. Early investigations into plant UPR primarily started in the early 2000s, with a surge of substantial research outputs emerging a decade later (Koizumi et al., 2001; Noh et al., 2002; Deng et al., 2011; Nagashima et al., 2011; Moreno et al., 2012). With the advent of omics technologies, the field of plant UPR research is now transitioning into a new era characterized by big data. Two ER stress-transducing pathways have been identified in plants: IRE1a and b, which are functional homologs of IRE1 in mammals, and basic leucine zipper protein 17 and 28 (bZIP17 and bZIP28), which are functional homologs of ATF6 in mammals (Kim et al., 2022a). These pathways are involved in the UPR and perform similar functions to their counterparts in mammals. However, the existence of the PERK branch in plants, which is present in mammalian cells, is still unknown (Bao and Howell, 2017).

In sessile plants, the inherent inability to evade unfavorable environmental conditions results in frequent exposure to various abiotic and biotic stresses, such as drought, temperature fluctuations, salinity, herbicidal exposure, and pathogen infection (Park and Park, 2019). These stresses detrimentally impact crop yields, posing significant challenges to global food security. Furthermore, climate change-induced alterations in pathogen and insect behavior contribute to substantial reductions in crop productivity worldwide (Anderegg et al., 2020; Hassani et al., 2020; Van Houtan et al., 2021; Von Der Gathen et al., 2021; Zandalinas et al., 2021). Therefore, it is important to better understand the mechanisms underlying the impacts of these stresses on various crops. This knowledge will facilitate the optimization of tolerance and resistance to both biotic and abiotic stresses, and will ultimately contribute to the optimization of plant growth, development, yield, and quality (Rivero et al., 2022).

In recent years, CRISPR-Cas-based precise genome editing has emerged as a powerful tool, enabling the study of molecular mechanisms associated with ER stress and crop improvements (Singh et al., 2019; Um et al., 2021). CRISPR-Cas9, initially discovered in bacteria, has been engineered for use in various plant species to improve yield, quality, and stress tolerance (El-Mounadi et al., 2020; Vu et al., 2020a). There are several prospective strategies in which the CRISPR-Cas-based genome editing technology can be applied to UPR research. For instance, researchers can employ CRISPR-Cas to knockout or knockdown UPR-related genes. By investigating how these engineered plants respond to ER stress and the phenotypes they exhibit, scientists can gain valuable insights into the role of the targeted genes in the UPR pathway (Mishiba et al., 2019; Liu et al., 2020). Furthermore, the CRISPR-Cas system holds potential for modifying *cis*-regulatory elements or promoter regions in the genome, which in turn control gene expression, to augment stress resilience and other desirable traits (Lim et al., 2022). In this review, we also discuss several promising applications and future prospects of employing the CRISPR-Cas-based genome editing technology for strategic modifications of genes associated with ER stress responses, aiming to improve stress tolerance, productivity, and crop quality.

UPR in plant adaptation to biotic and abiotic stresses

Plant adaptation to environmental stress is a complex process that involves a range of molecular, physiological, and biochemical responses. In plant stress response research, the majority of investigations have focused on single biotic or abiotic elements; however, the simultaneous presence of both biotic and abiotic stresses can markedly influence plant growth, productivity, and viability (Park and Park, 2019). To cope with these multiple stresses, plants initiate a range of signaling pathways and regulatory processes to preserve homeostasis and adapt to changing environmental conditions. The UPR is one such mechanism; it is a conserved response found across eukaryotic organisms, including plants, and plays a critical role in cellular adaptation to stress (Chakraborty et al., 2016). The UPR has emerged as a crucial regulatory mechanism in plant adaptation to combined biotic and abiotic stresses, allowing plants to cope with the challenges posed by their environment.

Under heat stress conditions, protein folding becomes perturbed, and several ER membrane-associated transcription factors relay stress signals to the nucleus, which in turn activates stress-responsive genes (Fragkostefanakis et al., 2016; Reyes-Impellizzeri and Moreno, 2021). It has been discovered that in diverse plant species, such as *Arabidopsis thaliana* (Arabidopsis)

and *Zea mays* (maize), heat stimulation causes IRE1 to splice *bZIP60* mRNA (Deng et al., 2011; Li et al., 2012; Neill et al., 2019). The expression of active bZIP60 also elevates the transcription of heat shock protein (HSP) genes, suggesting a link between the UPR and the heat shock response mechanism (Li et al., 2020c). The transcription factor bZIP28 regulates the expression of UPR-related genes in response to heat stress conditions via a proteolytic mechanism, which triggers the translocation of bZIP28 to the nucleus (Iwata et al., 2017). Drought and salt significantly impact plant development and yield. In response to salt stress, bZIP17 is cleaved by site-1 proteases (S1P) and translocated to the nucleus to activate UPR genes (Liu et al., 2007b). It has been shown that the transcription factors bZIP60 and bZIP17 orchestrate the expression of the molecular chaperone gene, luminal-binding protein 3 (BiP3), as well as several genes implicated in the response to salt stress conditions (Henriquez-Valencia et al., 2015). Elevated expression of BiP has been observed to augment drought tolerance in a variety of plant species, such as *Glycine max* (soybean), *Nicotiana tabacum* (tobacco), and Arabidopsis (Coutinho et al., 2019). Infection by pathogens has been shown to induce ER stress in plants, with the IRE1-bZIP60 signaling pathway playing a crucial role in mounting a defense against the fungal pathogen, *Alternaria alternate* (Xu et al., 2019). Plants with mutations in *IRE1* and *bZIP60* are more susceptible to bacterial and viral infections (Moreno et al., 2012). In *Nicotiana benthamiana*, UPR was activated by the Geminivirus satellite-encoded βC1, which induces the nuclear export of NbbZIP60 to evade the plant defense response (Zhang et al., 2023). Overall, these findings indicate that various biotic and abiotic factors can disrupt protein folding capacity and activate the UPR in plants. As environmental stresses continue to impact global agriculture, the role of the UPR in facilitating plant adaptation to combined biotic and abiotic stresses is becoming a more significant area of research. Investigating the function of the UPR in plants has the potential to enhance crop improvement and sustainable agriculture practices, making the study of UPR activation in response to stress in plants increasingly important for agricultural research.

UPR pathways in plant cells: mechanisms and regulation

The UPR constitutes a crucial regulatory process in plant cells, which is activated upon the presence of misfolded or unfolded proteins stress (Howell, 2021). Comprehensive investigations have been conducted to elucidate the UPR pathways in a range of plant species, encompassing maize, *Oryza sativa* (rice), *Solanum lycopersicum* (tomato), soybean, tobacco, and Arabidopsis (Lu et al., 2012; Czekus et al., 2020; Pastor-Cantizano et al., 2020; Czekus et al., 2022; Yang et al., 2022). In plants, the UPR is mediated through two distinct signal transduction pathways. The initial pathway, referred to as the IRE1 pathway, is facilitated by IRE1 and involves two isoforms in Arabidopsis, IRE1a and IRE1b (Figure 1) (Koizumi et al., 2001; Noh et al., 2002; Moreno et al., 2012). IRE1a and IRE1b possess homologous cytoplasmic regions characterized by the presence of a kinase domain, but exhibit functional divergence (Noh et al., 2002). IRE1a is primarily required for biotic stresses, while IRE1b plays a predominant role in abiotic stresses (Deng et al., 2011; Afrin et al., 2020). An additional isoform of IRE1, designated as IRE1C, has been identified as unique to plants (Mishiba et al., 2019). Recent evidence has shown that IRE1 promotes balanced cell expansion by restricting the Target of Rapamycin (TOR) kinase-dependent control of cellular differentiation (Angelos and Brandizzi, 2022). However, its precise role in the UPR remains to be elucidated. Under ER stress conditions, BiP binds to unfolded proteins, dissociating IRE1, which then undergoes trans-autophosphorylation and dimerization (Figure 1). The endonucleases IRE1a and IRE1b facilitate the removal of a 26-nucleotide intron from *bZIP60* mRNA, resulting in the production of the spliced variant *bZIP60s*, which encodes an active transcription factor (Figure 1) (Deng et al., 2011; Nagashima et al., 2011; Li et al., 2012; Moreno et al., 2012; Li et al., 2022). Upon activation, bZIP60s translocates to the nucleus, where it promotes the expression of genes associated with ER stress (Figure 1) (Iwata et al., 2008; Deng et al., 2011; Nagashima et al., 2011; Moreno et al., 2012; Hayashi et al., 2013; Sun et al., 2013; Xu et al., 2019). Under severe or prolonged ER stress, IRE1 also degrades many mRNAs on the ER membrane encoding secretory pathway proteins through a selective cleavage mechanism referred to as regulated IRE1-dependent decay (RIDD) (Figure 1) (Mishiba et al., 2013; Hayashi et al., 2016). The recent findings indicate that AtIRE1 determines cell fate during ER stress by balancing the UPR and the ubiquitin-proteasome system (UPS) via a key pro-death component, phosphatase type 2CA (PP2CA)-interacting finger protein 1 (PIR1). However, the mechanism by which AtIRE1 regulates PIR1 remains unknown (Ko et al., 2023).

The second pathway involves membrane-associated bZIP transcription factors bZIP17 and bZIP28, which are functional homologs of mammalian ATF6 (Liu et al., 2007a; Liu et al., 2007b). Under normal conditions, bZIP17/28 is retained in the ER due to its binding to the BiP protein (Figure 1) (Liu et al., 2007a; Srivastava et al., 2013; Srivastava et al., 2014; Henriquez-Valencia et al., 2015). Under ER stress conditions, bZIP17 and bZIP28 dissociate from BiP, become mobilized, and undergo translocation to the Golgi apparatus through coat protein complex II (COPII) vesicle-mediated transport (Figure 1) (Srivastava et al., 2012). In the Golgi apparatus, they undergo proteolytic processing by two resident site proteases, S1P and S2P, releasing their transcription factor (TF) domains (Figure 1) (Manghwar and Li, 2022). Subsequently, these TF domains translocate to the nucleus, where they act as transcription factors, enhancing the expression of ER stress-associated genes (Figure 1) (Liu et al., 2007b; Li et al., 2010; Liu et al., 2022). Nonetheless, a recent investigation has demonstrated that the activation of bZIP28 occurs through a sequential process involving S2P and as-yet-unidentified proteases, rather than S1P-mediated cleavage (Sun et al., 2015; Iwata et al., 2017). Both bZIP17 and bZIP28 can bind to ER stress response elements (ERSEs) and unfolded protein response elements (UPREs) at the promoter region of UPR-related genes, including BiPs (Figure 1) (Liu and Howell, 2010; Gao et al., 2022). Moreover, bZIP28 can interact with Nuclear

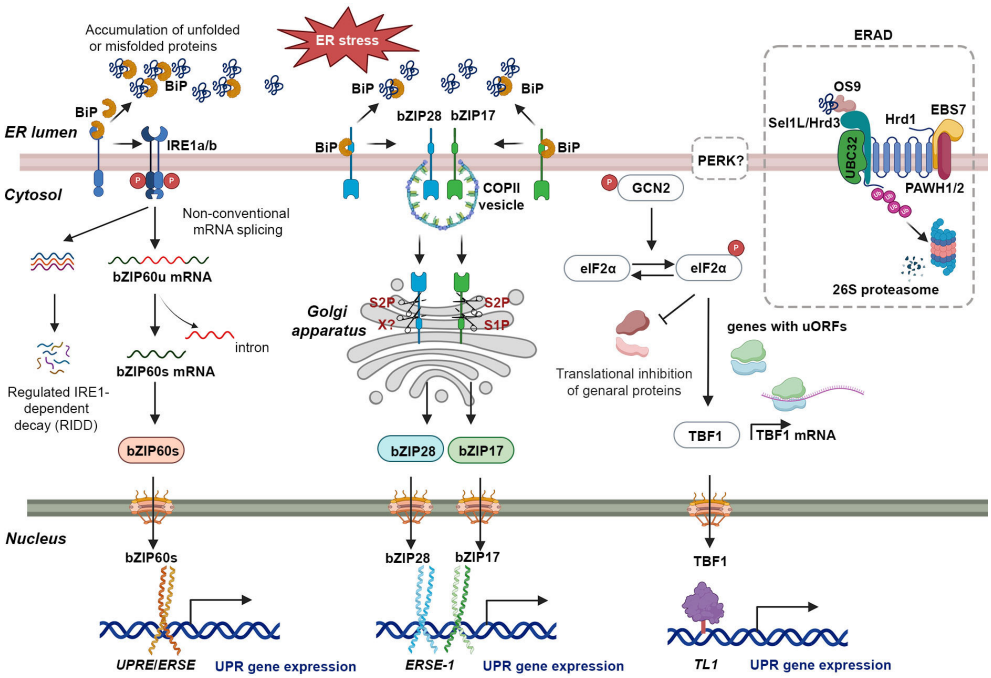


FIGURE 1
The UPR in plants: a signaling network coordinating ER homeostasis and stress adaptation. The UPR is activated by the accumulation of unfolded proteins in the ER due to various factors (top left of figure). BiP binds to unfolded proteins, leading to dissociation of IRE1a and IRE1b. Activated IRE1 then cleaves a specific intron from bZIP60u mRNA, generating bZIP60s mRNA. bZIP60s, a potent transcriptional activator, translocates to the nucleus and binds to UPREs and ERSEs in target gene promoters, inducing the expression of stress-responsive genes. In addition to its role in splicing bZIP60 mRNA, activated IRE1 is also involved in a process called Regulated IRE1-dependent decay (RIDD). Under conditions of chronic stress, IRE1 hyper-activates and cleaves additional mRNAs through RIDD. The bZIP17 and bZIP28 pathway is activated by ER stress in plants. Unfolded proteins bind to BiP, causing bZIP17 and bZIP28 to dissociate from the ER membrane. These transcription factors are transported to the Golgi, where proteolytic cleavage mediated by S1P and S2P enzymes releases their N-terminal domains. The N-terminal domains contain the necessary domains for their function as transcription factors. Upon translocation to the nucleus, they bind to ERSE-1 sequences in target gene promoters, inducing the expression of UPR-associated genes encoding ER chaperones and ERAD proteins involved in protein folding, quality control, and degradation within the ER. GCN2 is a kinase activated by dimerization and autophosphorylation in response to endoplasmic reticulum stress. It phosphorylates eIF2α, leading to widespread inhibition of mRNA translation. However, a specific group of uncapped mRNAs with upstream open reading frames (uORFs), such as TBF1 mRNA, are selectively translated. TBF1, a heat-shock factor-like transcription factor, binds to the *TL1 cis*-element, crucial for inducing BiP2 and CRT3. TBF1 also plays a role in coordinating developmental processes with stress responses, particularly in the growth-to-defense transition. During ER stress, ER chaperones assist in proper protein folding, while ERAD proteins eliminate irreversibly misfolded proteins. ERAD initiation involves OS9 recognizing the N-glycan on a misfolded protein and associating with Sel1L/Hrd3. The Hrd1-Sel1L/Hrd3-OS9 complex, along with UBC32, the E2 enzyme, promotes ubiquitination (Ub) of the misfolded protein for subsequent cytosolic degradation. This process helps restore ER homeostasis by removing unfolded proteins that could disrupt cellular functions.

transcription factor Y (NF-Y) and form a transcriptional complex to upregulate UPR-related genes (Liu and Howell, 2010). Typically, bZIP17 and bZIP28 exhibit comparable activation patterns in response to ER stress inducers, including chemicals like tunicamycin (TM) or dithiothreitol (DTT), cadmium (Cd) as well as environmental stresses such as heat stress and viral infections (Liu et al., 2007b; Li et al., 2010; Gao et al., 2022; Li et al., 2022; De Benedictis et al., 2023). However, they show differences in sensitivity in certain environmental stresses. For instance, under salt stress conditions, bZIP17 elevates the expression of the chaperone BiP3, whereas bZIP28 participates in responses to pathogen infections (Henriquez-Valencia et al., 2015; Qiang et al., 2021).

Although the PERK pathway, which is present in mammals, has not been identified in plants, General Control Non-repressible 2 (GCN2) has been identified as an orthologue of the elF2α kinase that responds to both abiotic and biotic stresses (Yu et al., 2022). Recently, Arabidopsis GCN2 was shown to activate the translation of a heat-shock factor-like transcription factor, TL1-binding transcription factor 1 (TBF1), which contains upstream open reading frames (uORFs) within its 5' untranslated region (5' UTR) (Figure 1) (Liu et al., 2019). This process is initiated in response to pathogen invasion, subsequently triggering specific transcriptional reprogramming through the expression of target genes (Lageix et al., 2008; Pajerowska-Mukhtar et al., 2012; Liu et al., 2019).

Role in maintaining ER homeostasis and protein quality control

In situations of excessive or prolonged ER stress, where UPR mechanisms cannot restore protein folding, the ERAD system facilitates the clearance of terminally aberrant proteins, thus maintaining ER homeostasis (Vembar and Brodsky, 2008; Hwang and Qi, 2018). The ERAD comprises a multistep process, which

includes the identification of cargo proteins, retro-translocation of substrates to the cytoplasm through an ER membrane channel, ubiquitination of ER proteins by ubiquitin enzymes, and subsequent degradation of ubiquitinated substrates via the 26S proteasome (Figure 1) (Chen et al., 2020). The ERAD machinery has been extensively studied in yeast and mammals (Ye et al., 2001; Sato et al., 2009; Avci and Lemberg, 2015; Habeck et al., 2015; Schoebel et al., 2017). In recent years, several ERAD components have been identified and characterized in plants (Chen et al., 2022). The N-glycans of misfolded proteins are recognized by osteosarcoma amplified 9 (OS9), which associates with the suppressor enhancer Lin12 1 like (Sel1L)/HMG-CoA reductase degradation protein 3 (Hrd3)/HMG-CoA reductase degradation 1 (Hrd1) complex (Figure 1) (Duwi Fanata et al., 2013). Protein associated with Hrd1-1/2 (PAWH1/2) interaction with EMS-mutagenized Bri1 suppressor 7 (EBS7) indirectly associates with Hrd1, regulating the stability and activity of the E3 ligase (Figure 1) (Liu et al., 2015; Lin et al., 2019). Therefore, Hrd1 has the potential to target UBC32, an E2 enzyme located on the ER membrane of Arabidopsis. UBC32 is responsible for the ubiquitination of aberrant proteins that is induced by stress, leading to their subsequent degradation in the cytosol via the proteasome pathway (Figure 1) (Cui et al., 2012; Chen et al., 2017; Chen et al., 2020).

ERAD, an important proteolytic pathway crucial to protein quality control, appears as a key factor in various studies associated with the enhancement of plant resistance to environmental stresses, productivity increase, and quality improvement. ERAD is a significant mechanism in plants for responding to environmental stresses, showing resistance capabilities to heat stress, drought, and salinity (Li et al., 2017; Strasser, 2018). In various plant species, evolutionarily conserved homologous ERAD components appear to be associated with stress tolerance and plant defense pathways elucidated two evolutionarily conserved ERAD pathways, DOA10 and HRD1, responding to heat stress in Arabidopsis (Liu and Li, 2014; Li et al., 2017; Strasser, 2018; Huber et al., 2021). This study demonstrated that loss-of-function mutants exhibited a higher survival rate and lower electrolyte leakage compared to the wild-type plants, enhancing plant resistance to heat stress (Li et al., 2017; Strasser, 2018). ERAD influences plant productivity by managing ER stress caused by protein misfolding. In this context, Ohta and Takaiwa (2015) showed that OsHrd3 is necessary for maintaining the quality of ER-derived protein bodies in rice endosperm (Ohta and Takaiwa, 2015). Additionally, Wakasa et al. (2011) proposed the possibility of improving the protein quality of rice through the role of ER stress response and ERAD (Wakasa et al., 2011). However, further research is necessary for a comprehensive understanding of ERAD associated with enhancing plant stress resistance, productivity, and quality.

CRISPR-Cas system as a versatile genome editing tool in plants

The CRISPR-Cas system is an adaptive immune mechanism used by bacteria to defend against the invasion of bacteriophages (Mojica et al., 2005). The system comprises an endonuclease (Cas) and a guide RNA (gRNA) that together form a ribonucleoprotein complex. The Cas complex locates and binds to a target dsDNA with the help of the guidance of gRNA. Once the complex is activated, the Cas enzyme cleaves the phosphodiester bonds of both strands, creating a double-stranded break (DSB) in the target DNA (Barrangou et al., 2007). The cells repair the DSB using nonhomologous end-joining (NHEJ) or homologous recombination (HR) (Figure 2A), which may result in mutations or modifications in the DNA sequence, thereby achieving gene editing (Jinek et al., 2012; Cong et al., 2013; Dickinson et al., 2013; Schwank et al., 2013; Van Vu et al., 2019).

Since its repurposing for customized DNA cleavages and subsequent gene editing in 2012, the CRISPR era has begun (Jinek et al., 2012). The technology, recognized for its potential in precise genome engineering, has proven valuable in agriculture and various other fields, paving the way for numerous applications and advancements (Chen et al., 2019). As of today, there are two classes, six types, and over 30 subtypes of the CRISPR-Cas system that function in DNA or RNA targeting or other activities (Koonin and Makarova, 2022). The system has not only been repurposed as molecular scissors but also for other applications such as transcriptional regulations (Cheng et al., 2013; Qi et al., 2013). Furthermore, the editing scope has expanded from single bases (Figure 2B) (Komor et al., 2016; Gaudelli et al., 2017; Chen et al., 2021; Kurt et al., 2021; Tong et al., 2023) to small DNA changes with prime editing (Figure 2C) (Anzalone et al., 2019; Lin et al., 2020) and microhomology-mediated precision short DNA replacement (Tien Van et al., 2022), extending to kilobase-gene targeting, thereby establishing it as a versatile tool for genome editing (Dickinson et al., 2013; Schwank et al., 2013; Chen et al., 2019; Vu et al., 2020b).

The CRISPR-Cas system has been extensively utilized in plant biology and crop engineering, with a wide range of applications developed for editing target genes in both monocot and dicot species (Chen et al., 2019; Zhu et al., 2020). These applications include simple edits with indel mutations as well as HR-based precise gene replacement (Feng et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Chen et al., 2019; Vu et al., 2020b). Due to the versatility of the CRISPR system, it has emerged as a valuable tool for achieving high efficiency in gene editing in plants, proving to be a significant asset in advancing plant biology and crop improvement research (Chen et al., 2019; Zhu et al., 2020; Tien Van et al., 2022).

Exploring the versatility of CRISPR-Cas system in UPR pathways

The CRISPR-Cas system is a versatile tool with numerous applications, including gene functionalization and regulation. Cas9 complexes act like molecular scissors and can theoretically cleave any genomic site of interest if a PAM motif is present. Previously, researchers relied on knockout lines generated by T-DNA insertion, random mutagenesis, or RNA interference (RNAi)-based downregulation lines to assess gene function (Howell, 2013; Pucker et al., 2021). However, these methods have limitations such

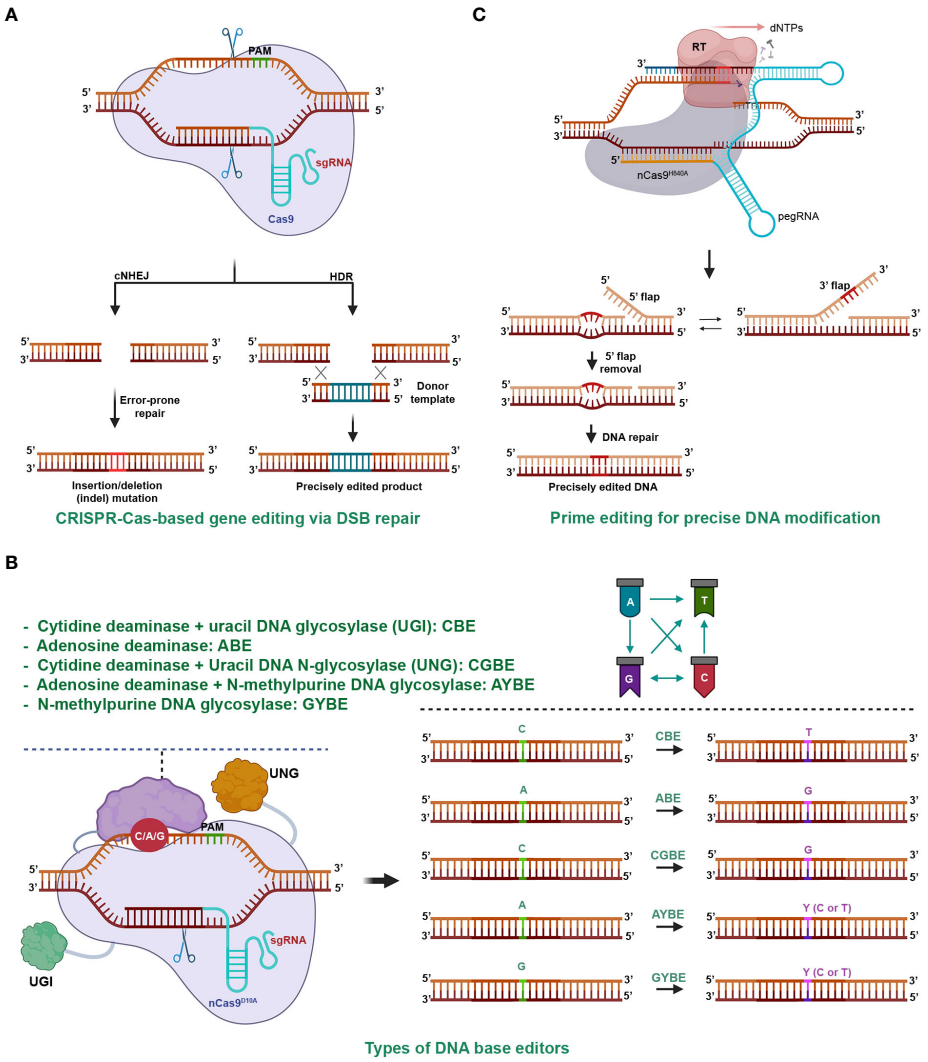


FIGURE 2
CRISPR-Cas-Mediated Gene Editing. **(A)** CRISPR-Cas9-Mediated Gene Editing through Double-Stranded Break (DSB) Repair. The CRISPR-Cas complex cleaves both strands of the target DNA, resulting in a DSB. The repair of the DSB predominantly occurs through two pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ usually restores the original DNA sequence, but it can lead to imperfect repair and DNA insertion or deletion mutations, particularly during intense DSB formation. HR precisely inserts the desired sequence (green sticks) into the genomic region using a DNA donor template with homologous ends to the DSB terminals. **(B)** DNA base editors for genome editing. Base editing involves a deaminase, usually fused with a Cas9 (nCas9^{D10A}) nickase, to remove an amino group from a nucleobase on the non-target strand. The deaminated base is then repaired via base excision or nucleotide excision repair, resulting in base transitions or transversions. Depending on the type of deaminase used, base conversion can lead to transitions, such as cytidine deaminase for C/G to A/T in cytosine base editors (CBE), or A/T to G/C in adenine base editors (ABE). Adding uracil DNA N-glycosylase inhibitors (UGI) enhances CBE efficiency. Base transversions can be achieved by adding uracil DNA N-glycosylase (UNG) to CBE (C/G to G/C in CGBE), N-methylpurine N-glycosylase to ABE (A/T to C/G or T/A), or by using UNG alone (G/C to C/G or T/A with GYBE). **(C)** Prime editing for precise DNA modification. The prime editing utilizes a pegRNA and a reverse transcriptase (RT) enzyme fused to the C-terminal of a nCas9^{H840A}. It copies genetic information from the 3′ extension of the pegRNA into the nicked end on the non-target strand. By introducing desired genetic changes within the RT template of the 3′ extension, prime editing enables precise genetic modifications at the target site. Prime editing allows for a wide range of precise DNA changes within a genome, including various types of base conversion, DNA insertion, and deletion.

as complex T-DNA integration events (Tamura et al., 2016) or residual gene activity in downregulated lines (Santillan Martinez et al., 2020), which lead to complicated analysis. The emergence of CRISPR-Cas technology has revolutionized the study of gene function in plants, as knockout lines generated by CRISPR-Cas tools are more precise and cleaner than traditional methods (Tien Van et al., 2022). The CRISPR-Cas system is highly efficient, customizable, simple, and cost-effective, making it an accessible tool for labs worldwide (Ahmadzadeh et al., 2019; Chen et al., 2019; He et al., 2023).

Although CRISPR-Cas has been used extensively for gene regulation and functionalization since its discovery in 2012, its application in studying ER stress response is relatively recent and began in 2019 (Mishiba et al., 2019; Liu et al., 2020). Even though a few CRISPR-Cas-related studies have been conducted in this field, there is still considerable potential for further functional exploration

of genes involved in ER stress signaling using this technology (Tables 1, 2). Early research using CRISPR-Cas to study ER stress response focused on the Arabidopsis genes Protein Associated with PAWH1 and PAWH2 (Table 1) (Lin et al., 2019). These genes are essential components of the ERAD pathway (Lin et al., 2019) and play vital roles in mitigating environmental stress such as salinity (Liu et al., 2011). The PAWH genes were induced by ER stress and contributed to the stabilization of the UPR sensing complexes mediated by the EBS7 and Hrd1 (Lin et al., 2019). Additionally, the IRE1-mediated RNA splicing of *AtbZIP60* is a critical aspect of the UPR pathway in ER stress responses (Yu et al., 2022). To investigate the role of IRE1b in the signaling arm, the sensor domain-coding region of the gene was deleted using dual gRNA CRISPR-Cas9 complexes in the *ire1a*/*c* mutant background (Table 1) (Mishiba et al., 2019). The IRE1b-edited lines exhibited similar effects on BiP3 and PR-4 transcription as the *ire1a*/*b* mutant lines and bZIP60 RNA splicing as the *ire1a*/*c* mutants. However, there was no evidence of growth defects or seed set reductions in the mutant lines (Table 1) (Mishiba et al., 2019).

In rice, the IRE1-mediated RNA splicing in ER stress responses is also conserved, and the OsbZIP74 (also known as OsbZIP50) is the ortholog of AtbZIP60 (Lu et al., 2012). Modified OsbZIP74 is transported to the nucleus and upregulates UPR-related genes, including several membrane-associated NAC transcription factors. In a recent study, researchers utilized the CRISPR-Cas9 gene-editing system to generate knockout mutants of OsNTL3, confirming its role as a transcriptional activator of OsbZIP74 (Table 1) (Liu et al., 2020). The investigation also showed that OsbZIP74 positively regulates the transcription of OsNTL3 under heat stress conditions. Additionally, the study revealed that the loss of OsNTL3 function results in increased heat sensitivity in rice seedlings (Table 1) (Liu et al., 2020). *OsbZIP60* has been identified as a critical regulator of grain chalkiness, a stress-related phenotype in rice (Hayashi et al., 2012; Takahashi et al., 2012; Yang et al., 2022). To understand the role of *OsbZIP60* in managing this response, *osbzip60* knockout mutants were created using CRISPR-Cas9 gene-editing technology. In these mutants, upregulation of several chaperone genes, including *OsbZIP50*, *OsBiP1*, *OsBiP2*, and

TABLE 1 Recent studies related to the UPR that used CRISPR-Cas9 tools.

No.	Target gene	Plant species	Gene function	Impact	Reference
1	*PAWH1* and *PAWH2*	*Arabidopsis thaliana*	Plant-specific components of ERAD complex	The *pawh1pawh2* double mutants suppressed the dwarf phenotype of the corresponding *bri1-5*	(Lin et al., 2019)
2	*IRE1B*	*Arabidopsis thaliana*	Splicing of bZIP60-encoding mRNA	Deletion of the IRE1B's sensor domain by CRISPR-Cas9 showed no growth defect and seed set reduction	(Mishiba et al., 2019)
3	*OsNTL3*	*Oryza sativa*	Regulation the expression of OsbZIP74 and other UPR related-genes involved under heat stress conditions	The *ntl3* mutant plants showed more sensitive phenotype to heat stress treatment	(Liu et al., 2020)
4	*IAN2*, *IAN3*, *IAN4*, *IAN5*, *IAN6*, *OsIAN1*, and *OsIAN2*	*Arabidopsis thaliana* and *Oryza sativa*	Regulation of the HSR, UPR, and cell death	The single mutants (*ian3*, *ian5*, *ian6*) and double mutants (*ian2ian3*, *ian4ian5*) showed less barren siliques along their main inflorescences than wild type	(Lu et al., 2021)
5	*Sec23* isoforms	*Physcomitrium patens*	Influencing ER to Golgi apparatus trafficking and secretion to the plasma membrane	The *sec23d* mutant showed smaller phenotype and fewer gametophores than the wild type, while the quintuple *sec23abcfg* mutant have no detectable growth defects	(Chang et al., 2022)
6	*OsHLP1*	*Oryza sativa*	OsHLP1 promotes disease resistance by compromising ER homeostasis when plants are infected by pathogens	The *oshlp1* mutant showed compromising blast disease resistance in rice	(Meng et al., 2022)
	OsNTL6		ER homeostasis in rice during infection of *Magnaporthe oryzae*	The *osntl6* mutant plants showed enhanced disease resistance compared with wild type plants	
7	*OsbZIP60*	*Oryza sativa*	OsbZIP60 regulates the formation of grain chalkiness in rice via UPR	The *osbzip60* mutant plants showed high grain chalkiness rate and white floury endosperm	(Yang et al., 2022)
	OsbZIP50		OsbZIP50 played an important role in the formation of grain chalkiness	The *osbzip50* mutant plants had high grain chalkiness rates	
8	*NOBIRO6/TAF12b*	*Arabidopsis thaliana*	NOBIRO6/TAF12b contributes to UPR-associated root growth control	The *nobiro6* mutant plants rescue the root growth defect characteristic of the *bzip17bzip28* double mutant	(Kim et al., 2022a)
9	*NbbZIP60*	*Nicotiana benthamiana*	Geminivirus satellite-encode βC1 activates UPR, induces bZIP60 nuclear export, and manipulates the expression of bZIP60 downstream genes to benefit virus infection	The *nbbzip60* mutant plants showed milder curling symptoms than the wild type plants after inoculating these plants with TYLCCNV/TYLCCNB through agro-infiltration.	(Zhang et al., 2023)

TABLE 2 Recent promoter editing studies that used CRISPR-Cas9 tools.

No.	Target gene	Plant species	Promoter edited regions	Impact	Reference
1	*OsRAV2*	*Oryza sativa*	Mutation in the *GT-1* element regions of *OsRAV2*	Induced salt stress response	(Duan et al., 2016)
2	*ARGOS8*	*Maize*	Mutation of negative maize GOS2 promoter of *ARGOS8*	Enhanced drought stress conditions in the field	(Shi et al., 2017)
3	*SWEET11/13/14*	*Oryza sativa*	Mutation in the *EBEs* in the *SWEET11/13/14* promoters	Improved *Xanthomonas oryzae* pv. *Oryzae* resistance	(Oliva et al., 2019)
4	*LsGGP2*	*Lactuca sativa*	Mutation in the uORFs of *LsGGP2*	Increased tolerance to oxidative stress and ascorbate content	(Zhang et al., 2018)
5	*AtTBF1*	*Arabidopsis thaliana*	Strategy of mutation in the uORFs of *AtTBF1*	Expectation in response to pathogen attack	(Vuong et al., 2023)

OsBiP3, was observed, leading to varying degrees of grain chalkiness. This result indicates that *OsbZIP60* plays a critical role in regulating rice grain chalkiness and maintaining ER homeostasis (Table 1) (Yang et al., 2022). In another study, the roles of two bZIP transcription factors, bZIP17 and bZIP28, in the UPR pathway were explored (Liu et al., 2007a; Liu et al., 2007b). The *bzip17bzip28* double mutant displayed stress-related phenotypes, including severe dwarfism, low germination rate, and short roots, compared to the wild-type plant (Kim et al., 2018). To unravel the underlying mechanisms associated with the observed stress-related phenotypes, a suppressor mutant named *nobiro6* was created within the *bzip17bzip28* background using CRISPR-Cas9 technology. This triple mutant, *bzip17bzip28nbr6*, demonstrated a partial rescue of root growth, highlighting the role of NOBIRO6/TAF12b as a transcription cofactor in UPR-associated root growth control (Table 1) (Kim et al., 2022b). In a recent study aiming to understand the role of NbbZIP60 in plant defense responses to pathogens, *nbbzip60* knockout mutants were generated using CRISPR/Cas9-based technology (Zhang et al., 2023). These knockout mutant plants showed a reduced amount of viral DNA, leading to milder leaf curling symptoms compared to wild-type plants under virus infection (Table 1). These findings highlight how CRISPR-Cas9 technology is enabling a deeper understanding of UPR in plants, opening avenues for enhancing plant stress resistance.

CRISPR-Cas mediated dissection of ER stress and plant responses to biotic stresses

The CRISPR-Cas system offers a significant advantage in generating multiple gene mutations through multiplexing methods with multiple gRNAs. For instance, it was used to generate various mutated variants in single and combinations of UPR-related genes encoding for immune-associated nucleotide-binding (IAN) proteins in Arabidopsis (Table 1) (Lu et al., 2021). In the study, IAN2 to IAN6 were found to be located at a single locus on chromosome 1 by genome-wide association study (GWAS) (Lu et al., 2021). The efficient CRISPR-Cas9 system was used to create single, double, triple, and quadruple IAN gene mutations that were crucial for gene functionalization. Interestingly, the *ian* mutants, particularly the *ian6* knockout lines, exhibited enhanced heat tolerance during the reproductive stage in both Arabidopsis and rice (Table 1) (Lu et al., 2021). The IAN6 protein has been discovered to localize to the ER, where it suppresses HSP and UPR-related gene expression and promotes programmed cell death during the reproductive stage (Table 1) (Lu et al., 2021). In a similar manner, multiplexed editing has been effectively used to generate combined knockout mutations in genes associated with COPII-mediated vesicle trafficking from the ER to the Golgi apparatus (Chang et al., 2022). The COPII complex is involved in transporting bZIP28 proteins to the Golgi for processing and subsequent release into the cytosol during ER stress (Srivastava et al., 2012; Howell, 2013). In plants, Sec23 and Sec24 combine with Sar1 to form the inner layer of COPII vesicles. Several isoforms of the Sec23 were shown to form distinct ER exit sites with differential effects on protein trafficking and growth (Yoshihisa et al., 1993; Zeng et al., 2015). The *sec23d* mutant significantly hindered ER-to-Golgi transport, whereas the quintuple *sec23abcfg* mutant primarily impacted protein secretion to the plasma membrane (Table 1) (Chang et al., 2022).

Plant responses to biotic stress, such as those induced by phytopathogens, require the maintenance of ER homeostasis. In rice, OsHLP1, which is induced by Magnaporthe oryzae infection, has been shown to interact with OsNTL6. This interaction suppresses the accumulation of OsNTL6, leading to the activation of genes involved in plant immunity and resulting in enhanced disease resistance (Meng et al., 2022). CRISPR-Cas9 knockout mutants of OsHLP1 displayed reduced disease resistance, suggesting that OsHLP1 positively regulates blast resistance in rice (Table 1) (Meng et al., 2022). In contrast, the OsNTL6 protein acts as a negative regulator of blast disease resistance; overexpression lines led to increased *Magnaporthe oryzae* infection, while knockout lines generated using CRISPR-Cas9 showed reduced infection compared to wild-type plants (Table 1) (Meng et al., 2022). Recent application of CRISPR-Cas technology in studying ER stress and plant responses to biotic stress has

provided valuable insights into critical components of these pathways (Lin et al., 2019; Mishiba et al., 2019; Liu et al., 2020; Lu et al., 2021; Chang et al., 2022; Meng et al., 2022). By using CRISPR-Cas to edit the genome of plants, researchers can generate mutations in UPR-related genes to study their function and determine their role in the UPR pathway. Overall, the CRISPR-Cas system has revolutionized the field of genetics and has significant implications for the study of gene function in plants, including UPR-related genes.

Enhancing stress tolerance and crop improvement through the CRISPR-Cas mediated modification of *cis*-regulatory elements

Cis-regulatory elements (CREs) are noncoding DNA sequences that incorporate transcription factors and other molecular binding sites, such as promoters and enhancers, which influence transcription (Wittkopp and Kalay, 2011; Wolter et al., 2019). The promoter regions of most plant UPR-related genes contain a consensus *cis*-acting element known as the ERSE and/or the UPRE (Liu and Howell, 2010; Howell, 2013; Nawkar et al., 2017). Several studies have underscored the significant potential of crop improvement through the editing of regulatory sequences to adjust gene expression levels, thereby generating novel phenotypic variants (Wolter and Puchta, 2018; Wolter et al., 2019). For instance, the *RAV2* gene, which is transcriptionally induced by salt stress in rice, was subjected to CRISPR-Cas-mediated engineering to modify the *GT-1* element in the promoter, revealing that the *GT-1* element directly governs the salt stress response (Table 2) (Duan et al., 2016; Li et al., 2020b). In maize, ARGOS8, a negative regulator of ethylene responses, enhances drought tolerance (Table 2) (Shi et al., 2015). Plants edited with CRISPR-Cas9 to replace the native promoter region of the *ARGOS8* gene with the *GOS2* promoter demonstrated increased grain yield under drought stress conditions in the field (Table 2) (Shi et al., 2015; Shi et al., 2017; Wang et al., 2022). Similarly, the removal of a regulatory fragment containing a transcription-activator-like effector (TALe)-Binding Element (EBE) in the promoter of *SWEET11* via CRISPR/Cas resulted in improved disease resistance in rice, without affecting fertility (Table 2) (Li et al., 2020a). This development presents a clear advantage over the sterile phenotype of the *Ossweet11* knockout mutant, which is unsuitable for crop improvement. Recently, genome editing of EBEs in *SWEET* promoter genes led to broad-spectrum bacterial blight resistance in rice (Table 2) (Oliva et al., 2019). While predicting the effects of gene expression regulation by manipulation of various CREs, including ERSEs and UPREs, and the resulting phenotypic changes could be difficult, the modification of CREs by CRISPR-Cas holds the potential to be a critical strategy not only for studying UPR signaling pathways, but also for breeding plants with stress tolerance and desirable traits (Figure 3A).

Upstream open reading frames as regulatory elements and CRISPR-Cas9 applications for crop improvement

The uORFs are essential regulatory elements located in the 5' UTR of main open reading frames (mORFs). Recent bioinformatics analyses estimate that approximately 35% of total plant transcripts contain uORFs (Silva et al., 2019; Li et al., 2021). These uORFs are known to act as inhibitors, repressing the initiation of mORF translation via ribosome stalling (Silva et al., 2019; Zhang et al., 2020). A recent research indicates that uORFs possess the ability to regulate gene expression in response to environmental stresses, as they control specific master regulators involved in stress responses (Zhang et al., 2020). Under adverse environmental conditions, stress-responsive transcripts containing uORFs are upregulated, suggesting that CRISPR-Cas9-mediated uORF editing could be a promising approach to enhance gene expression for crop trait improvement (Figure 3B) (Um et al., 2021). For example, the application of CRISPR-Cas9 editing to the uORF of LsGGP2 in *Lactuca sativa* (lettuce) has yielded promising results, demonstrating increased tolerance to oxidative stress and a substantial 150% increase in ascorbate content (Table 2 and Figure 3B) (Zhang et al., 2018). Similarly, in Arabidopsis, TBF1 plays a critical role in the growth-to-defense transition in response to pathogen attack (Table 2) (Traubenik et al., 2021). Under normal conditions, two uORFs in TBF1 inhibit AtTBF1 translation; however, upon pathogen infection, these inhibitory effects are relieved, allowing TBF1 to regulate and induce the expression of defense-related genes (Table 2 and Figure 3B) (Pajerowska-Mukhtar et al., 2012). These findings suggest that CRISPR-Cas-based gene editing can be used to remove or generate uORF sequences in target genes. This strategy can be used to increase or decrease protein translation levels, and applied to develop crops with improved traits, including stress resistance (Vuong et al., 2023). In conclusion, CRISPR-Cas9-mediated uORF editing represents a promising avenue for enhancing plant resilience to environmental stresses and advancing crop trait improvement.

Strategies for enhancing ER stress tolerance in plants using CRISPR-Cas9

Regulating plant responses to ER stress is essential for enhancing crop productivity and survival rates. To address this gap, we propose several strategies for using CRISPR-Cas tools to enhance ER stress tolerance in plants. One approach involves editing genes that directly or indirectly regulate the UPR signaling pathway. However, it is important to note that a thorough understanding of the roles of the targeted genes is necessary for designing an effective editing strategy. The most straightforward method for breeding ER stress tolerance using CRISPR-Cas complexes involves introducing indel mutations into the coding sequences of targeted genes and selecting knockout lines. The fundamental concept behind employing simple indel mutations to improve crop performance is to target genes known to regulate a

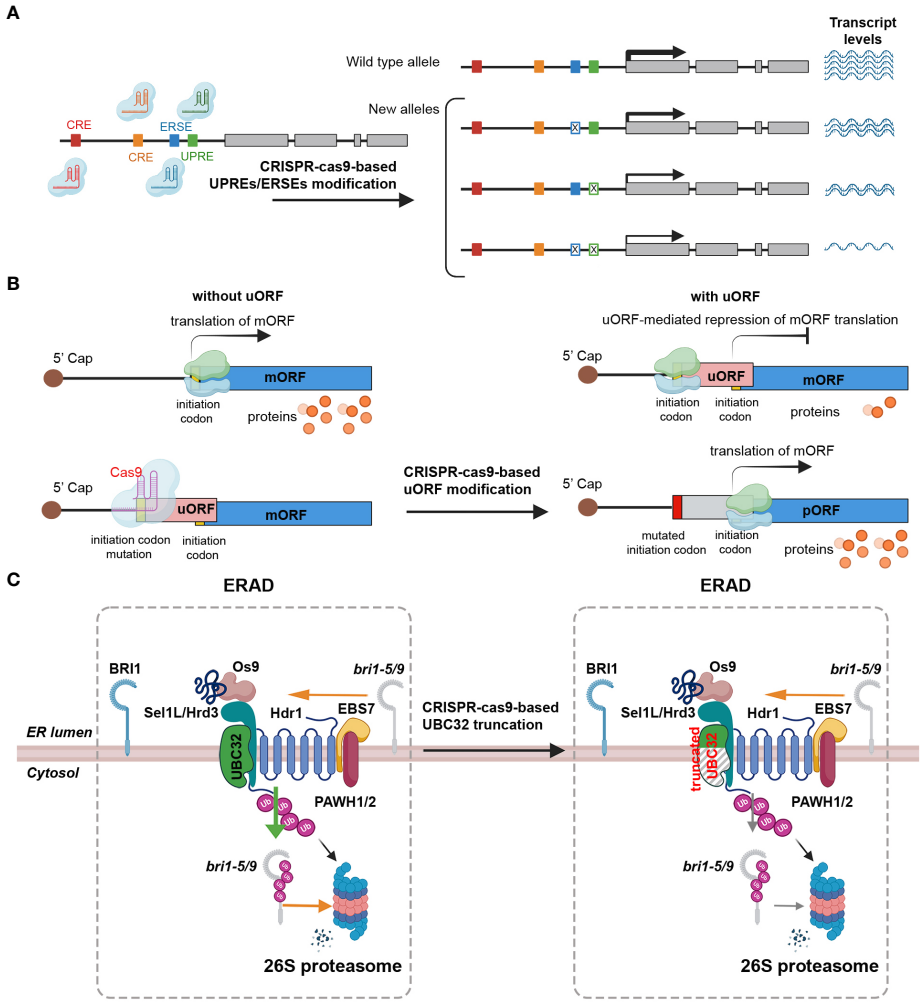


FIGURE 3
Strategies for prospective editing of ER stress signaling components to enhance stress tolerance. **(A)** CRISPR-Cas9 can be effectively employed to edit the promoter region of target genes in order to modulate gene expression. The promoter region contains important *cis*-regulatory elements (CRE, brown and ocher boxes), endoplasmic reticulum stress response elements (ERSEs, blue boxes), and unfolded protein response elements (UPREs, green box). These elements act as enhancers or repressors, playing a crucial role in regulating the transcriptional activity of the gene. By utilizing a multiplex genome editing approach, multiple single-guide RNAs (sgRNAs) can be designed to specifically target distinct ERSEs and UPREs within the promoter region. The CRISPR-Cas9 system, guided by these sgRNAs, induces double-strand breaks at the desired sites in the promoter region, leading to DNA repair mechanisms that can introduce stochastic mutations. These stochastic mutations occurring in the promoter region lead to the generation of alleles with diverse patterns and levels of gene expression. Certain mutations may enhance gene expression, while others may repress it. Implementing this method has the potential to generate a spectrum of phenotypic variations across different lines. **(B)** CRISPR-Cas9 can be used to manipulate gene translation by targeting upstream open reading frames (uORFs). By utilizing the CRISPR-Cas9 system, specific mutations can be introduced into the start codon region of uORFs, disrupting their inhibitory effects on translation. The translation process of messenger RNA (mRNA) begins when small (light blue) and large (light green) ribosomal subunits scan the mRNA from its 5′ cap (represented by a dark brown circle). The initiation codon, represented by a yellow box, serves as the starting point for translation. However, if the mRNA contains an upstream open reading frame (uORF) represented by a pink rectangle, the ribosome can stall at the uORF. This stalling event leads to the repression of translation of the main open reading frame (mORF) indicated by a blue rectangle. Consequently, the reduced translation of the mORF results in a decreased production of protein products, represented by orange circles. The mutated initiation codon (red rectangle) within uORF regions using the CRISPR-Cas9 inhibits ribosome stalling, resulting in increased production of proteins encoded by the mORF. **(C)** Strategy to generate truncated UBC32 using CRISPR-Cas9-mediated knockout to enhance BR signaling by stabilizing structurally imperfect, yet biochemically active, bri1 peptides to achieve stress tolerance. The strategy to enhance brassinosteroid (BR) signaling and improve stress tolerance involves the generation of a truncated form of the *ubiquitin-conjugating enzyme 32* (*UBC32*) gene using CRISPR-Cas9-mediated knockout. *UBC32* is responsible for encoding an E2 ubiquitin-conjugating enzyme that plays a crucial role in the degradation of the biochemically active but structurally incomplete brassinosteroid insensitive 1 (*bri1*: *bri1-5* or *bri1-9*) peptide. Through the process of ubiquitination, UBC32 targets the *bri1-5* or *bri1-9* peptide for 26S proteasome-mediated degradation in the cytosol. However, utilizing the CRISPR-Cas9 to disrupt UBC32 allows for a reduction in the ubiquitination of *bri1-5* or *bri1-9* peptide, leading to increased stability of the peptide. This enhanced stability contributes to the amplification of BR signaling, thereby improving stress tolerance in plants.

specific response or trait. This method has been proven to be effective in improving crop performance (Chen et al., 2019; Vu et al., 2020a; Zhu et al., 2020). However, it is necessary to ensure that the targeted knockout mutants have minimal negative impact on plant morphology, agronomical traits, growth, development, and yield. A minimal trade-off in growth, development, and yield may be acceptable if the benefits to other agronomic traits are highly significant. Genes that directly or indirectly regulate the UPR signaling pathway could be targets for gene editing to confer ER stress tolerance in plants. However, fundamental studies are necessary to identify the specific genes that are suitable for the editing approach, and it is crucial to ensure that the benefits outweigh any potential trade-offs.

UPR regulators play a crucial role in maintaining ER homeostasis in both normal and stress conditions. Identifying genes involved in ERAD pathways is of great importance. One such gene is *UBC32*, which is involved in the ERAD-mediated quality control process through ubiquitination-associated protein degradation. The mutation of *UBC32* results in the accumulation of structurally abnormal *bri1-5* and *bri1-9* mutant forms of brassinosteroid insensitive 1 (BRI1). Despite their structural abnormalities, these mutant forms still retain the biochemical activity of the BRI1 receptor, subsequently enhancing brassinosteroid (BR) signaling (Cui et al., 2012). The *ubc32* single and *bri1-5/9 ubc32* double mutant lines shows improved salt stress tolerance compared to the wild-type control (Figure 3C) (Cui et al., 2012; Zhou et al., 2021). Interestingly, the *ubc32* single mutant Arabidopsis demonstrates a phenotype similar to the wild-type (WT) Col-0 when grown under normal conditions (Figure 3C) (Zhou et al., 2021). These findings suggest that *UBC32* could be a promising target for enhancing stress tolerance via CRISPR-Cas9-mediated knockout. By employing gRNAs to direct CRISPR-Cas9, cleavage of the UBC32 coding sequence can be achieved. This results in indel mutations that cause premature termination of translation and truncation of the UBC32 polypeptide chain, which may ultimately enhance salt tolerance by affecting BR signaling.

Enhancing plant stress tolerance through CRISPR-Cas editing of key regulatory genes

Plant growth and development are significantly influenced by environmental conditions such as light (Bae and Choi, 2008; Kami et al., 2010; Paik and Huq, 2019). Suboptimal lighting conditions can trigger ER stress responses, which can lead to cellular dysfunction and ultimately affect plant growth and survival (Mawphlang and Kharshiing, 2017; Ahn et al., 2022). The connection between light and UPR has been shown to be mediated by ELONGATED HYPOCOTYL 5 (HY5), a bZIP factor previously known as a master regulator of light signaling (Gangappa and Botto, 2016). HY5 acts as an important transcription factor in both light signaling and the UPR pathway. In the dark, HY5 is targeted for degradation by the E3 ubiquitin ligase COP1, which marks HY5 with ubiquitin and targets it for degradation by the 26S proteasome (Ang et al., 1998; Xu et al., 2016). However, in the presence of light or under ER stress conditions, HY5 is stabilized and can regulate the expression of UPR-related genes (Nawkar et al., 2017). The *HY5* gene has been identified as a critical regulator of stress resistance in plants (Xiao et al., 2021). Therefore, the utilization of CRISPR-Cas-based gene editing technology for the generation and characterization of crops carrying HY5 alleles may present a promising and compelling direction for further scientific investigation. Under various stress conditions, the levels and activity of HY5 protein increase, leading to enhanced expression of downstream genes. Overexpression or complete removal of the *HY5* gene may result in significant trade-off in the phenotypes of edited lines, due to the important role of HY5 in multiple processes, such as photomorphogenesis. Consequently, generating mutants with HY5 alleles that maintain a certain level of expression could be an interesting approach for developing crops that efficiently respond to various stresses. The CRISPR-Cas-mediated generation of crops with HY5 alleles could be achieved by targeting the *cis*-regulatory elements of the *HY5* gene. This approach has already been shown to be effective in previous studies (Rodriguez-Leal et al., 2017). Additionally, introducing CRISPR-Cas-mediated precise modification of the DNA binding bZIP domain or the COP1 binding domain in HY5 may be alternative strategies to alter HY5 functions. Such a strategy could be readily implemented through CRISPR-Cas-based gene targeting or prime editing (Van Vu et al., 2019). In summary, the regulatory function of HY5 in various stress responses makes it a promising target for enhancing stress tolerance in plants via CRISPR-Cas-based gene editing. By generating mutant alleles of HY5, it might be possible to indirectly alter the expression of downstream genes, including UPR-related genes, and consequently enhance the resistance of plants to various stresses.

Cadmium, a hazardous heavy metal, significantly impacts plants by interfering with crucial processes such as water and nutrient uptake, photosynthesis, calcium signaling, and genome maintenance. This interference leads to stunted growth, diminished yield, and in severe cases, plant death (Xi et al., 2016; De Benedictis et al., 2023). Recent studies have demonstrated that knockout of the supernumerary aleurone 1 (SAL1) enzyme can mitigate the toxicity of cadmium in Arabidopsis plants (Xi et al., 2016). SAL1, also known as FIERY1, is a well-established regulator of stress response signaling. This enzyme possesses 3'(2'),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase functions (Quintero et al., 1996; Xiong et al., 2001; Wilson et al., 2009), and is implicated in leaf morphogenesis (Robles et al., 2010). These findings suggest that targeting SAL1 using CRISPR-Cas-based techniques could be a promising approach to alleviating cadmium toxicity and other environmental stresses in crop plants. By knocking out SAL1, plants may be better equipped to cope with environmental stresses, leading to improved crop yields and sustainability. In conclusion, the recent discovery of the role of SAL1 in mitigating cadmium-induced toxicity and ER stress responses offers an exciting opportunity for enhancing the sustainability of crop production. By utilizing CRISPR-Cas-based techniques to target SAL1 and other regulators of ER stress

responses, crop plants may exhibit increased resilience to environmental stresses, ultimately leading to enhanced crop yield and quality. Taken together, the proposed strategies for improving crop stress tolerance through genetic modification present a promising opportunity for enhancing plant tolerance and agricultural productivity. By targeting key genes involved in stress responses, such as *UBC32*, *HY5*, and *SAL1*, it may be possible to develop crops that are better adapted to challenging environmental conditions. However, further research is necessary to identify the optimal genes for modification and ensure that the benefits outweigh any potential drawbacks.

Concluding remarks

The UPR mechanism plays a vital role in facilitating the growth and survival of plants under unfavorable environmental conditions (Figure 1). Despite the extensive research on the molecular mechanism of plant UPR, the adoption of CRISPR-Cas-based gene editing technology has been slow. This has limited the potential for developing crops with resistance to a variety of adverse biological and non-biological environmental conditions, including ER stress. Consequently, it is imperative to actively consider the use of CRISPR-Cas technology to study the functions of genes involved in ER stress responses and expand the scope of plant gene editing (Figure 2). Understanding how UPR is activated and regulated, as well as the consequences of such regulation, can provide valuable insights into the development of crops with resistance to various stresses. As plants continue to face unpredictable environmental stresses that can significantly impact crop yield and quality, the adoption of CRISPR-Cas-based gene knockout of UPR-related genes is of great importance. In this review, we summarize the current understanding of ER stress signaling and regulation, as well as the recent advances in CRISPR-Cas technology for ER stress research (Tables 1, 2). Additionally, we discuss the prospects of using CRISPR-Cas-based gene editing for crop breeding, particularly in the development of crop varieties with enhanced ER stress tolerance (Figure 3). We hope that our review will help expand research in this field and attract attention to the potential of CRISPR-Cas technology for studying gene functions related to ER stress responses and expanding the scope of plant gene editing. In this review, we summarize the current understanding of ER stress signaling and regulation, as well as the recent progress made in CRISPR-Cas technology for ER stress studies (Tables 1, 2). We also discuss the future prospects of using CRISPR-Cas-based gene editing for crop breeding, particularly in the development of crop varieties with enhanced stress tolerance We hope that our review will help propel the field and attract attention to the potential of CRISPR-Cas technology for studying gene functions related to ER stress responses.

Author contributions

BV: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. TV: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. JY: Writing – original draft. NN: Writing – original draft. KK: Writing – original draft. J-YK: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. KL: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Research Foundation of Korea (2020R1I1A1A01072130, 2020M3A9I4038352, 2020R1A6A1A03044344, 2021R1A5A8029490, 2022R1A2C3010331, 2021R1A2C1013516, and 2022R1I1A1A01071947), the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ016236), and the Program for New Plant Breeding Techniques (NBT, Grant PJ016867022022), Rural Development Administration (RDA), Korea. BV and NN were supported by the BK4 program funded by the Ministry of Education of Korea.

Acknowledgments

We apologize to colleagues whose work could not be included owing to space constraints. All the figures were created with BioRender under a paid subscription.

Conflict of interest

Author J-YK is employed by Nulla Bio Inc.

The remaining 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.

Publisher's note

References

Afrin, T., Seok, M., Terry, B. C., and Pajerowska-Mukhtar, K. M. (2020). Probing natural variation of IRE1 expression and endoplasmic reticulum stress responses in Arabidopsis accessions. *Sci. Rep.* 10, 19154. doi: 10.1038/s41598-020-76114-1

Ahmadzadeh, V., Farajnia, S., Baghban, R., Rahbarnia, L., and Zarredar, H. (2019). CRISPR-Cas system: Toward a more efficient technology for genome editing and beyond. *J. Cell Biochem.* 120, 16379–16392. doi: 10.1002/jcb.29140

Ahn, G., Jung, I. J., Cha, J. Y., Jeong, S. Y., Shin, G. I., Ji, M. G., et al. (2022). Phytochrome B positively regulates red light-mediated ER stress response in arabidopsis. *Front. Plant Sci.* 13, 846294. doi: 10.3389/fpls.2022.846294

Anderegg, W. R. L., Trugman, A. T., Badgley, G., Anderson, C. M., Bartuska, A., Ciais, P., et al. (2020). Climate-driven risks to the climate mitigation potential of forests. *Science* 368, eaaz7005. doi: 10.1126/science.aaz7005

Ang, L. H., Chattopadhyay, S., Wei, N., Oyama, T., Okada, K., Batschauer, A., et al. (1998). Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development. *Mol. Cell* 1, 213–222. doi: 10.1016/S1097-2765(00)80022-2

Angelos, E., and Brandizzi, F. (2022). The UPR regulator IRE1 promotes balanced organ development by restricting TOR-dependent control of cellular differentiation in Arabidopsis. *Plant J.* 109, 1229–1248. doi: 10.1111/tpj.15629

Angelos, E., Ruberti, C., Kim, S. J., and Brandizzi, F. (2017). Maintaining the factory: the roles of the unfolded protein response in cellular homeostasis in plants. *Plant J.* 90, 671–682. doi: 10.1111/tpj.13449

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., et al. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157. doi: 10.1038/s41586-019-1711-4

Avci, D., and Lemberg, M. K. (2015). Clipping or extracting: two ways to membrane protein degradation. *Trends Cell Biol.* 25, 611–622. doi: 10.1016/j.tcb.2015.07.003

Bae, G., and Choi, G. (2008). Decoding of light signals by plant phytochromes and their interacting proteins. *Annu. Rev. Plant Biol.* 59, 281–311. doi: 10.1146/annurev.arplant.59.032607.092859

Bao, Y., and Howell, S. H. (2017). The unfolded protein response supports plant development and defense as well as responses to abiotic stress. *Front. Plant Sci.* 8, 344. doi: 10.3389/fpls.2017.00344

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140

Chakrabarti, A., Chen, A. W., and Varner, J. D. (2011). A review of the mammalian unfolded protein response. *Biotechnol. Bioeng.* 108, 2777–2793. doi: 10.1002/bit.23282

Chakraborty, R., Baek, J. H., Bae, E. Y., Kim, W.-Y., Lee, S. Y., and Kim, M. G. (2016). Comparison and contrast of plant, yeast, and mammalian ER stress and UPR. *Appl. Biol. Chem.* 59, 337–347. doi: 10.1007/s13765-016-0167-6

Chang, M., Wu, S. Z., Ryken, S. E., O'sullivan, J. E., and Bezanilla, M. (2022). COPII Sec23 proteins form isoform-specific endoplasmic reticulum exit sites with differential effects on polarized growth. *Plant Cell* 34, 333–350. doi: 10.1093/plcell/koab229

Chen, K., Wang, Y., Zhang, R., Zhang, H., and Gao, C. (2019). CRISPR/cas genome editing and precision plant breeding in agriculture. *Annu. Rev. Plant Biol.* 70, 667–697. doi: 10.1146/annurev-arplant-050718-100049

Chen, L., Park, J. E., Paa, P., Rajakumar, P. D., Prekop, H. T., Chew, Y. T., et al. (2021). Programmable C:G to G:C genome editing with CRISPR-Cas9-directed base excision repair proteins. *Nat. Commun.* 12, 1384. doi: 10.1038/s41467-021-21559-9

Chen, Q., Liu, R., Wang, Q., and Xie, Q. (2017). ERAD tuning of the HRD1 complex component atOS9 is modulated by an ER-bound E2, UBC32. *Mol. Plant* 10, 891–894. doi: 10.1016/j.molp.2016.12.011

Chen, Q., Wu, Y., Yu, F., and Xie, Q. (2022). Coordinative regulation of ERAD and selective autophagy in plants. *Essays Biochem.* 66, 179–188. doi: 10.1042/EBC20210099

Chen, Q., Yu, F., and Xie, Q. (2020). Insights into endoplasmic reticulum-associated degradation in plants. *New Phytol.* 226, 345–350. doi: 10.1111/nph.16369

Cheng, A. W., Wang, H., Yang, H., Shi, L., Katz, Y., Theunissen, T. W., et al. (2013). Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* 23, 1163–1171. doi: 10.1038/cr.2013.122

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Coutinho, F. S., Dos Santos, D. S., Lima, L. L., Vital, C. E., Santos, L. A., Pimenta, M. R., et al. (2019). Mechanism of the drought tolerance of a transgenic soybean overexpressing the molecular chaperone BiP. *Physiol. Mol. Biol. Plants* 25, 457–472. doi: 10.1007/s12298-019-00643-x

Cui, F., Liu, L., Li, Q., Yang, C., and Xie, Q. (2012). UBC32 mediated oxidative tolerance in Arabidopsis. *J. Genet. Genomics* 39, 415–417. doi: 10.1016/j.jgg.2012.05.005

Czekus, Z., Csikos, O., Ordog, A., Tari, I., and Poor, P. (2020). Effects of jasmonic acid in ER stress and unfolded protein response in tomato plants. *Biomolecules* 10, 1031. doi: 10.3390/biom10071031

Czekus, Z., Szalai, G., Tari, I., Khan, M. I. R., and Poor, P. (2022). Role of ethylene in ER stress and the unfolded protein response in tomato (Solanum lycopersicum L.) plants. *Plant Physiol. Biochem.* 181, 1–11. doi: 10.1016/j.plaphy.2022.03.031

De Benedictis, M., Gallo, A., Migoni, D., Papadia, P., Roversi, P., and Santino, A. (2023). Cadmium treatment induces endoplasmic reticulum stress and unfolded protein response in Arabidopsisthaliana. *Plant Physiol. Biochem.* 196, 281–290. doi: 10.1016/j.plaphy.2023.01.056

Deng, Y., Humbert, S., Liu, J. X., Srivastava, R., Rothstein, S. J., and Howell, S. H. (2011). Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 108, 7247–7252. doi: 10.1073/pnas.1102117108

Dickinson, D. J., Ward, J. D., Reiner, D. J., and Goldstein, B. (2013). Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nat. Methods* 10, 1028–1034. doi: 10.1038/nmeth.2641

Duan, Y. B., Li, J., Qin, R. Y., Xu, R. F., Li, H., Yang, Y. C., et al. (2016). Identification of a regulatory element responsible for salt induction of rice OsRAV2 through ex situ and in *situ* promoter analysis. *Plant Mol. Biol.* 90, 49–62. doi: 10.1007/s11103-015-0393-z

Duwi Fanata, W. I., Lee, S. Y., and Lee, K. O. (2013). The unfolded protein response in plants: a fundamental adaptive cellular response to internal and external stresses. *J. Proteomics* 93, 356–368. doi: 10.1016/j.jprot.2013.04.023

El-Mounadi, K., Morales-Floriano, M. L., and Garcia-Ruiz, H. (2020). Principles, applications, and biosafety of plant genome editing using CRISPR-cas9. *Front. Plant Sci.* 11, 56. doi: 10.3389/fpls.2020.00056

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., et al. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23, 1229–1232. doi: 10.1038/cr.2013.114

Fragkostefanakis, S., Mesihovic, A., Hu, Y., and Schleiff, E. (2016). Unfolded protein response in pollen development and heat stress tolerance. *Plant Reprod.* 29, 81–91. doi: 10.1007/s00497-016-0276-8

Gangappa, S. N., and Botto, J. F. (2016). The multifaceted roles of HY5 in plant growth and development. *Mol. Plant* 9, 1353–1365. doi: 10.1016/j.molp.2016.07.002

Gao, J., Wang, M. J., Wang, J. J., Lu, H. P., and Liu, J. X. (2022). bZIP17 regulates heat stress tolerance at reproductive stage in Arabidopsis. *aBIOTECH* 3, 1–11. doi: 10.1007/s42994-021-00062-1

Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D. I., et al. (2017). Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* 551, 464–471. doi: 10.1038/nature24644

Habeck, G., Ebner, F. A., Shimada-Kreft, H., and Kreft, S. G. (2015). The yeast ERAD-C ubiquitin ligase Doa10 recognizes an intramembrane degron. *J. Cell Biol.* 209, 261–273. doi: 10.1083/jcb.201408088

Harding, H. P., Calfon, M., Urano, F., Novoa, I., and Ron, D. (2002). Transcriptional and translational control in the Mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.* 18, 575–599. doi: 10.1146/annurev.cellbio.18.011402.160624

Hassani, A., Azapagic, A., and Shokri, N. (2020). Predicting long-term dynamics of soil salinity and sodicity on a global scale. *Proc. Natl. Acad. Sci. U. S. A.* 117, 33017–33027. doi: 10.1073/pnas.2013771117

Hayashi, S., Takahashi, H., Wakasa, Y., Kawakatsu, T., and Takaiwa, F. (2013). Identification of a cis-element that mediates multiple pathways of the endoplasmic reticulum stress response in rice. *Plant J.* 74, 248–257. doi: 10.1111/tpj.12117

Hayashi, S., Wakasa, Y., Ozawa, K., and Takaiwa, F. (2016). Characterization of IRE1 ribonuclease-mediated mRNA decay in plants using transient expression analyses in rice protoplasts. *New Phytol.* 210, 1259–1268. doi: 10.1111/nph.13845

Hayashi, S., Wakasa, Y., Takahashi, H., Kawakatsu, T., and Takaiwa, F. (2012). Signal transduction by IRE1-mediated splicing of bZIP50 and other stress sensors in the endoplasmic reticulum stress response of rice. *Plant J.* 69, 946–956. doi: 10.1111/j.1365-313X.2011.04844.x

He, Y., Yan, W., Long, L., Dong, L., Ma, Y., Li, C., et al. (2023). The CRISPR/cas system: A customizable toolbox for molecular detection. *Genes (Basel)* 14, 850. doi: 10.3390/genes14040850

Henriquez-Valencia, C., Moreno, A. A., Sandoval-Ibanez, O., Mitina, I., Blanco-Herrera, F., Cifuentes-Esquivel, N., et al. (2015). bZIP17 and bZIP60 Regulate the Expression of BiP3 and Other Salt Stress Responsive Genes in an UPR-Independent Manner in Arabidopsis thaliana. *J. Cell Biochem.* 116, 1638–1645. doi: 10.1002/jcb.25121

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 13, 89–102. doi: 10.1038/nrm3270

Howell, S. H. (2013). Endoplasmic reticulum stress responses in plants. *Annu. Rev. Plant Biol.* 64, 477–499. doi: 10.1146/annurev-arplant-050312-120053

Howell, S. H. (2021). Evolution of the unfolded protein response in plants. *Plant Cell Environ.* 44, 2625–2635. doi: 10.1111/pce.14063

Huber, M., Armbruster, L., Etherington, R. D., de la Torre, C., Hawkesford, M. J., Sticht, C., et al. (2021). Disruption of the N(alpha)-acetyltransferase natB causes

sensitivity to reductive stress in arabidopsis thaliana. *Front. Plant Sci.* 12, 799954. doi: 10.3389/fpls.2021.799954

Hwang, J., and Qi, L. (2018). Quality control in the endoplasmic reticulum: crosstalk between ERAD and UPR pathways. *Trends Biochem. Sci.* 43, 593–605. doi: 10.1016/j.tibs.2018.06.005

Iwata, Y., Ashida, M., Hasegawa, C., Tabara, K., Mishiba, K. I., and Koizumi, N. (2017). Activation of the Arabidopsis membrane-bound transcription factor bZIP28 is mediated by site-2 protease, but not site-1 protease. *Plant J.* 91, 408–415. doi: 10.1111/tpj.13572

Iwata, Y., Fedoroff, N. V., and Koizumi, N. (2008). Arabidopsis bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. *Plant Cell* 20, 3107–3121. doi: 10.1105/tpc.108.061002

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Kami, C., Lorrain, S., Hornitschek, P., and Fankhauser, C. (2010). Light-regulated plant growth and development. *Curr. Top. Dev. Biol.* 91, 29–66. doi: 10.1016/S0070-2153(10)91002-8

Kim, J. S., Mochida, K., and Shinozaki, K. (2022a). ER stress and the unfolded protein response: homeostatic regulation coordinate plant survival and growth. *Plants (Basel)* 11, 3197. doi: 10.3390/plants11233197

Kim, J. S., Sakamoto, Y., Takahashi, F., Shibata, M., Urano, K., Matsunaga, S., et al. (2022b). Arabidopsis TBP-ASSOCIATED FACTOR 12 ortholog NOBIRO6 controls root elongation with unfolded protein response cofactor activity. *Proc. Natl. Acad. Sci. U. S. A.* 119, e2120219119. doi: 10.1073/pnas.2120219119

Kim, J. S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2018). ER-Anchored Transcription Factors bZIP17 and bZIP28 Regulate Root Elongation. *Plant Physiol.* 176, 2221–2230. doi: 10.1104/pp.17.01414

Ko, D. K., Kim, J. Y., Thibault, E. A., and Brandizzi, F. (2023). An IRE1-proteasome system signalling cohort controls cell fate determination in unresolved proteotoxic stress of the plant endoplasmic reticulum. *Nat. Plants* 9, 1333–1346. doi: 10.1038/s41477-023-01480-3

Koizumi, N., Martinez, I. M., Kimata, Y., Kohno, K., Sano, H., and Chrispeels, M. J. (2001). Molecular characterization of two Arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiol.* 127, 949–962. doi: 10.1104/pp.010636

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., and Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424. doi: 10.1038/nature17946

Koonin, E. V., and Makarova, K. S. (2022). Evolutionary plasticity and functional versatility of CRISPR systems. *PloS Biol.* 20, e3001481. doi: 10.1371/journal.pbio.3001481

Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988). The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 332, 462–464. doi: 10.1038/332462a0

Kurt, I. C., Zhou, R., Iyer, S., Garcia, S. P., Miller, B. R., Langner, L. M., et al. (2021). CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nat. Biotechnol.* 39, 41–46. doi: 10.1038/s41587-020-0609-x

Lageix, S., Lanet, E., Pouch-Pelissier, M. N., Espagnol, M. C., Robaglia, C., Deragon, J. M., et al. (2008). Arabidopsis eIF2alpha kinase GCN2 is essential for growth in stress conditions and is activated by wounding. *BMC Plant Biol.* 8, 134. doi: 10.1186/1471-2229-8-134

Li, Y., Humbert, S., and Howell, S. H. (2012). ZmbZIP60 mRNA is spliced in maize in response to ER stress. *BMC Res. Notes* 5, 144. doi: 10.1186/1756-0500-5-144

Li, C., Li, W., Zhou, Z., Chen, H., Xie, C., and Lin, Y. (2020a). A new rice breeding method: CRISPR/Cas9 system editing of the Xa13 promoter to cultivate transgene-free bacterial blight-resistant rice. *Plant Biotechnol. J.* 18, 313–315. doi: 10.1111/pbi.13217

Li, L. M., Lu, S. Y., and Li, R. J. (2017). The Arabidopsis endoplasmic reticulum associated degradation pathways are involved in the regulation of heat stress response. *Biochem. Biophys. Res. Commun.* 487, 362–367. doi: 10.1016/j.bbrc.2017.04.066

Li, J. F., Norville, J. E., Aach, J., Mccormack, M., Zhang, D., Bush, J., et al. (2013). Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat. Biotechnol.* 31, 688–691. doi: 10.1038/nbt.2654

Li, Q., Sapkota, M., and van der Knaap, E. (2020b). Perspectives of CRISPR/Cas-mediated cis-engineering in horticulture: unlocking the neglected potential for crop improvement. *Hortic. Res.* 7, 36. doi: 10.1038/s41438-020-0258-8

Li, Z., Tang, J., Srivastava, R., Bassham, D. C., and Howell, S. H. (2020c). The transcription factor bZIP60 links the unfolded protein response to the heat stress response in maize. *Plant Cell* 32, 3559–3575. doi: 10.1105/tpc.20.00260

Li, C., Zhang, T., Liu, Y., Li, Z., Wang, Y., Fu, S., et al. (2022). Rice stripe virus activates the bZIP17/28 branch of the unfolded protein response signalling pathway to promote viral infection. *Mol. Plant Pathol.* 23, 447–458. doi: 10.1111/mpp.13171

Li, J., Zhang, J., Wang, X., and Chen, J. (2010). A membrane-tethered transcription factor ANAC089 negatively regulates floral initiation in Arabidopsis thaliana. *Sci. China Life Sci.* 53, 1299–1306. doi: 10.1007/s11427-010-4085-2

Li, Z., Fu, Y., Shen, J., and Liang, J. (2021). Upstream open reading frame mediated translation of WNK8 is required for ABA response in arabidopsis. *Int. J. Mol. Sci.* 22, 10683. doi: 10.3390/ijms221910683

Lim, C. K. W., Mccallister, T. X., Saporito-Magrina, C., Mcpheron, G. D., Krishnan, R., Zeballos, C. M., et al. (2022). CRISPR base editing of cis-regulatory elements enables the perturbation of neurodegeneration-linked genes. *Mol. Ther.* 30, 3619–3631. doi: 10.1016/j.ymthe.2022.08.008

Lin, L., Zhang, C., Chen, Y., Wang, Y., Wang, D., Liu, X., et al. (2019). PAWH1 and PAWH2 are plant-specific components of an Arabidopsis endoplasmic reticulum-associated degradation complex. *Nat. Commun.* 10, 3492. doi: 10.1038/s41467-019-11480-7

Lin, Q., Zong, Y., Xue, C., Wang, S., Jin, S., Zhu, Z., et al. (2020). Prime genome editing in rice and wheat. *Nat. Biotechnol.* 38, 582–585. doi: 10.1038/s41587-020-0455-x

Liu, X., Afrin, T., and Pajerowska-Mukhtar, K. M. (2019). Arabidopsis GCN2 kinase contributes to ABA homeostasis and stomatal immunity. *Commun. Biol.* 2, 302. doi: 10.1038/s42003-019-0544-x

Liu, L., Cui, F., Li, Q., Yin, B., Zhang, H., Lin, B., et al. (2011). The endoplasmic reticulum-associated degradation is necessary for plant salt tolerance. *Cell Res.* 21, 957–969. doi: 10.1038/cr.2010.181

Liu, J. X., and Howell, S. H. (2010). bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in Arabidopsis. *Plant Cell* 22, 782–796. doi: 10.1105/tpc.109.072173

Liu, Y., and Li, J. (2014). Endoplasmic reticulum-mediated protein quality control in Arabidopsis. *Front. Plant Sci.* 5, 162. doi: 10.3389/fpls.2014.00162

Liu, Y., Lv, Y., Wei, A., Guo, M., Li, Y., Wang, J., et al. (2022). Unfolded protein response in balancing plant growth and stress tolerance. *Front. Plant Sci.* 13, 1019414. doi: 10.3389/fpls.2022.1019414

Liu, X. H., Lyu, Y. S., Yang, W., Yang, Z. T., Lu, S. J., and Liu, J. X. (2020). A membrane-associated NAC transcription factor OsNTL3 is involved in thermotolerance in rice. *Plant Biotechnol. J.* 18, 1317–1329. doi: 10.1111/pbi.13297

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007a). An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. *Plant Cell* 19, 4111–4119. doi: 10.1105/tpc.106.050021

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007b). Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J.* 51, 897–909. doi: 10.1111/j.1365-313X.2007.03195.x

Liu, Y., Zhang, C., Wang, D., Su, W., Liu, L., Wang, M., et al. (2015). EBS7 is a plant-specific component of a highly conserved endoplasmic reticulum-associated degradation system in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 112, 12205–12210. doi: 10.1073/pnas.1511724112

Lu, S. J., Yang, Z. T., Sun, L., Song, Z. T., and Liu, J. X. (2012). Conservation of IRE1-regulated bZIP74 mRNA unconventional splicing in rice (Oryza sativa L.) involved in ER stress responses. *Mol. Plant* 5, 504–514. doi: 10.1093/mp/ssr115

Lu, S., Zhu, T., Wang, Z., Luo, L., Wang, S., Lu, M., et al. (2021). Arabidopsis immune-associated nucleotide-binding genes repress heat tolerance at the reproductive stage by inhibiting the unfolded protein response and promoting cell death. *Mol. Plant* 14, 267–284. doi: 10.1016/j.molp.2020.11.010

Manghwar, H., and Li, J. (2022). Endoplasmic reticulum stress and unfolded protein response signaling in plants. *Int. J. Mol. Sci.* 23, 828. doi: 10.3390/ijms23020828

Marciniak, S. J. (2019). Endoplasmic reticulum stress: a key player in human disease. *FEBS J.* 286, 228–231. doi: 10.1111/febs.14740

Mawphlang, O. I. L., and Kharshiing, E. V. (2017). Photoreceptor mediated plant growth responses: implications for photoreceptor engineering toward improved performance in crops. *Front. Plant Sci.* 8, 1181. doi: 10.3389/fpls.2017.01181

Meng, F., Zhao, Q., Zhao, X., Yang, C., Liu, R., Pang, J., et al. (2022). A rice protein modulates endoplasmic reticulum homeostasis and coordinates with a transcription factor to initiate blast disease resistance. *Cell Rep.* 39, 110941. doi: 10.1016/j.celrep.2022.110941

Mishiba, K. I., Iwata, Y., Mochizuki, T., Matsumura, A., Nishioka, N., Hirata, R., et al. (2019). Unfolded protein-independent IRE1 activation contributes to multifaceted developmental processes in Arabidopsis. *Life Sci. Alliance* 2, e201900459. doi: 10.26508/lsa.201900459

Mishiba, K., Nagashima, Y., Suzuki, E., Hayashi, N., Ogata, Y., Shimada, Y., et al. (2013). Defects in IRE1 enhance cell death and fail to degrade mRNAs encoding secretory pathway proteins in the Arabidopsis unfolded protein response. *Proc. Natl. Acad. Sci. U. S. A.* 110, 5713–5718. doi: 10.1073/pnas.1219047110

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., and Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol.* 60, 174–182. doi: 10.1007/s00239-004-0046-3

Moreno, A. A., Mukhtar, M. S., Blanco, F., Boatwright, J. L., Moreno, I., Jordan, M. R., et al. (2012). IRE1/bZIP60-mediated unfolded protein response plays distinct roles in plant immunity and abiotic stress responses. *PloS One* 7, e31944. doi: 10.1371/journal.pone.0031944

Nagashima, Y., Mishiba, K., Suzuki, E., Shimada, Y., Iwata, Y., and Koizumi, N. (2011). Arabidopsis IRE1 catalyses unconventional splicing of bZIP60 mRNA to produce the active transcription factor. *Sci. Rep.* 1, 29. doi: 10.1038/srep00029

Nawkar, G. M., Kang, C. H., Maibam, P., Park, J. H., Jung, Y. J., Chae, H. B., et al. (2017). HY5, a positive regulator of light signaling, negatively controls the unfolded protein response in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 114, 2084–2089. doi: 10.1073/pnas.1609844114

Neill, E. M., Byrd, M. C. R., Billman, T., Brandizzi, F., and Stapleton, A. E. (2019). Plant growth regulators interact with elevated temperature to alter heat stress signaling via the Unfolded Protein Response in maize. *Sci. Rep.* 9, 10392. doi: 10.1038/s41598-019-46839-9

Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D., and Kamoun, S. (2013). Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 691–693. doi: 10.1038/nbt.2655

Noh, S. J., Kwon, C. S., and Chung, W. I. (2002). Characterization of two homologs of Ire1p, a kinase/endoribonuclease in yeast, in Arabidopsis thaliana. *Biochim. Biophys. Acta* 1575, 130–134. doi: 10.1016/S0167-4781(02)00237-3

Ohta, M., and Takaiwa, F. (2015). OsHrd3 is necessary for maintaining the quality of endoplasmic reticulum-derived protein bodies in rice endosperm. *J. Exp. Bot.* 66, 4585–4593. doi: 10.1093/jxb/erv229

Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A., Li, T., et al. (2019). Broad-spectrum resistance to bacterial blight in rice using genome editing. *Nat. Biotechnol.* 37, 1344–1350. doi: 10.1038/s41587-019-0267-z

Paik, I., and Huq, E. (2019). Plant photoreceptors: Multi-functional sensory proteins and their signaling networks. *Semin. Cell Dev. Biol.* 92, 114–121. doi: 10.1016/j.semcdb.2019.03.007

Pajerowska-Mukhtar, K. M., Wang, W., Tada, Y., Oka, N., Tucker, C. L., Fonseca, J. P., et al. (2012). The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. *Curr. Biol.* 22, 103–112. doi: 10.1016/j.cub.2011.12.015

Park, C. J., and Park, J. M. (2019). Endoplasmic reticulum plays a critical role in integrating signals generated by both biotic and abiotic stress in plants. *Front. Plant Sci.* 10, 399. doi: 10.3389/fpls.2019.00399

Pastor-Cantizano, N., Ko, D. K., Angelos, E., Pu, Y., and Brandizzi, F. (2020). Functional diversification of ER stress responses in arabidopsis. *Trends Biochem. Sci.* 45, 123–136. doi: 10.1016/j.tibs.2019.10.008

Pucker, B., Kleinbolting, N., and Weisshaar, B. (2021). Large scale genomic rearrangements in selected Arabidopsis thaliana T-DNA lines are caused by T-DNA insertion mutagenesis. *BMC Genomics* 22, 599. doi: 10.1186/s12864-021-07877-8

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183. doi: 10.1016/j.cell.2013.02.022

Qiang, X., Liu, X., Wang, X., Zheng, Q., Kang, L., Gao, X., et al. (2021). Susceptibility factor RTP1 negatively regulates Phytophthora parasitica resistance via modulating UPR regulators bZIP60 and bZIP28. *Plant Physiol.* 186, 1269–1287. doi: 10.1093/plphys/kiab126

Quintero, F. J., Garciadeblas, B., and Rodriguez-Navarro, A. (1996). The SAL1 gene of Arabidopsis, encoding an enzyme with 3'(2'),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. *Plant Cell* 8, 529–537. doi: 10.1105/tpc.8.3.529

Reyes-Impellizzeri, S., and Moreno, A. A. (2021). The endoplasmic reticulum role in the plant response to abiotic stress. *Front. Plant Sci.* 12, 755447. doi: 10.3389/fpls.2021.755447

Rivero, R. M., Mittler, R., Blumwald, E., and Zandalinas, S. I. (2022). Developing climate-resilient crops: improving plant tolerance to stress combination. *Plant J.* 109, 373–389. doi: 10.1111/tpj.15483

Robles, P., Fleury, D., Candela, H., Cnops, G., Alonso-Peral, M. M., Anami, S., et al. (2010). The RON1/FRY1/SAL1 gene is required for leaf morphogenesis and venation patterning in Arabidopsis. *Plant Physiol.* 152, 1357–1372. doi: 10.1104/pp.109.149369

Rodriguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., and Lippman, Z. B. (2017). Engineering quantitative trait variation for crop improvement by genome editing. . *Cell* 171, 470–480.e478. doi: 10.1016/j.cell.2017.08.030

Santillan Martinez, M. I., Bracuto, V., Koseoglou, E., Appiano, M., Jacobsen, E., Visser, R. G. F., et al. (2020). CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew. *BMC Plant Biol.* 20, 284. doi: 10.1186/s12870-020-02497-y

Sato, B. K., Schulz, D., Do, P. H., and Hampton, R. Y. (2009). Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. *Mol. Cell* 34, 212–222. doi: 10.1016/j.molcel.2009.03.010

Schoebel, S., Mi, W., Stein, A., Ovchinnikov, S., Pavlovicz, R., Dimaio, F., et al. (2017). Cryo-EM structure of the protein-conducting ERAD channel Hrd1 in complex with Hrd3. *Nature* 548, 352–355. doi: 10.1038/nature23314

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404, 770–774. doi: 10.1038/35008096

Schuldiner, M., and Schwappach, B. (2013). From rags to riches - the history of the endoplasmic reticulum. *Biochim. Biophys. Acta* 1833, 2389–2391. doi: 10.1016/j.bbamcr.2013.03.005

Schwank, G., Koo, B. K., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., et al. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13, 653–658. doi: 10.1016/j.stem.2013.11.002

Shi, J., Gao, H., Wang, H., Lafitte, H. R., Archibald, R. L., Yang, M., et al. (2017). ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnol. J.* 15, 207–216. doi: 10.1111/pbi.12603

Shi, J., Habben, J. E., Archibald, R. L., Drummond, B. J., Chamberlin, M. A., Williams, R. W., et al. (2015). Overexpression of ARGOS genes modifies plant sensitivity to ethylene, leading to improved drought tolerance in both arabidopsis and maize. *Plant Physiol.* 169, 266–282. doi: 10.1104/pp.15.00780

Silva, J., Fernandes, R., and Romao, L. (2019). Translational regulation by upstream open reading frames and human diseases. *Adv. Exp. Med. Biol.* 1157, 99–116. doi: 10.1007/978-3-030-19966-1_5

Singh, B., Salaria, N., Thakur, K., Kukreja, S., Gautam, S., and Goutam, U. (2019). Functional genomic approaches to improve crop plant heat stress tolerance. *F1000Res* 8, 1721. doi: 10.12688/f1000research.19840.1

Srivastava, R., Chen, Y., Deng, Y., Brandizzi, F., and Howell, S. H. (2012). Elements proximal to and within the transmembrane domain mediate the organelle-to-organelle movement of bZIP28 under ER stress conditions. *Plant J.* 70, 1033–1042. doi: 10.1111/j.1365-313X.2012.04943.x

Srivastava, R., Deng, Y., and Howell, S. H. (2014). Stress sensing in plants by an ER stress sensor/transducer, bZIP28. *Front. Plant Sci.* 5, 59. doi: 10.3389/fpls.2014.00059

Srivastava, R., Deng, Y., Shah, S., Rao, A. G., and Howell, S. H. (2013). BINDING PROTEIN is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in Arabidopsis. *Plant Cell* 25, 1416–1429. doi: 10.1105/tpc.113.110684

Strasser, R. (2018). Protein quality control in the endoplasmic reticulum of plants. *Annu. Rev. Plant Biol.* 69, 147–172. doi: 10.1146/annurev-arplant-042817-040331

Sun, L., Yang, Z. T., Song, Z. T., Wang, M. J., Lu, S. J., and Liu, J. X. (2013). The plant-specific transcription factor gene NAC103 is induced by bZIP60 through a new cis-regulatory element to modulate the unfolded protein response in Arabidopsis. *Plant J.* 76, 274–286. doi: 10.1111/tpj.12287

Sun, L., Zhang, S. S., Lu, S. J., and Liu, J. X. (2015). Site-1 protease cleavage site is important for the ER stress-induced activation of membrane-associated transcription factor bZIP28 in Arabidopsis. *Sci. China Life Sci.* 58, 270–275. doi: 10.1007/s11427-015-4807-6

Takahashi, H., Kawakatsu, T., Wakasa, Y., Hayashi, S., and Takaiwa, F. (2012). A rice transmembrane bZIP transcription factor, OsbZIP39, regulates the endoplasmic reticulum stress response. *Plant Cell Physiol.* 53, 144–153. doi: 10.1093/pcp/pcr157

Tamura, K., Kawabayashi, T., Shikanai, T., and Hara-Nishimura, I. (2016). Decreased expression of a gene caused by a T-DNA insertion in an adjacent gene in arabidopsis. *PloS One* 11, e0147911. doi: 10.1371/journal.pone.0147911

Tien Van, V., Gah-Hyun, L., Seung Hee, C., Ju Yeon, M., Ngan Thi, N., Swati, D., et al. (2022). CRISPR/Cas-based precision gene replacement in plants via microhomology-mediated end joining. *bioRxiv* 2022, 2008.2027.505510. doi: 10.1101/2022.08.27.505510

Tong, H., Liu, N., Wei, Y., Zhou, Y., Li, Y., Wu, D., et al. (2023). Programmable deaminase-free base editors for G-to-Y conversion by engineered glycosylase. *Natl. Sci. Rev.* 10, nwad143. doi: 10.1093/nsr/nwad143

Traubenik, S., Ferrari, M., Blanco, F. A., and Zanetti, M. E. (2021). Translational regulation in pathogenic and beneficial plant-microbe interactions. *Biochem. J.* 478, 2775–2788. doi: 10.1042/BCJ20210066

Um, T., Park, T., Shim, J. S., Kim, Y. S., Lee, G. S., Choi, I. Y., et al. (2021). Application of upstream open reading frames (uORFs) editing for the development of stress-tolerant crops. *Int. J. Mol. Sci.* 22, 3743. doi: 10.3390/ijms22073743

Van Houtan, K. S., Tanaka, K. R., Gagne, T. O., and Becker, S. L. (2021). The geographic disparity of historical greenhouse emissions and projected climate change. *Sci. Adv.* 7, eabe4342. doi: 10.1126/sciadv.abe4342

Van Vu, T., Sung, Y. W., Kim, J., Doan, D. T. H., Tran, M. T., and Kim, J. Y. (2019). Challenges and perspectives in homology-directed gene targeting in monocot plants. *Rice (N Y)* 12, 95. doi: 10.1186/s12284-019-0355-1

Vembar, S. S., and Brodsky, J. L. (2008). One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* 9, 944–957. doi: 10.1038/nrm2546

Von Der Gathen, P., Kivi, R., Wohltmann, I., Salawitch, R. J., and Rex, M. (2021). Climate change favours large seasonal loss of Arctic ozone. *Nat. Commun.* 12, 3886. doi: 10.1038/s41467-021-24089-6

Vu, T. V., Das, S., Tran, M. T., Hong, J. C., and Kim, J. Y. (2020a). Precision genome engineering for the breeding of tomatoes: recent progress and future perspectives. *Front. Genome Ed* 2, 612137. doi: 10.3389/fgeed.2020.612137

Vu, T. V., Sivankalyani, V., Kim, E. J., Doan, D. T. H., Tran, M. T., Kim, J., et al. (2020b). Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. *Plant Biotechnol. J.* 18, 2133–2143. doi: 10.1111/pbi.13373

Vuong, U. T., Iswanto, A. B. B., Nguyen, Q. M., Kang, H., Lee, J., Moon, J., et al. (2023). Engineering plant immune circuit: walking to the bright future with a novel toolbox. *Plant Biotechnol. J.* 21, 17–45. doi: 10.1111/pbi.13916

Wakasa, Y., Yasuda, H., Oono, Y., Kawakatsu, T., Hirose, S., Takahashi, H., et al. (2011). Expression of ER quality control-related genes in response to changes in BiP1 levels in developing rice endosperm. *Plant J.* 65, 675–689. doi: 10.1111/j.1365-313X.2010.04453.x

Wallin, E., and Von Heijne, G. (1998). Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci.* 7, 1029–1038. doi: 10.1002/pro.5560070420

Wang, Y., Zafar, N., Ali, Q., Manghwar, H., Wang, G., Yu, L., et al. (2022). CRISPR/cas genome editing technologies for plant improvement against biotic and abiotic stresses: advances, limitations, and future perspectives. *Cells* 11, 3928. doi: 10.3390/cells11233928

Wilson, P. B., Estavillo, G. M., Field, K. J., Pornsiriwong, W., Carroll, A. J., Howell, K. A., et al. (2009). The nucleotidase/phosphatase SAL1 is a negative regulator of drought tolerance in Arabidopsis. *Plant J.* 58, 299–317. doi: 10.1111/j.1365-313X.2008.03780.x

Wittkopp, P. J., and Kalay, G. (2011). Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* 13, 59–69. doi: 10.1038/nrg3095

Wolter, F., and Puchta, H. (2018). Application of CRISPR/cas to understand cis- and trans-regulatory elements in plants. *Methods Mol. Biol.* 1830, 23–40. doi: 10.1007/978-1-4939-8657-6_2

Wolter, F., Schindele, P., and Puchta, H. (2019). Plant breeding at the speed of light: the power of CRISPR/Cas to generate directed genetic diversity at multiple sites. *BMC Plant Biol.* 19, 176. doi: 10.1186/s12870-019-1775-1

Xi, H., Xu, H., Xu, W., He, Z., and Ma, M. (2016). A SAL1 loss-of-function arabidopsis mutant exhibits enhanced cadmium tolerance in association with alleviation of endoplasmic reticulum stress. *Plant Cell Physiol.* 57, 1210–1219. doi: 10.1093/pcp/pcw069

Xiao, Y., Chu, L., Zhang, Y., Bian, Y., Xiao, J., and Xu, D. (2021). HY5: A pivotal regulator of light-dependent development in higher plants. *Front. Plant Sci.* 12, 800989. doi: 10.3389/fpls.2021.800989

Xiong, L., Lee, B., Ishitani, M., Lee, H., Zhang, C., and Zhu, J. K. (2001). FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes Dev.* 15, 1971–1984. doi: 10.1101/gad.891901

Xu, D., Jiang, Y., Li, J., Lin, F., Holm, M., and Deng, X. W. (2016). BBX21, an Arabidopsis B-box protein, directly activates HY5 and is targeted by COP1 for 26S proteasome-mediated degradation. *Proc. Natl. Acad. Sci. U. S. A.* 113, 7655–7660. doi: 10.1073/pnas.1607687113

Xu, Z., Song, N., Ma, L., and Wu, J. (2019). IRE1-bZIP60 pathway is required for nicotiana attenuata resistance to fungal pathogen alternaria alternata. *Front. Plant Sci.* 10, 263. doi: 10.3389/fpls.2019.00263

Yang, W., Xu, P., Zhang, J., Zhang, S., Li, Z., Yang, K., et al. (2022). OsbZIP60-mediated unfolded protein response regulates grain chalkiness in rice. *J. Genet. Genomics* 49, 414–426. doi: 10.1016/j.jgg.2022.02.002

Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* 414, 652–656. doi: 10.1038/414652a

Yoshihisa, T., Barlowe, C., and Schekman, R. (1993). Requirement for a GTPase-activating protein in vesicle budding from the endoplasmic reticulum. *Science* 259, 1466–1468. doi: 10.1126/science.8451644

Yu, C. Y., Cho, Y., Sharma, O., and Kanehara, K. (2022). What's unique? The unfolded protein response in plants. *J. Exp. Bot.* 73, 1268–1276. doi: 10.1093/jxb/erab513

Zandalinas, S. I., Fritschi, F. B., and Mittler, R. (2021). Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. *Trends Plant Sci.* 26, 588–599. doi: 10.1016/j.tplants.2021.02.011

Zeng, Y., Chung, K. P., Li, B., Lai, C. M., Lam, S. K., Wang, X., et al. (2015). Unique COPII component AtSar1a/AtSec23a pair is required for the distinct function of protein ER export in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* 112, 14360–14365. doi: 10.1073/pnas.1519333112

Zhang, M., Cao, B., Zhang, H., Fan, Z., Zhou, X., and Li, F. (2023). Geminivirus satellite-encoded betaC1 activates UPR, induces bZIP60 nuclear export, and manipulates the expression of bZIP60 downstream genes to benefit virus infection. *Sci. China Life Sci.* 66, 1408–1425. doi: 10.1007/s11427-022-2196-y

Zhang, H., Si, X., Ji, X., Fan, R., Liu, J., Chen, K., et al. (2018). Genome editing of upstream open reading frames enables translational control in plants. *Nat. Biotechnol.* 36, 894–898. doi: 10.1038/nbt.4202

Zhang, T., Wu, A., Yue, Y., and Zhao, Y. (2020). uORFs: important cis-regulatory elements in plants. *Int. J. Mol. Sci.* 21, 6238. doi: 10.3390/ijms21176238

Zhou, B., Wang, C., Chen, X., Zhang, Y., and Zeng, L. (2021). Two ubiquitin-activating systems occur in plants with one playing a major role in plant immunity. *bioRxiv* 2021.09.02, 458739. doi: 10.1101/2021.09.02.458739

Zhu, H., Li, C., and Gao, C. (2020). Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nat. Rev. Mol. Cell Biol.* 21, 661–677. doi: 10.1038/s41580-020-00288-9

10.3389/fpls.2023.1271368

Glossary

ATF6	activating transcription factor 6
BiP3	luminal-binding protein 3
BR	brassinosteroid
BRI1	brassinosteroid insensitive 1
bZIP	basic leucine zipper protein
Cd	cadmium
COPII	coat protein complex II
CREs	*cis*-regulatory elements
Hrd1	HMG-CoA reductase degradation protein 1
DSB	double-stranded break
DTT	dithiothreitol
EBS7	EMS-mutagenized Bri1 suppressor 7
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
ERSE	ER stress response element
GCN2	general control non-repressible 2
gRNA	guide RNA
GWAS	genome-wide association study
HR	homologous recombination
Hrd1	HMG-CoA reductase degradation protein 1
Hrd1	HMG-CoA reductase degradation 3
HSP	heat shock protein
IRE1	inositol requiring enzyme 1
IAN	immune-associated nucleotide-binding
HY5	elongated hypocotyl 5
mORFs	main open reading frames
NHEJ	nonhomologous end-joining
NBT	new breeding techniques
NF-Y	Nuclear transcription factor Y
OS9	osteosarcoma amplified 9
PAWH1	protein associated with Hrd1-1
PERK	double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase
PIR1	phosphatase type 2CA (PP2CA)-interacting finger protein 1
RIDD	regulated IRE1-dependent decay
RNAi	RNA interference
SAL1	supernumerary aleurone 1
Sel1L	suppressor enhancer Lin12 1 like
S1P	site-1 proteases
TBF1	TL1-binding transcription factor 1
TF	transcription factor
TOR	Target of rapamycin
TM	tunicamycin
UBC32	ubiquitin-conjugating enzyme 32
uORFs	upstream open reading frames
UPR	unfolded protein response
UPRE	unfolded protein response element
UPS	ubiquitin-proteasome system
UTR	untranslated region
XBP1.	X-box binding protein 1

frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 04 March 2024
DOI 10.3389/fpls.2024.1329697

OPEN ACCESS

EDITED BY
Litao Yang,
Shanghai Jiao Tong University, China

REVIEWED BY
Prathima Perumal Thirugnanasambandam,
Indian Council of Agricultural Research, India
Lin Shao,
Yunnan University, China

*CORRESPONDENCE
Changjun Huang
cjhuang@zju.edu.cn

†These authors have contributed equally to this work and share first authorship

RECEIVED 29 October 2023
ACCEPTED 16 February 2024
PUBLISHED 04 March 2024

CITATION
Yuan C, Zeng J, Liu Y, Yu H, Tong Z, Zhang J, Gao Q, Wang Z, Sui X, Xiao B and Huang C (2024) Establishment and application of Agrobacterium-delivered CRISPR/Cas9 system for wild tobacco (*Nicotiana alata*) genome editing.
Front. Plant Sci. 15:1329697.
doi: 10.3389/fpls.2024.1329697

Establishment and application of Agrobacterium-delivered CRISPR/Cas9 system for wild tobacco (*Nicotiana alata*) genome editing

Cheng Yuan[1†], Jianmin Zeng[1†], Yong Liu[1], Haiqin Yu[1], Zhijun Tong[1], Jianduo Zhang[2], Qian Gao[2], Zhong Wang[3], Xueyi Sui[1], Bingguang Xiao[1] and Changjun Huang[1*]

[1]Yunnan Academy of Tobacco Agricultural Sciences, Key Laboratory of Tobacco Biotechnological Breeding, National Tobacco Genetic Engineering Research Center, Kunming, China, [2]Technology Center, China Tobacco Yunnan Industrial Co. LTD, Kunming, China, [3]China Tobacco Gene Research Center, Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9) system has been widely applied in cultivated crops, but limited in their wild relatives. *Nicotiana alata* is a typical wild species of genus *Nicotiana* that is globally distributed as a horticultural plant and well-studied as a self-incompatibility model. It also has valuable genes for disease resistance and ornamental traits. However, it lacks an efficient genetic transformation and genome editing system, which hampers its gene function and breeding research. In this study, we developed an optimized hypocotyl-mediated transformation method for CRISPR-Cas9 delivery. The genetic transformation efficiency was significantly improved from approximately 1% to over 80%. We also applied the CRISPR-Cas9 system to target the *phytoene desaturase* (*NalaPDS*) gene in *N. alata* and obtained edited plants with PDS mutations with over 50% editing efficiency. To generate self-compatible *N. alata* lines, a polycistronic tRNA-gRNA (PTG) strategy was used to target exonic regions of allelic *S-RNase* genes and generate targeted knockouts simultaneously. We demonstrated that our system is feasible, stable, and high-efficiency for *N. alata* genome editing. Our study provides a powerful tool for basic research and genetic improvement of *N. alata* and an example for other wild tobacco species.

KEYWORDS

genome editing, CRISPR/Cas9, wild tobacco, *Nicotiana alata*, self-incompatibility

1 Introduction

Crop wild relatives (CWRs) harbor untapped genetic diversity and resilience, serving as a genetic reservoir to improve resistance, yield, quality and adaptability of cultivated crops. However, during the process of domestication, with the main purpose of increasing crop yield and biomass, a treasure trove of genes was lost in cultivated crops (Sang et al., 2011; Tirnaz et al., 2022). As the demands of a burgeoning global population escalate alongside the multifaceted challenges posed by environmental stresses, scientists have turned to explore the key functional genes that control beneficial traits in CWRs and are lost in cultivated crops, and applied them to molecular breeding (Bohra et al., 2022). Consequently, the modern agricultural paradigm is undergoing a transformation from concentrating on staple crops to interplaying between cultivated crops and their wild progenitors. In recent decades, several 'game-changing' genes that can offer beneficial traits, such as resistance to pests and diseases, abiotic stress tolerance, quality improvements and yield increases, have been reintroduced into cultivated varieties to enhance their resilience and yield (Engels and Thormann, 2020). Growing evidence has also demonstrated the value of CWRs in agricultural breeding programs, with the annual contribution of these traits to agriculture estimated at USD 115 billion globally, but they still remain a relatively under-utilized and under-estimated resource (Dempewolf et al., 2017). Many valuable traits have not yet been explored and most of the important functional genes in CWRs have not yet been studied.

With the rapid development of sequencing technology and the sharp decrease of sequencing cost, the genomic information of important CWRs has been uncovered in the last decade (Weigel and Nordborg, 2015; Purugganan and Jackson, 2021). Integrated with large-scale transcriptome, metabolome and/or proteome information, candidate genes that determine important traits are now readily predicted. To maximize these potential valuable assets, candidate gene validation and function identification by using genetic engineering approaches, such as gene overexpression, gene silencing and gene knockout mediated by genetic transformation methods, could facilitate the deployment of wild alleles into new cultivars. However, in contrast to the well-developed systems in the cultivars, efficient genetic transformation systems have not yet been established in amounts of the CWRs (Chen et al., 2022).

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR-Cas) based genome editing technology is a recently developed powerful tool and has been widely utilized in plant molecular biology (Manghwar et al., 2019; Zhu et al., 2020). Compared to the conventional strategies used for functional characterization of plant genes and genetic improvement of agricultural crops, which are complicated and laborious-, time-consuming, CRISPR-Cas, especially CRISPR-Cas9 from *Streptococcus pyogenes*, has been proved in plant biology and provides more effective and time-saving methods with precise genetic manipulation of target genes (Hsu et al., 2014; Liu et al., 2016). However, while CRISPR-Cas9 has enabled various genome editing applications, such as indels production, precise nucleotide substitution, gene-expression regulation, multiplexed and high-throughput gene editing in a variety of model plants and crop species, the recalcitrance of efficient genetic transformation severely hinders the application of CRISPR-Cas9 in the CWRs (Rahman et al., 2023). Consequently, due to the lack of efficient CRISPR-Cas9 editing system, gene function verification of most CWRs *in situ* still cannot be carried out.

Nicotiana alata is a perennial species native to Brazil, Paraguay, and northeastern Argentina, belonging to the genus *Nicotiana* section Alatea. It has been introduced to other regions as an ornamental plant for its attractive and fragrant flowers that vary in color (Zheng et al., 2021; Zhang et al., 2023). As an important horticultural plant, the genes involved in the secondary metabolism pathway that regulate the fragrance and color of the flowers need to be further addressed (Raguso et al., 2003, 2006; Fahnrich et al., 2011; Guo et al., 2020; Zheng et al., 2021). In its native wild habitats, *N. alata* encounters a range of biotic and abiotic stresses. To survive these challenges, it has evolved a suite of adaptive systems that confer protection against specific threats. Several resistance loci have been identified in *N. alata*, including but not limited to, resistance to *Tomato spotted wilt virus* (*RTSW*), *Tobacco mosaic virus* (*N'ala*) and black root rot (Yuan et al., 2015; Trojak-Goluch et al., 2016; Huang et al., 2018; Berbeć and Doroszewska, 2020; Czubacka, 2022; Li et al., 2023). More important, *N. alata* is a model species used to study gametophytic self-incompatibility (SI), controlled by the *S*-locus (Bredemeijer and Blaas, 1981). The first cloning and sequencing of a cDNA encoding an S-RNase protein that co-segregates with an *S* allele was obtained in *N. alata* (Anderson et al., 1986; Mcclure et al., 1989). The *S*-locus gene of *N. alata* has subsequently been extensively studied by molecular biologists and geneticists, as it provides a model system to understand the molecular mechanisms of SI, as well as its evolution, diversity, and plasticity (Cruz-Garcia et al., 2003, 2005; Roldan et al., 2015; Fujii et al., 2016). In addition, the interaction between *N. alata* and its pollinator, the hawkmoth *Manduca sexta*, is also a fascinating example of coevolution and mutualism (Kessler et al., 2015; Haverkamp et al., 2016). In summary, the diverse roles and significance of *N. alata* in research, from reproductive biology to environmental interactions, underline it is not only an ornamental plant but also a model organism.

Despite its pivotal role in scientific inquiries, researchers working with *N. alata* faced a significant technological hurdle. The most significant challenge posed by *N. alata*, particularly its recalcitrance to conventional genetic transformation techniques like the leaf disk method (Ebert and Clarke, 1990; Taheri-Dehkordi et al., 2017), has been a major obstacle for researchers. The lack of highly efficient genetic transformation has led to the revolutionary CRISPR/Cas9 genome-editing system, which has transformed genetic research in myriad organisms, but has not been established in *N. alata*. In this study, we optimized the approach centered on hypocotyl transformation. This innovative shift not only circumvented the longstanding challenges but also set the stage for new genome-editing endeavors in *N. alata*. By using the *phytoene desaturase* (*PDS*) gene as a proof of concept, we validated the efficacy of our CRISPR-Cas9 system in *N. alata*. For further breeding and basic research in future, we aimed to disrupt the *S-RNase* gene and thereby obtain self-compatible *N. alata*.

Therefore, we not only established an efficient genome editing system but also obtained an important germplasm for future study.

2 Materials and methods

2.1 Cloning of the first exon of the *N. alata PDS* (*NalaPDS*) coding sequence

The annotated *PDS* sequences of *N. tabacum* (XM_016642616), *N. benthamiana* (EU165355), and *N. attenuata* (JX185751) were retrieved from the National Centre for Biotechnology Information (NCBI) database and used as queries for a Basic Local Alignment Search Tool-Nucleotide (BLASTn) search against the draft genome sequences of *N. tabacum*, *N. benthamiana*, and *N. attenuata*, respectively (available at https://solgenomics.net). The obtained genomic DNA and coding sequences (CDs) were aligned (Supplementary Figure 1) using the MEGA software (Tamura et al., 2021). Primer pairs, NalaPDS1stExon-F/-R (Supplementary Table 1), were designed based on the conserved regions in the exonic region to isolate the partial *PDS* coding sequence from *N. alata*. Total RNA was extracted from *N. alata* leaf tissue using the RNeasy Plant Mini Kit (Qiagen, CA, USA) and the first strand of cDNA was synthesized using The PrimeScript 1st strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was carried out using 100 ng of cDNA as a template and the Q5 High-Fidelity DNA Polymerase, following the recommended cycling conditions provided by the manufacturer (NEB, MA, USA). The PCR amplification consisted of an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. The amplified product was recovered from a low-melting point agarose gel and subsequently sequenced.

2.2 *S-RNase* allele identification in *N. alata*

To identify the *S-RNase* allele in *N. alata*, degenerate primers C2F/C4R (Supplementary Table 1) were used to amplify the *S-RNase* candidates. Genomic DNA was extracted from *N. alata* leaf tissue using the Plant Genomic DNA Miniprep Kit protocol (Tiangen, Tianjin, China). Genomic PCR was performed using the Q5 High-Fidelity DNA Polymerase, as described above. The amplified PCR product was cloned into the pCE2-TA-Blunt-Zero vector (Vazyme, Nanjing, China) following the manufacturer's instructions. 10 Clones of each plant were sequenced using Sanger sequencing platform (Generay, Shanghai, China), and the obtained sequences were subjected to BLAST search against the non-redundant NCBI database to determine the candidate *S-RNase* allele.

To confirm the *S2/Sc10* bi-allele distribution result in the family, specific primer pairs S2F/R and Sc10F/R (Supplementary Table 1) were used to examine the same population. The PCR products displaying expected size unique bands were subjected to electrophoresis using the ZAG DNA Analyzer (Agilent, Santa Clara, USA).

2.3 Guide RNA design and plasmid construction

Cas9-PF vector was digested using the *Bsa*I enzyme (NEB) for vector linearization. The online CRISPR software (http://crispor.tefor.net/) was utilized to identify sgRNA sequences in the gene's first exon (Concordet and Haeussler, 2018). For *NalaPDS*, the oligonucleotides of sgRNA (Supplementary Table 1) were annealed and ligated into *Bsa*I-digested Cas9-PF plasmids to generate the Cas9-PF-NalaPDS structure. For *S-RNase*, a polycistronic tRNA-gRNA (PTG) cassette (Supplementary Table 1) containing S2-RNase and Sc10-RNase gRNAs was synthesized (Generay, Shanghai, China). The synthesized PTG polynucleotides were inserted into *Bsa*I-digested Cas9-PF plasmids through homologous recombination to generate the Cas9-PF-SRNase structure.

2.4 Sterilization seeds preparation of *N. alata* plants

The *N. alata* plants (accession PI42334), used in this study, were provided by Prof. Hanhui Kuang (Huazhong Agriculture University) (Yuan et al., 2015). The seeds of the *N. alata* line were harvested after crossing from different plants. Sterilization of the seeds was carried out by placing them in a 1.5-mL microfuge tube with 70% ethanol for 1 minute, followed by treatment with a bleach solution containing 10% sodium hypochlorite for 10 minutes. After five rinses with sterilized deionized water, the sterilized seeds were sown on agar plates containing plant growth media (Table 1).

2.5 *Agrobacterium* culture preparation

Target plasmids were transformed into *A. tumefaciens* using electroporation. For each transformation, 100 µl of competent cells were mixed with 50 ng of plasmid DNA and suspended in an electroporation cuvette with an electrode distance of 1 mm (Bio-Rad, USA). Electroporation was carried out using MicroPulser Electroporator (Bio-Rad, USA) with 2.5 kV, 25 µF capacitance, and 400 Ohm resistance. To screen for transformants of the target plasmid, colony PCR was performed using specific primers. Each colony was suspended in 20 µl of 1 X Taq DNA polymerase Master Mix (Vazyme, Nanjing, China) and subjected to PCR amplification. Positive single colonies were transferred to 50 mL centrifuge tubes containing 5 mL of liquid LB medium supplemented with 50 mg/L rifampicin and 100 mg/L kanamycin. The bacterial cultures were incubated on a shaker overnight at 220 rpm and 28°C until the OD at 600 nm (OD600) reached 0.8. The *Agrobacterium* cultures were then centrifuged at 2500 × g for 5 minutes, washed two times with MS medium containing 30 g/L sucrose. The bacterial pellet was resuspended in 4 mL of MS medium containing 30 g/L sucrose and supplemented with 100 µM acetosyringone to achieve an OD600 of 0.3 - 0.4.

TABLE 1 Optimized steps, medium used and culture conditions in *Agrobacterium*-mediated transformation of *Nicotiana alata*.

Type of Medium	Medium content	Antibiotics applicaiton	Culture conditions	Culture time
Washing medium	MS basal medium (4.41 g/L), 6-Benzyladenine (6-BA) 1 mg/L, IAA 0.02 mg/l, 30 g/L sucrose, adjust the pH to 5.8 with 1 M KOH.	None	None	None
Co-cultivation	MS basal medium (4.41 g/L), 6-BA 1 mg/L, IAA 0.02 mg/l, 30 g/L sucrose, and 5 g/L Bacto agar, adjust the pH to 5.8 with 1 M KOH.	None	Dark, 25°C	3 days
Callus induction	MS basal medium (4.41 g/L), 6-BA 1 mg/L, IAA 0.02 mg/l, 30 g/L sucrose, and 5 g/L Bacto agar, adjust the pH to 5.8 with 1 M KOH.	Timentin (125 mg/L) for *Agrobacterium* inhibition and hygromycin (35 mg/L) for selectable antibiotic	Long-day condition (16-h light/8-h dark, 25 °C)	3-5 weeks
Shoot regeneration	MS basal medium (4.41 g/L), 6-BA 1 mg/L, 30 g/L sucrose, and 5 g/L Bacto agar, adjust the pH to 5.8 with 1 M KOH.	Timentin (125 mg/L) for *Agrobacterium* inhibition and hygromycin (35 mg/L) for selectable antibiotic	Long-day condition (16-h light/8-h dark, 25 °C)	3 weeks
Rooting	1/4 X MS basal medium (4.41 g/L), 0.8 mg/L 4-(3-Indolyl) butyric acid, 30 g/L sucrose, and 6 g/L Bacto agar, adjust the pH to 5.8 with 1 M KOH	Timentin (125 mg/L) for Agrobacterium inhibition	Long-day condition (16-h light/8-h dark, 25 °C)	3-5 weeks

2.6 *Agrobacterium* strains screening

To screen for the most efficient *Agrobacterium* strain, we performed the transformation using four *A. tumefaciens* strains: GV3101, C58C1, EHA105 and LBA4404. All strains harbored the same Cas9-PF-SRNase plasmid as described above. This plasmid contained the *hygromycin B phosphotransferase* gene as a selectable marker for plant selection (conferring hygromycin resistance) under the control of the 35S promoter and CaMV poly(A) signal terminator. We evaluated the transformation efficiency by measuring the callus proliferation and shoot regeneration rates.

2.7 Explants preparing and *Agrobacterium* infection

The sterilized seeds were germinated for 3 days in the dark (25 °C) and then transferred to long-day condition (16-h light/8-h dark, 25 °C) for another 7 days. The seedlings (15-20 mm in length) were excised below the apical meristem and above the roots. The hypocotyl part was subsequently cut into 4-5 segments (about 3–5 mm in length) and the segments were placed on cocultivation medium (Table 1). The hypocotyl preparing and *Agrobacterium* infection could be achieved simultaneously by dipping the scalpel into *Agrobacterium* suspension before each cut.

2.8 Callus induction and shoots regeneration

After 3 days of co-cultivation with *Agrobacterium*, explants were taken out and transferred to callus induction medium (Table 1) containing the antibiotic (cefotaxime or timentin) to inhibit growth of *Agrobacterium* and subsequently sub-cultured at second week. After 2-3 weeks, when explants had developed swelling callus with shoot primordia, the calli were transferred onto regeneration medium (Table 1), which contained both *Agrobacterium* and plant antibiotics. The calli with shoots were subsequently sub-cultured every 3 weeks until plantlets were formed. These plantlets were separated into single plants and cultured on rooting medium without selectable antibiotics (Table 1). At this stage, clean cut of callus from the base of the plantlet was essential. Plantlets were sub-cultured on rooting media every 3 weeks until roots developed. When the roots are 2-3 cm long, plants were carefully removed from the gel and transplanted into soil. These transgenic plants were subsequently maintained in growth chambers at long-day condition (16-h light/8-h dark, 25 °C).

2.9 Effect of antibacterial antibiotics on *Agrobacterium* growth

To identify the most suitable antibiotic and its concentrations for the *Agrobacterium* growth inhibition, different ranges of cefotaxime and timentin concentrations were evaluated according to the ones described in the literature for other *nicotiana* species. *Agrobacterium* infected hypocotyl explants were transferred into co-cultivation and callus induction plates containing MS medium with cefotaxime alone at concentrations of 50, 100, 200 and 300 mg/L and timentin alone at concentrations of 50, 125, 200 and 300 mg/L. Data were statement as the callus proliferation and shoot regeneration rate.

2.10 Detection of genome editing events

The genomic DNA of the stable transgenic *N. alata* plants from hygromycin selection and wild-type plants were extracted from leaf tissue using the Plant Genomic DNA Miniprep Kit protocol (Tiangen) to assess targeted mutagenesis using PCR amplification and Sanger sequencing. The genomic region spanning the CRISPR

target sequences were amplified by PCR (primer sequences in Supplementary Table 1) using Q5 High-Fidelity DNA Polymerase (NEB). The PCR products were sequenced and then subjected to SnapGene software assay (www.snapgene.com) according to the manufacturer's instructions. PCR fragments of putative editing events were then cloned into pCE2-TA-Blunt-Zero vector (Vazyme) and 10 clones each plant were sequenced to further measure the frequencies of CRISPR induced mutations. The mutation rate was calculated based on the ratio of mutated plants versus total transgenic plants.

3 Results

3.1 Optimization of *Agrobacterium*-mediated transformation and regeneration

Two methods of Agrobacterium-mediated transformation in *N. alata* have been previously described (Ebert and Clarke, 1990; Schroeder and Stimart, 1996). Leaf discs and seedling hypocotyls were utilized as explants for plantlet regeneration, respectively. Initially, we strictly followed the methodology described by Schroeder and Stimart (1996) for leaf discs-mediated transformation, as it is a conventional method applied in multiple *Nicotiana* species. However, our attempts were unsuccessful. We made efforts to adjust various parameters, including different *Agrobacterium* strains, selectable antibiotic concentrations, and callus and shoot induction media compositions, in order to achieve improved outcomes. Unfortunately, none of the tested parameters resulted in the formation of well-developed callus and regeneration shoots in *N. alata*. Instead, friable and disorganized tumors were observed (data not shown). Therefore, we speculated that the use of leaf explants may not be suitable for generating transgenic lines of our owned *N. alata* (PI42334) line.

Although the regeneration of transformed *N. alata* using hypocotyl explants was established, the low transformation frequency (<1%) was identified as a bottleneck (Ebert and Clarke, 1990). To enhance the transformation frequency, we aimed to optimize the *Agrobacterium* strains, co-culture reagents. Four *Agrobacterium* tumefaciens strains, namely GV3101, C58C1, EHA105, and LBA4404, were tested to evaluate the effects of strain types on the transformation efficiency in *N. alata*. Hypocotyls from 10-day-old seedlings were cut into 3-5 mm segments as explants. The explants were then transferred to callus-induction medium (Figures 1A, B) and cultured in darkness for 2 days to increase infection efficiency before being transferred to a long-day condition (16-hour light/8-hour darkness, 26°C). After 2 weeks on the callus induction plates and one month on the regeneration induction plates, containing 100 mg/L cefotaxime and 35 mg/L hygromycin, the hypocotyl segments gave rise to calli and shoots. The results demonstrated successful transformation of *N. alata* using GV3101, EHA105, and LBA4404 (Table 2). *A. tumefaciens* EHA105 and GV3101 exhibited higher transformation efficiency, with 12.5% and 8.33% regeneration shoot, respectively. Consistent with previous studies by Ebert and Clarke (1990), LBA4404 showed successful transformation but low efficiency (2.08%) among the four strains, whereas C58C1 failed to transform *N. alata*.

During the screening of *A. tumefaciens* strains, we found that the primary cause of low transformation efficiency was *Agrobacterium* contamination (Figure 1A). Therefore, we next investigated the impact of antibiotics on *Agrobacterium* during the transformation process. Cefotaxime, commonly used in tissue culture of *N. tabacum* and *N. benthamiana*, is known to effectively eliminate any remaining *Agrobacterium* after co-cultivation. However, our study on *N. alata* transformation revealed a conflicting relationship between calli/shoots induction and the anti-agrobacterial effect of cefotaxime (Table 3). Upon screening, bacterial growth was observed at concentrations below 200 mg/L of cefotaxime (Table 3). Conversely, concentrations higher than 200 mg/L exhibited effective antibacterial properties against surface-growing bacteria, but severely impaired calli induction and shoots regeneration (Table 3). Recent studies have demonstrated the efficacy of timentin in suppressing *A. tumefaciens*, comparable to carbenicillin and cefotaxime, with minimal impact on shoot regeneration (Cheng et al., 1998; Krügel et al., 2002). Hence, we explored the effects of timentin on calli induction, shoot regeneration of *N. alata* hypocotyls, and its inhibitory ability on *Agrobacterium* growth. As presented in Table 3, timentin alone exhibited more efficient bacteria elimination than cefotaxime. After two weeks in the presence of these antibiotics, calli induction on timentin plates with concentrations higher than 125 mg/L proliferated normally without observable negative effects. Our findings indicate that timentin, with its broad spectrum of concentrations for suppressing *A. tumefaciens* in *Agrobacterium*-mediated genetic transformation, enabled successful regeneration, whereas cefotaxime significantly hindered regeneration.

After investigating different *Agrobacterium* strains and antibacterial antibiotics, we determined that genetic transformation using the EHA105 strain and timentin at a concentration of 125 mg/L resulted in higher values for *N. alata* calli induction and shoot regeneration. Ultimately, we summarized the optimized *Agrobacterium*-mediated transformation of *N. alata* with media composition used, culture conditions, and average duration in different steps in Table 1.

3.2 Cas9–induced mutagenesis of *NalaPDS* in *N. alata*

The *PDS* gene is responsible for encoding one of the crucial enzymes involved in the carotenoid biosynthesis pathway. Mutant plants with an albino phenotype, resulting from disruptions in this gene, have been widely utilized as a model gene for virus-induced gene silencing and CRISPR/Cas9-mediated gene editing (Qin et al., 2007; Huang et al., 2009; Ma et al., 2020). Due to the absence of sequence information for the *PDS* gene in *N. alata* (referred to *NalaPDS*), conserved primers were designed based on the homologous nucleotide sequences of the *PDS* gene in *N. tabacum*, *N. benthamiana*, and *N. attenuate* (Edwards et al., 2017; Xu et al., 2017; Ranawaka et al., 2023). These primers were employed to amplify the *NalaPDS* partial CDs region from total RNA.

10.3389/fpls.2024.1329697

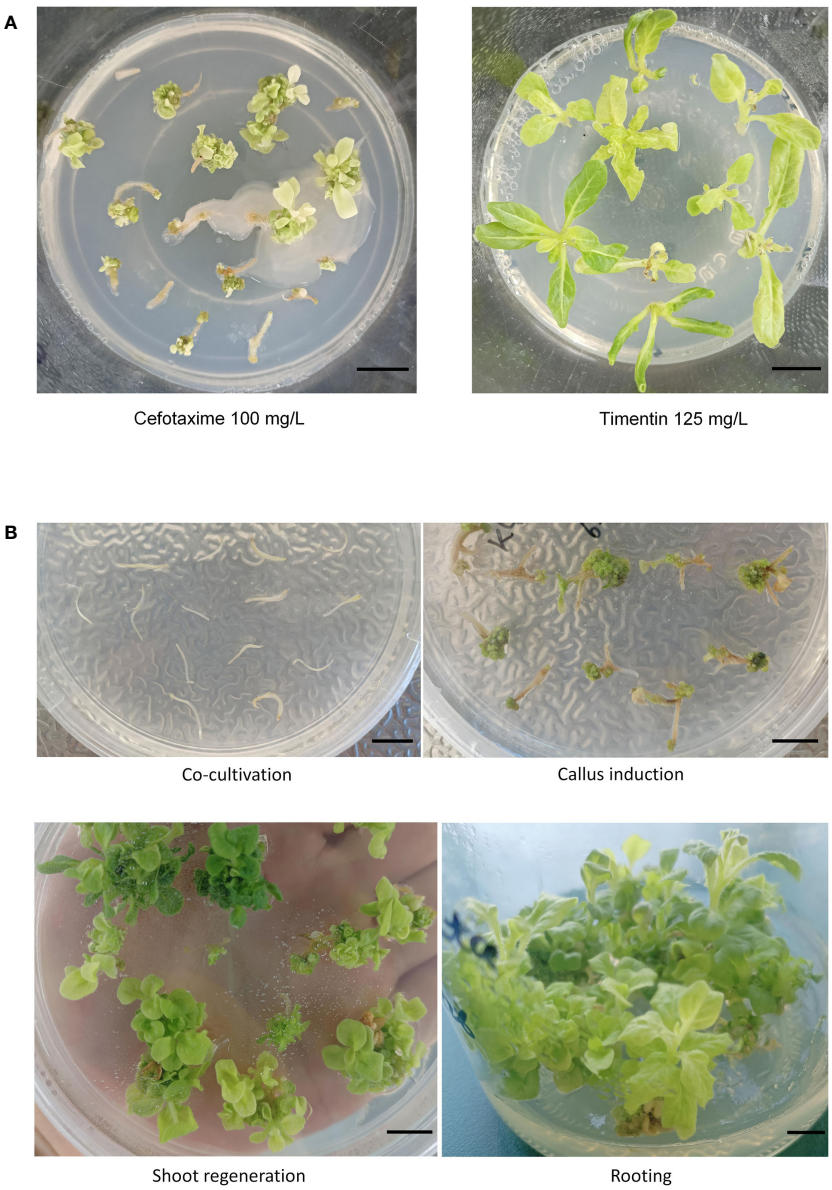


FIGURE 1
Agrobacterium-mediated transformation of *Nicotiana alata* using hypocotyl explants. **(A)** Inhibition of *Agrobacterium* growth by 100 mg/L cefotaxime and 125 mg/L timentin. **(B)** Regeneration frequency of *N. alata* based on optimized transformation method. Representative transformation steps are shown: hypocotyl segments were co-cultured with Cas9-PF-SRNase, callus formation and shoot regeneration on selection medium with hygromycin (35 mg/L) and timentin (125 mg/L), *in vitro* culture and transgenic seedling on root induction medium. Scale bars = 5 mm.

TABLE 2 Transformation efficiency of callus and regenerated shoots by different *Agrobacterium* strain.

Agrobacterium strain	No. of explants	No. of resistant calli induction	No. of resistant shoots induction
GV3101	48	20	4
C58C1	48	0	0
EHA105	48	23	6
LBA4404	48	12	1

Subsequently, a guide RNA (gRNA) targeting the first exon of *NalaPDS* was selected and integrated into the Cas9-PF system (Liu et al., 2019). The resulting construct (Figure 2A) was then introduced into *N. alata* plants using the optimized *Agrobacterium*-mediated genetic transformation protocol mentioned earlier.

Total 43 transformed seedlings were recovered with hygromycin selecting from 50 explants. Among them, 9 plants exhibited albino leaf phenotype which was recognized as completely disrupted *NalaPDS*, loss-of-function mutants (Figure 2B). Interestingly, the visual selection marker system mediated by the PAP gene in this CRISPR system (Liu et al., 2019) was ineffective in

10.3389/fpls.2024.1329697

TABLE 3 Antibiotic effect screening on *Agrobacterium* EHA105.

Antibiotics	Concentrations (mg/L)	No. of explants	No of calli induction	No of calli without *Agrobacterium* growth	No of shoots induction	No. of resistant shoots without Agrobacterium growth
Cefotaxime	50	48	26	0	0	0
	100	48	32	11	8	5
	200	48	8	8	3	1
	300	48	0	0	0	0
Timentin	50	48	34	26	18	18
	125	48	43	43	40	40
	200	48	38	38	38	38
	300	50	24	24	16	16

N. alata, none of the transformed seedlings exhibited purple phenotype in this study (Figure 2B). Specific primers were designed to amplify the target regions and sequenced to detect the mutant plants with *NalaPDS*. Mutations in *NalaPDS* were identified in 23 of 43 independent transgenic plants with the percentage of 53.5%. To further dissect the type of mutation, we also cloned and sequenced (10 random selected clones each plant) from the amplification of genomic DNA extracted from mutants (Figure 2C). We found that two and seven of nine displayed albino leaves are homozygous editing and bi-allele gene editing respectively and remaining 14 mutations in *NalaPDS* which show green leaf as wild type (WT) are heterozygous editing with indel on one allele (Figure 2D). Notably, the efficient mutations were generated in the *NalaPDS* locus of transgenic plants with indel rates more than 50% at T0 generation. These results showed that the CRISPR/Cas9 system could be used to modify genome and have high efficiency for targeted mutagenesis in *N. alata*.

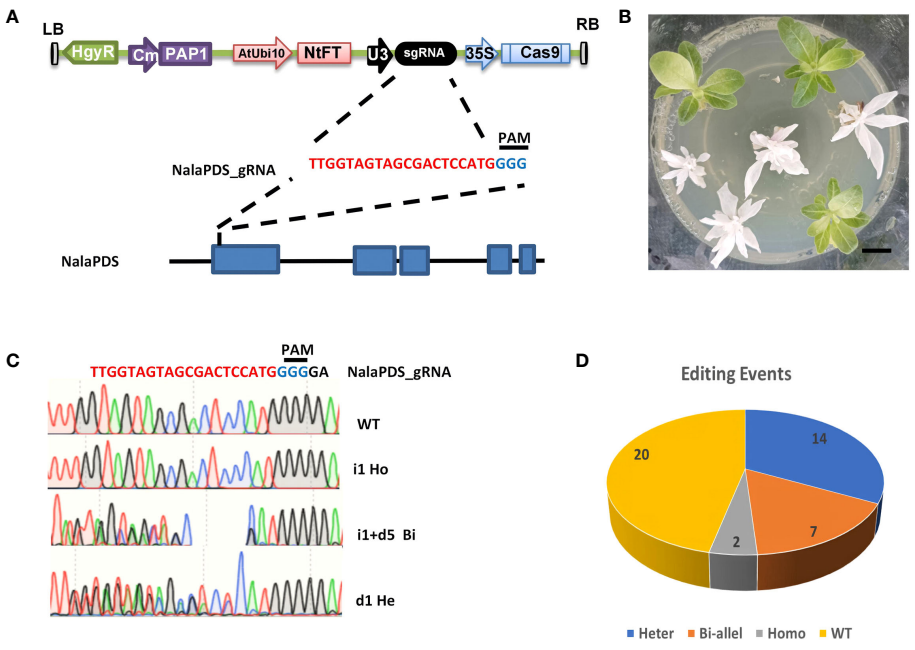


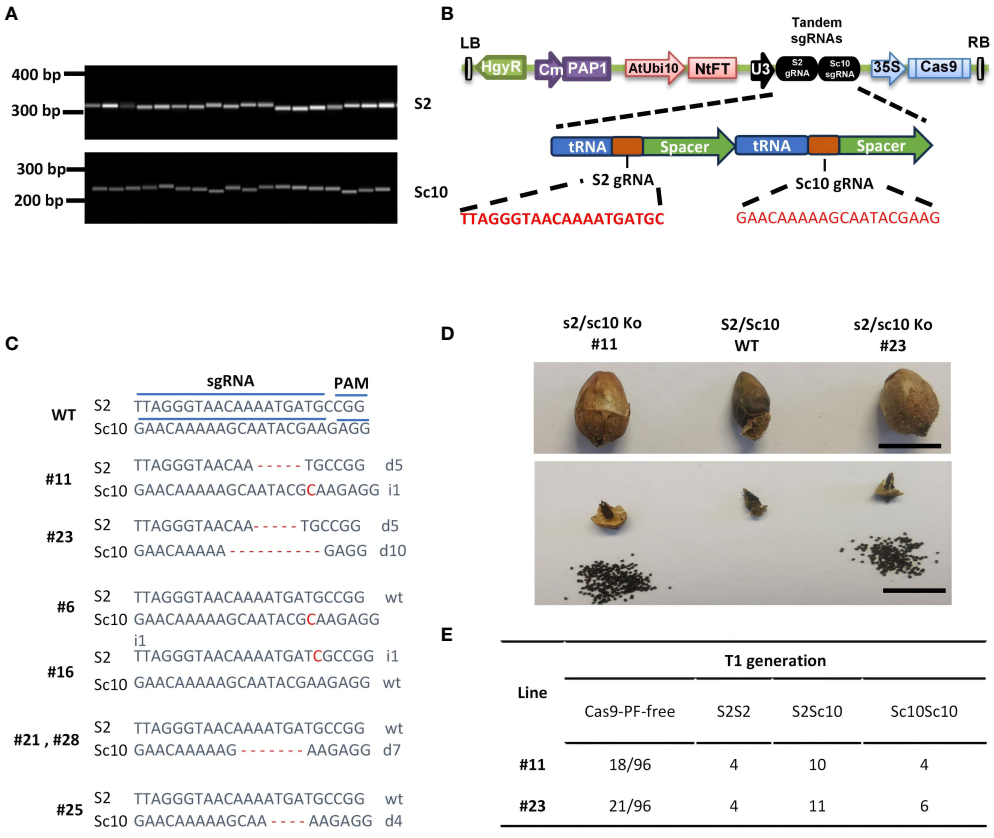
FIGURE 2
Agrobacterium-mediated CRISPR/Cas9 genome editing of *NalaPDS* in *N. alata*. **(A)** T-DNA constructs for Cas9-PF-NalaPDS. Cas9-PF-NalaPDS consists of a PF Cassette, which contains anthocyanin synthesis regulation gene *PAP1* and early flower *NtFT5* gene, as a visible marker, a *Cas9* gene driven by 35S promoter, an sgRNA targeting the *NalaPDS* gene under the control of a U3 promoter, and a hygromycin resistance gene. The first exon of the *N. alata* PDS gene was selected as the target site for the gRNA. **(B)** Phenotypes of the *pds* mutants. Seedlings displayed albino phenotype are homozygous or bi-allele gene editing of *NalaPDS*. Scale bar=1 cm. **(C)** PCR product sequencing results of PDS mutant plants in **(B)** are aligned to the reference genome sequence. Different chromatograms of target position indicate different types of editing events, e.g., homozygous (Ho), bi-allele (Bi) and heterozygous (He) type editing. WT, wild-type; inserts and deletions are indicated as 'i' and 'd' respectively. **(D)** Total precise editing events categorized by different editing types, including homozygous (Homo), bi-allele (Bi-allel) and heterozygous (Heter) and wild-type (WT).

3.3 Targeted mutagenesis of *S-RNase* results in self-compatibility

The SI in *N. alata* is controlled by the highly polymorphic *S*-locus. To identify the *S* allele in our owned line, the degenerate primer pair C2f/C4r (Supplementary Table 1) was used to amplify the conserved domains of S-RNases (Roldan et al., 2010). Fragments amplified from random selected 7 plants of the population of *N. alata* were cloned and sequenced. Among 70 sequences (10 clones per plant), 29 and 41 were 100% identical to *Sc10-* and *S2-RNase* respectively, which were previously characterized as functional *SI* alleles (Anderson et al., 1986; Murfett et al., 1996). To further confirm the allelic variants of *S-RNases*, specific primers for *Sc10-* and *S2-RNase* were used to examine the same population. As Figure 3A shown, a single band with the expected size was amplified in each case, suggesting the presence of *Sc10-* and *S2-RNases* alleles in plants.

To test whether the developed CRISPR/Cas9 system could be used to target two *S-RNases* alleles simultaneously, we designed and armed pairs of gRNA targeting the *Sc10-* and *S2-RNase* first exon to an endogenous tRNA processing system (Figure 3B). This system could dramatically enhance the processing efficiency and function of dual sgRNAs transcribed from a single transcript (Xie et al., 2015). According to our optimized transformation method, we generated 26 regeneration plants from 30 explants. To evaluate the efficiency of the target gene editing, we amplified and sequenced the first exons sequences of *Sc10-* and *S2-RNases* gene in all T0 plants. In total, we obtained 12 plants containing mutations in *Sc10-and S2-RNases*. Among them, two plants contain bi-allelic mutations of both *S-RNases*, one contains mutations in *S2-RNase* and four contain mutations in *Sc10-RNase* allele (Figure 3C). However, the remaining five mutations were heterozygous in *Sc10-* or *S2-RNases*, which showed overlapping peaks starting at 17th nucleotide of gRNA. We speculated these plants are chimeric editing, which is a common phenomenon in somatic cells gene editing.

To investigate the self-compatibility of the *S-RNase* gene editing plants, we transferred two bi-allelic mutants to the greenhouse under a 16-h light/8-h dark cycle. We performed artificial pollinations at the open flower stage. After self-pollination, compare to the small and shriveled fruit of WT plants, two bi-allelic mutant lines (#11 and #23) produced big and well-stacked fruits (Figure 3D). In contrast to the complete sterility of WT plants, self-pollination of two mutant lines produces large amounts of seeds



Line	T1 generation			
	Cas9-PF-free	S2S2	S2Sc10	Sc10Sc10
#11	18/96	4	10	4
#23	21/96	4	11	6

FIGURE 3
CRISPR/Cas9-mediated *S-RNase* mutations result in self-compatible *N. alata*. **(A)** *S-RNase* allele identification in *N. alata*. Primer sets S2F/R and Sc10F/R were used for PCR analysis to determine the presence of *S2* and *Sc10* alleles in the population used for gene editing. **(B)** Cas9-PF was armed with a polycistronic tRNA-gRNA (PTG) cassette consisting of S2 and Sc10 gRNAs. **(C)** Mutation patterns in T0 transgenic plants. sgRNA, single guide RNA; PAM, the protospacer adjacent motif; WT, wild type; inserts and deletions are indicated as 'i' and 'd' respectively. **(D)** compared to the sterility of WT, well-stacked fruits and mounts of seeds were harvested in two bi-allele edited *N. alata* plants. Scale bar=1 cm. **(E)** Segregation ratio of Cas9-PF-free plants and *S2* and *Sc10* alleles in T1 generation of two bi-allele edited *N. alata* lines respectively.

per fruit, ranging from 137 to 349 seeds per fruit. These results demonstrate that inducing loss-of-function mutations in *S-RNase* genes resulted in self-compatibility in *N. alata*.

To seek for the Cas9 cassette free offspring and determine whether the mutations could be inherited to the next generation, we sowed the T1 seeds. The germination rate was > 80% for two lines. The segregation ratio of T1 plants of lines 11 and 23 without the Cas9 cassette is 18.7% and 21.8% respectively (Figure 3E). The Chi-square test showed that these two lines contained a single copy of the Cas9 cassette. We further detected mutations in the *S-RNase* genes and genotyping of *Sc10-* and *S2-* allele in all Cas9-free T1 generation plants. The sequencing result showed that bi-allele mutations of *Sc10-* or *S2-RNases* in lines 11 and 23 were transmitted to the T1 generation and all T1 plants harbored mutations in *S2-* and/or *Sc10*-allele. The ratios of *S2S2*, *S2Sc10* and *Sc10Sc10* in the two lines are 19-22%, 52-56% and 22-29%, corresponding to a predictable Mendelian ratio of 1:2:1 (Figure 3E). In addition, these mutants have similar plant morphology and growth vigor as the WT (Supplementary Figure 3), indicating that they can directly be used for breeding or basic research.

4 Discussion

Leaf disk transformation is a widely used method for introducing foreign DNA into plant cells, leading to the development of transgenic plants (Curtis et al., 1995). However, this method may not work for all plant species, even within a plant species, different genotypes or varieties may respond differently to leaf disk transformation. The success of leaf disk transformation is sensitive to the specific parameters of the protocol, such as the choice of tissue culture medium, antibiotics, hormones, and environmental conditions. Variations in these parameters can affect the outcome, and it may be difficult to find the optimal conditions for recalcitrant species. The hypocotyl, located below the cotyledons in the embryonic stem region of a plant, often has characteristics that make it more amenable to transformation. Therefore, hypocotyl transformation is considered a valuable alternative to leaf disk transformation for several reasons. It tends to be less recalcitrant than leaves, making it easier for *Agrobacterium* to infect and introduce foreign DNA. Hypocotyl tissue typically exhibits a higher regeneration potential than leaf tissue in many plant species (Wang and Xu, 2008; Mahto et al., 2018; Muto et al., 2021; Xiao et al., 2022). In this study, we failed to induce callus formation by using leaf disks as explants. However, we observed abundant swelling tissue in *Agrobacterium*-infected hypocotyl segments. By screening different strains of *A. tumefaciens*, we found that EHA105 and GV3101 had relatively high infection efficiency, which is consistent with previous reports in other species.

During the hypocotyl-mediated transformation using EHA105, another challenge was to control *Agrobacterium* contamination in the induced callus and regenerated shoots. Induced calli were easily obtained when we used low concentration cefotaxime, but most of these calli did not survive to the rooting stage due to the incomplete inhibition of *Agrobacterium*. On the other hand, high concentration cefotaxime to control *Agrobacterium* infestation after transformation dramatically suppressed callus induction and shoot regeneration. As timentin is a novel antibiotic that has been utilized in other species' genetic transformation (Cheng et al., 1998; Krügel et al., 2002), we tested whether timentin could replace cefotaxime in our *N. alata* transformation. We found that timentin dramatically inhibited *Agrobacterium* growth and allowed for regeneration within a wide range of concentrations (Table 1). After optimization, the efficiency of genetic transformation achieved more than 80% from <1% as described (Ebert and Clarke, 1990). These results indicate that efficient and repeatable transformation systems were established in *N. alata*. More important, the efficiencies of our optimized transformation system should be high enough to test *Agrobacterium*-delivered CRISPR/Cas9 system for *N. alata* genome editing.

Due to the obvious albino phenotypes associated with *pds* mutation, we selected the first exon and designed a single guide RNA (sgRNA) targeting *NalaPDS* to arm our Cas9-PF vector. According to our optimized transformation method, 23 *NalaPDS* editing events with different size indels were obtained from 43 independent transgenic lines. The percentage of the independent T0 transgenic lines that generated mutations in *NalaPDS* was more than 50% (Figure 2D). Moreover, 9 transgenic lines of Cas9-PF-NalaPDS showed obvious albino leaf phenotypes. Sequencing results demonstrated that 2 plants harbored homozygous mutations and 7 plants harbored biallelic mutations (Figure 2D). These results indicated that targeted mutagenesis using *Agrobacterium*-delivered CRISPR/Cas9 system in *N. alata* is feasible.

N. alata is a model species to study gametophytic SI, which is a reproductive barrier that prevents self-fertilization and promotes outcrossing and genetic diversity. SI is controlled by a single polymorphic *S*-locus, which encodes two products: the *S-RNase* in the pistil and the *SLF* in the pollen. If the pollen and pistil share the same *S*-haplotype, the pollen tube growth is arrested by the cytotoxic effect of the S-RNase, resulting in an incompatible reaction. SI plays an important role in genetic diversity in flowering plant evolution, but poses some challenges and limitations to fix useful genetic variation. SI makes the plant always have a highly heterozygous genome, which presents a big challenge to perform high quality haploid genome assembly. Moreover, SI makes it difficult to produce pure lines, which interferes with both basic research and breeding. SI prevents the plant from producing homozygotes, which hampers the development of transgenic or genome-edited plants. SI also complicates the genetic analysis and functional characterization of plant genes, as it can affect the segregation and inheritance patterns of genes in self-incompatible populations (Ye et al., 2018; Enciso-Rodriguez et al., 2019; Lee et al., 2023). Therefore, beyond the *NalaPDS* gene, our research ventured into breaking the SI in *N. alata* by targeting the *S-RNase* gene. As it has at least two different *S-RNases* in *S*-alleles, we additionally wanted to test whether we can edit two genes simultaneously in *N. alata* by using our gene editing system. With a PTG system, we armed the Cas9-PF vector with both *S2* and *Sc10* gRNAs. Ultimately, we obtained two lines with bi-allelic editing events at *S2-* and *Sc10-RNase* from 30 explants.

To test whether the bi-allelic *S-RNase* mutations are enough to break the SI and whether the targeted gene mutagenesis passes to the next generations, two lines were artificially pollinated. Well-stacked fruits and abundant seeds were harvested from both two *s2/sc10* mutation lines, indicating that the mutations were self-compatible. Further analysis of the target gene demonstrated that the mutations had been stably inherited to T1 generation in *N. alata*. The successful knockout of *S-RNase* and the subsequent breakdown of SI in *N. alata* is particularly noteworthy. These *S-RNase* edited lines will be a powerful tool to accelerate basic research and breeding. These self-compatible diploid *N. alata* can be used to produce inbred lines, such as recombinant inbred lines (RILs) or near isogenic lines (NILs), which can be further used for gene mapping and breeding. As more than half of the species of genus *Nicotiana* are SI, the strategy presented should be beneficial for researchers and breeders of other SI wild tobacco.

It is worth mentioning that unlike gene editing in *N. tabacum* and other species by using Cas9-PF, purple phenotype did not occur in *N. alata* (Figures 1, 2B; Supplementary Figure 2). As Cas9-PF vector contains a visual selection marker mediated by the constitutive overexpression of PAP1 gene, it was supposed to display distinct purple color in leaf, stem and flower (Liu et al., 2019). However, our results indicated that the *PAP1* gene-mediated visual selection marker was ineffective in *N. alata*. Compared with other *N. alata* lines that showed colorful flowers, the line (PI42334) we used had pure white flowers. Therefore, we speculate that this *N. alata* line may have intrinsic genetic mutation in anthocyanidin synthase pathway that renders the *PAP1*-mediated phenotype less effective. Considering that the anthocyanidin synthase pathway has been proved to be important for biotic and abiotic stress tolerance, it is worth exploring which gene controls the anthocyanidin defect phenotype and whether this gene plays a role in biotic and abiotic stress tolerance in *N. alata*.

The aim of this study was to demonstrate the applicability of the CRISPR-Cas9 system by performing gene knockout of the single *NalaPDS* and allelic *S-RNase* genes using an improved *N. alata* transformation protocol for the first time. A single gRNA was found to be successful to achieve high efficiency editing, resulting in *PDS* mutants with albino phenotypes. Dual gRNA was also proved to be efficient to produce bi-allelic editing, resulting in self-compatible *N. alata*. A rapid, easily operated, highly reproducible, and stable transformation and CRISPR-Cas9-based genome editing system for *N. alata* was established. We expect that the established CRISPR-Cas9 system, with self-compatible lines and improved genetic transformation approach, will enable functional genomics and trait improvement in *N. alata*. In conclusion, this study lays the foundation for a new era of genetic research in *N. alata*.

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

CY: Data curation, Validation, Writing – review & editing. JMZ: Data curation, Validation, Writing – review & editing. YL: Investigation, Writing – review & editing. HY: Investigation, Writing – review & editing. ZT: Methodology, Writing – review & editing. JDZ: Resources, Writing – review & editing. QG: Resources, Writing – review & editing. ZW: Resources, Writing – review & editing. XS: Formal analysis, Writing – review & editing. BX: Formal analysis, Writing – review & editing. CH: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by the key projects of YNTC (2021530000241015), the NSF of China (No. 31960530 and No. 31860499) and High-Level Talent Cultivation Support Plan of Yunnan Province (YNWR-QNBJ-2018-079).

Acknowledgments

We thank Prof. Hanhui Kuang (Huazhong Agriculture University, China) for providing the *N. alata* seeds.

Conflict of interest

Authors QG and JDZ were employed by the company China Tobacco Yunnan Industrial Co. LTD,.

The remaining 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.

The handling editor LY declared a past co-authorship with the author XS.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1329697/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Alignment of genomic DNA and CDs sequences of *PDS* gene in *N. tabacum*, *N. benthamiana*, and *N. attenuata* using the MEGA software. The position of the translation initiation codon is indicated by a black arrow. Conserved primers used for amplifying the first exon of *NalaPDS* are shown by blue arrows. gRNA and the first intron of *NalaPDS* are indicated by red and black underlines, respectively.

SUPPLEMENTARY FIGURE 2
Genetic transformation of the same Cas9-PF-SRNase plasmid in *N. tabacum* by leaf disk and *N. alata* by hypocotyl segments. In contrast to *N. tabacum* overexpressing *PAP1*, which results in purple-colored calli, leaf and shoot tissue due to the accumulation of anthocyanin, *Agrobacterium*-mediated Cas9-PF-SRNase in *N. alata* does not exhibit any purple coloration. Scale bars = 5 mm.

SUPPLEMENTARY FIGURE 3
Comparison of morphology and growth vigor between wild type and *S-RNase* gene-edited *Nicotiana alata* plants. WT shows a representative wild type *N. alata* plant, highlighting its standard morphology and growth characteristics under our experimental conditions. *S2Sc10* Gene-edited *N. alata* (*s2sc10*) displays a representative *S-RNase* gene-edited *N. alata* plant, demonstrating the plant's morphology and growth vigor post-editing. The randomly selected T1 plants of line 11 harbored mutations both in *S2*- and *Sc10*-allele edited plant exhibits characteristics similar to the WT, indicating that the editing did not adversely affect its overall growth and development. Bar = 10 cm.

SUPPLEMENTARY TABLE 1
Oligonucleotides using in this study.

References

Anderson, M. A., Cornish, E. C., Mau, S. L., Williams, E. G., Hoggart, R., Atkinson, A., et al. (1986). Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in Nicotiana alata. *Nature* 321, 38–44. doi: 10.1038/321038a0

Berbeć, A., and Doroszewska, T. (2020). "The use of nicotiana species in tobacco improvement," in *The Tobacco Plant Genome*. Eds. N. V. Ivanov, N. Sierro and M. C. Peitsch (Springer International Publishing, Cham), 101–146.

Bohra, A., Kilian, B., Sivasankar, S., Caccamo, M., Mba, C., Mccouch, S. R., et al. (2022). Reap the crop wild relatives for breeding future crops. *Trends Biotechnol.* 40, 412–431. doi: 10.1016/j.tibtech.2021.08.009

Bredemeijer, G. M., and Blaas, J. (1981). S-specific proteins in styles of self-incompatible Nicotiana alata. *Theor. Appl. Genet.* 59, 185–190. doi: 10.1007/BF00264974

Chen, Z., Debernardi, J. M., Dubcovsky, J., and Gallavotti, A. (2022). Recent advances in crop transformation technologies. *Nat. Plants* 8, 1343–1351. doi: 10.1038/s41477-022-01295-8

Cheng, Z. M., Schnurr, J. A., and Kapaun, J. A. (1998). Timentin as an alternative antibiotic for suppression of Agrobacterium tumefaciens in genetic transformation. *Plant Cell Rep.* 17, 646–649. doi: 10.1007/s002990050458

Concordet, J. P., and Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res.* 46, W242–W245. doi: 10.1093/nar/gky354

Cruz-Garcia, F., Hancock, C. N., and Mcclure, B. (2003). S-RNase complexes and pollen rejection. *J. Exp. Bot.* 54, 123–130. doi: 10.1093/jxb/erg045

Cruz-Garcia, F., Nathan Hancock, C., Kim, D., and Mcclure, B. (2005). Stylar glycoproteins bind to S-RNase in *vitro*. *Plant J.* 42, 295–304. doi: 10.1111/j.1365-313X.2005.02375.x

Curtis, I. S., Davey, M. R., and Power, J. B. (1995). "Leaf disk transformation," in *Agrobacterium Protocols*. Eds. K. M. A. Gartland and M. R. Davey (Springer New York, Totowa, NJ), 59–70.

Czubacka, A. (2022). The use of the polish germplasm collection of Nicotiana tabacum in research and tobacco breeding for disease resistance. *Agriculture* 12, 1994. doi: 10.3390/agriculture12121994

Dempewolf, H., Baute, G., Anderson, J., Kilian, B., Smith, C., and Guarino, L. (2017). Past and future use of wild relatives in crop breeding. *Crop Sci.* 57, 1070–1082. doi: 10.2135/cropsci2016.10.0885

Ebert, P. R., and Clarke, A. E. (1990). Transformation and regeneration of the self-incompatible species Nicotiana alata Link & Otto. *Plant Mol. Biol.* 14, 815–824.

Edwards, K. D., Fernandez-Pozo, N., Drake-Stowe, K., Humphry, M., Evans, A. D., Bombarely, A., et al. (2017). A reference genome for Nicotiana tabacum enables map-based cloning of homeologous loci implicated in nitrogen utilization efficiency. *BMC Genomics* 18, 448. doi: 10.1186/s12864-017-3791-6

Enciso-Rodriguez, F., Manrique-Carpintero, N. C., Nadakuduti, S. S., Buell, C. R., Zarka, D., and Douches, D. (2019). Overcoming self-incompatibility in diploid potato using CRISPR-Cas9. *Front. Plant Sci.* 10, 376. doi: 10.3389/fpls.2019.00376

Engels, J. M. M., and Thormann, I. (2020). Main challenges and actions needed to improve conservation and sustainable use of our crop wild relatives. *Plants* 9, 968. doi: 10.3390/plants9080968

Fahnrich, A., Krause, K., and Piechulla, B. (2011). Product variability of the 'cineole cassette' monoterpene synthases of related Nicotiana species. *Mol. Plant* 4, 965–984. doi: 10.1093/mp/ssr021

Fujii, S., Kubo, K., and Takayama, S. (2016). Non-self- and self-recognition models in plant self-incompatibility. *Nat. Plants* 2, 16130. doi: 10.1038/nplants.2016.130

Guo, H., Lackus, N. D., Kollner, T. G., Li, R., Bing, J., Wang, Y., et al. (2020). Evolution of a novel and adaptive floral scent in wild tobacco. *Mol. Biol. Evol.* 37, 1090–1099. doi: 10.1093/molbev/msz292

Haverkamp, A., Bing, J., Badeke, E., Hansson, B. S., and Knaden, M. (2016). Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. *Nat. Commun.* 7, 11644. doi: 10.1038/ncomms11644

Hsu, P. D., Lander, E. S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157, 1262–1278. doi: 10.1016/j.cell.2014.05.010

Huang, C., Liu, Y., Yu, H., Yuan, C., Zeng, J., Zhao, L., et al. (2018). Non-structural protein NSm of tomato spotted wilt virus is an avirulence factor recognized by resistance genes of tobacco and tomato via different elicitor active sites. *Viruses* 10. doi: 10.3390/v10110660

Huang, C., Xie, Y., and Zhou, X. (2009). Efficient virus-induced gene silencing in plants using a modified geminivirus DNA1 component. *Plant Biotechnol. J.* 7, 254–265. doi: 10.1111/j.1467-7652.2008.00395.x

Kessler, D., Kallenbach, M., Diezel, C., Rothe, E., Murdock, M., and Baldwin, I. T. (2015). How scent and nectar influence floral antagonists and mutualists. *Elife* 4, e07641. doi: 10.7554/eLife.07641.009

Krügel, T., Lim, M., Gase, K., Halitschke, R., and Baldwin, I. T. (2002). Agrobacterium-mediated transformation of Nicotiana attenuata, a model ecological expression system. *Chemoecology* 12, 177–183. doi: 10.1007/PL00012666

Lee, S., Enciso-Rodriguez, F. E., Behling, W., Jayakody, T., Panicucci, K., Zarka, D., et al. (2023). HT-B and S-RNase CRISPR-Cas9 double knockouts show enhanced self-fertility in diploid Solanum tuberosum. *Front. Plant Sci.* 14, 1151347. doi: 10.3389/fpls.2023.1151347

Li, Y., Huang, C., Liu, Y., Zeng, J., Yu, H., Tong, Z., et al. (2023). CRISPR/Cas9-mediated seamless gene replacement in protoplasts expands the resistance spectrum to TMV-U1 strain in regenerated Nicotiana tabacum. *Plant Biotechnol. J.* 21(12), 2641–2653. doi: 10.1111/pbi.14159

Liu, D., Hu, R., Palla, K. J., Tuskan, G. A., and Yang, X. (2016). Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research. *Curr. Opin. Plant Biol.* 30, 70–77. doi: 10.1016/j.pbi.2016.01.007

Liu, Y., Zeng, J., Yuan, C., Guo, Y., Yu, H., Li, Y., et al. (2019). Cas9-PF, an early flowering and visual selection marker system, enhances the frequency of editing event occurrence and expedites the isolation of genome-edited and transgene-free plants. *Plant Biotechnol. J.* 17, 1191–1193. doi: 10.1111/pbi.13118

Ma, X., Zhang, X., Liu, H., and Li, Z. (2020). Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nat. Plants* 6, 773–779. doi: 10.1038/s41477-020-0704-5

Mahto, B. K., Sharma, P., Rajam, M. V., Reddy, P. M., and Dhar-Ray, S. (2018). An efficient method for Agrobacterium-mediated genetic transformation of chilli pepper (Capsicum annuum L.). *Indian J. Plant Physiol.* 23, 573–581. doi: 10.1007/s40502-018-0389-1

Manghwar, H., Lindsey, K., Zhang, X., and Jin, S. (2019). CRISPR/Cas system: recent advances and future prospects for genome editing. *Trends Plant Sci.* 24, 1102–1125. doi: 10.1016/j.tplants.2019.09.006

Mcclure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F., et al. (1989). Style self-incompatibility gene products of Nicotiana alata are ribonucleases. *Nature* 342, 955–957. doi: 10.1038/342955a0

Murfett, J., Strabala, T. J., Zurek, D. M., Mou, B., Beecher, B., and Mcclure, B. A. (1996). S RNase and interspecific pollen rejection in the genus nicotiana: multiple pollen-rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. *Plant Cell* 8, 943–958. doi: 10.2307/3870207

Muto, N., Komatsu, K., and Matsumoto, T. (2021). Efficient Agrobacterium-mediated genetic transformation method using hypocotyl explants of radish (Raphanus sativus L.). *Plant Biotechnol. (Tokyo)* 38, 457–461. doi: 10.5511/plantbiotechnology.21.1021b

Purugganan, M. D., and Jackson, S. A. (2021). Advancing crop genomics from lab to field. *Nat. Genet.* 53, 595–601. doi: 10.1038/s41588-021-00866-3

Qin, G., Gu, H., Ma, L., Peng, Y., Deng, X. W., Chen, Z., et al. (2007). Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Res.* 17, 471–482. doi: 10.1038/cr.2007.40

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W., and Mcdade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in Nicotiana. *Phytochemistry* 63, 265–284. doi: 10.1016/S0031-9422(03)00113-4

Raguso, R. A., Schlumpberger, B. O., Kaczorowski, R. L., and Holtsford, T. P. (2006). Phylogenetic fragrance patterns in Nicotiana sections Alatae and Suaveolentes. *Phytochemistry* 67, 1931–1942. doi: 10.1016/j.phytochem.2006.05.038

Rahman, S. U., Khan, M. O., Ullah, R., Ahmad, F., and Raza, G. (2023). Agrobacterium-mediated transformation for the development of transgenic crops; present and future prospects. *Mol. Biotechnol.* doi: 10.1007/s12033-023-00826-8

Ranawaka, B., An, J., Lorenc, M. T., Jung, H., Sulli, M., Aprea, G., et al. (2023). A multi-omic Nicotiana benthamiana resource for fundamental research and biotechnology. *Nat. Plants* 9, 1558–1571. doi: 10.1038/s41477-023-01489-8

Roldan, J. A., Quiroga, R., and Goldraij, A. (2010). Molecular and genetic characterization of novel S-RNases from a natural population of Nicotiana alata. *Plant Cell Rep.* 29, 735–746. doi: 10.1007/s00299-010-0860-6

Roldan, J. A., Rojas, H. J., and Goldraij, A. (2015). *In vitro* inhibition of incompatible pollen tubes in Nicotiana alata involves the uncoupling of the F-actin cytoskeleton and the endomembrane trafficking system. *Protoplasma* 252, 63–75. doi: 10.1007/s00709-014-0658-4

Sang, W., Ma, K., and Axmacher, J. C. (2011). Securing a future for China's wild plant resources. *BioScience* 61, 720–725. doi: 10.1525/bio.2011.61.9.11

Schroeder, K. R., and Stimart, D. P. (1996). Transformation of Nicotiana alata link and otto. with an autoregulatory senescence-inhibitor gene construct. *HortScience* 31, 617c–6617. doi: 10.21273/HORTSCI.31.4.617c

Taheri-Dehkordi, A., Khandan-Mirkohi, A., Kafi, M., and Salami, S. A. (2017). Exploring and optimising the conditions for virus-induced gene silencing in an ornamental tobacco,Nicotiana alata. *J. Hortic. Sci. Biotechnol.* 93, 377–384. doi: 10.1080/14620316.2017.1370986

Tamura, K., Stecher, G., and Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* 38, 3022–3027. doi: 10.1093/molbev/msab120

Tirnaz, S., Zandberg, J., Thomas, W. J. W., Marsh, J., Edwards, D., and Batley, J. (2022). Application of crop wild relatives in modern breeding: An overview of resources, experimental and computational methodologies. *Front. Plant Sci.* 13, 1008904. doi: 10.3389/fpls.2022.1008904

Trojak-Goluch, A., Laskowska, D., and Kursa, K. (2016). Morphological and chemical characteristics of doubled haploids of flue-cured tobacco combining resistance to Thielaviopsis basicola and TSWV. *Breed Sci.* 66, 293–299. doi: 10.1270/jsbbs.66.293

Wang, G., and Xu, Y. (2008). Hypocotyl-based Agrobacterium-mediated transformation of soybean (Glycine max) and application for RNA interference. *Plant Cell Rep.* 27, 1177–1184. doi: 10.1007/s00299-008-0535-8

Weigel, D., and Nordborg, M. (2015). Population genomics for understanding adaptation in wild plant species. *Annu. Rev. Genet.* 49, 315–338. doi: 10.1146/annurev-genet-120213-092110

Xiao, Y., Zhang, S., Liu, Y., Chen, Y., Zhai, R., Yang, C., et al. (2022). Efficient Agrobacterium-mediated genetic transformation using cotyledons, hypocotyls and roots of 'Duli' (Pyrus betulifolia Bunge). *Scientia Hortic.* 296, 110906. doi: 10.1016/j.scienta.2022.110906

Xie, K., Minkenberg, B., and Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3570–3575. doi: 10.1073/pnas.1420294112

Xu, S., Brockmoller, T., Navarro-Quezada, A., Kuhl, H., Gase, K., Ling, Z., et al. (2017). Wild tobacco genomes reveal the evolution of nicotine biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 114, 6133–6138. doi: 10.1073/pnas.1700073114

Ye, M., Peng, Z., Tang, D., Yang, Z., Li, D., Xu, Y., et al. (2018). Generation of self-compatible diploid potato by knockout of S-RNase. *Nat. Plants* 4, 651–654. doi: 10.1038/s41477-018-0218-6

Yuan, X., Yan, C., Wu, Z., Ren, F., Zhang, H., Baker, B., et al. (2015). Frequent gain and loss of resistance against tobacco mosaic virus in nicotiana species. *Mol. Plant* 8, 1813–1815. doi: 10.1016/j.molp.2015.09.001

Zhang, Y. F., Chen, S. T., Gao, Y., Yang, L., and Yu, H. (2023). Prediction of global potential suitable habitats of Nicotiana alata Link et Otto based on MaxEnt model. *Sci. Rep.* 13, 4851. doi: 10.1038/s41598-023-29678-7

Zheng, Y., Chen, Y., Liu, Z., Wu, H., Jiao, F., Xin, H., et al. (2021). Important roles of key genes and transcription factors in flower color differences of Nicotiana alata. *Genes* 12, 1976. doi: 10.3390/genes12121976

Zhu, H., Li, C., and Gao, C. (2020). Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nat. Rev. Mol. Cell Biol.* 21, 661–677. doi: 10.1038/s41580-020-00288-9

frontiers | Frontiers in Plant Science

TYPE Opinion
PUBLISHED 13 March 2024
DOI 10.3389/fpls.2024.1385037

OPEN ACCESS

EDITED BY
Cheng Yuan,
Yunnan Academy of Tobacco Agricultural Sciences, China

REVIEWED BY
Tong Zhang,
South China Agricultural University, China

*CORRESPONDENCE
SV Ramesh
ramesh.sv@icar.gov.in

RECEIVED 11 February 2024
ACCEPTED 28 February 2024
PUBLISHED 13 March 2024

CITATION
Ramesh SV, Rajesh MK, Das A and Hebbar KB (2024) CRISPR/Cas9 –based genome editing to expedite the genetic improvement of palms: challenges and prospects.
Front. Plant Sci. 15:1385037.
doi: 10.3389/fpls.2024.1385037

CRISPR/Cas9 –based genome editing to expedite the genetic improvement of palms: challenges and prospects

SV Ramesh[1]*, MK Rajesh[2], Alpana Das[3] and KB Hebbar[1]

[1]ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala, India, [2]ICAR-Central Plantation Crops Research Institute Regional Station, Vittal, Dakshina Kannada, Karnataka, India, [3]ICAR-Central Plantation Crops Research Institute, Research Centre, Kahikuchi, Assam, India

KEYWORDS

genomics assisted breeding, genome editing, palm improvement, palms, tree crops

1 Introduction

Palms encompass over 2,500 species, across 200 genera, ranking second only to grasses (Poaceae) and legumes (Fabaceae) in the realm of agricultural food production and industrial applications. The coconut (*Cocos nucifera* L.), arecanut (*Areca catechu* L.), oil palm (*Elaeis guineensis* Jacq.), and date palm (*Phoenix dactylifera* L.) are among the economically significant perennial species within the Arecaceae family. Coconut, often referred to as the "tree of life," is celebrated for its diverse range of applications in food, nutrition, medicine, and various industrial uses (Ramesh et al., 2021). Coconut products encompass edible oil derived from the kernel or testa, tender coconut water, kernel, copra, coconut shell, coconut cake, wood-based products, coir pith, and items resulting from various valorization processes. The unopened spathe is tapped to extract inflorescence sap (*neera*), which can be further processed into jaggery, sugar, vinegar, and a variety of secondary products (Hebbar et al., 2022).

Arecanut (*Areca catechu* L.) is a crop in tropical Asia and certain parts of East Africa. In India, it holds a prominent place as a major commercial crop and is also medically important, primarily grown in a few states of the country. Nevertheless, its commercial products are distributed throughout India, and the country undeniably leads in terms of both area under cultivation and production, accounting for 54% of the world's output. The fruit or nuts of the *Areca catechu* L. palm, commonly known as betel nut or *supari*, have a long history of use as a masticatory product by the Indian population, dating back to the Vedic period. As a result, arecanut is deeply intertwined with India's history and social heritage. On a global scale, the betel quid is used by as many as 600 million people in Asia alone.

Date palm, on the other hand, thrives in arid regions such as Egypt, Iran, Saudi Arabia, and the UAE, among others (Aljohi et al., 2016). In addition to its fruit, date palm seeds also serve as a novel source of edible oil, further expanding its industrial applications (Ali et al., 2015). Oil palm stands out as an economically vital palm species, supplying approximately 35% of the world's vegetable oil. The genetic improvement of oil palm could play a pivotal role in global nutritional security.

2 Palm problems

Economically important palm species worldwide are experiencing stagnation in yield, a rapid deceleration in land expansion, the effects of climate change, a surge in the cost of production due to agricultural inputs and labor, as well as biotic stressors like the emergence or reemergence of major pests and diseases, among other challenges (Figure 1). The long juvenile phase of palms, which can extend beyond 5-9 years, the time-consuming process of backcross breeding (30 years for date palms and 15-18 years for oil palms), and the reliance on seeds, as the primary propagule, severely hinder palm crop improvement programs. Therefore, expediting the adoption of novel breeding technologies, such as genome editing (GE), is imperative to ensure that missed opportunities in genomics-assisted breeding and genetic engineering, which have already revolutionized other cereal and legume crops, are not lost.

Genome editing (GE) approaches have been transforming the fields of plant breeding and genomics by enabling precise genetic manipulation of crop species. The utilization of CRISPR/Cas9 holds significant promise in revolutionizing agriculture and creating opportunities for innovative developments in plant gene editing systems (Chinnusamy et al., 2023; Saini et al., 2023). While the effectiveness of GE techniques has been demonstrated in other field crops and even in tree crops, it remains a relatively uncharted path for genetic improvement in palm species such as coconut, arecanut, or date palm, with the exception of oil palm. The utilization of genetic engineering technologies for commercial purposes in palms is currently quite limited. In addition to the complexity of palm genomes, significant obstacles related to genetic transformation and the development of efficient regeneration protocols pose major challenges to the widespread adoption of GE technologies.

3 Palm genomic resources

Continuous improvement of genomic resources for palm species is of paramount importance. Over the past decade, there has been a significant increase in the scale of genome assemblies for palms, opening the door to multi-omics studies. Genome assemblies are now available for economically important palm species such as oil palm (*Elaeis guineensis* Jacq), date palm (*Phoenix dactylifera* L), coconut (*Cocos nucifera* L), and arecanut (*Areca catechu* L), as well as related species like *Calamus simplicifolius* and *Daemonorops jenkinsiana*. These assemblies have been complemented by numerous resequencing and transcriptomics approaches (Al-Mssallem et al., 2013; Singh et al., 2013; Wang et al., 2021; Yang et al., 2021; Zhao et al., 2018). In this context, the development of the Arecaceae Multi-omics Database, ArecaceaeMDB (http://arecaceae-gdb.com), is a significant step forward. This database houses genomes of six economically important palms, along with resequencing data from 1631 different accessions, over 800 transcriptome sequences, and 138 metabolome datasets (Yang et al., 2023). However, unlike fruit and nut crops that benefit from well-assembled and high-quality genome sequences (Savadi et al., 2021), palms, such as date palm, present unique challenges due to their dioecious nature, requiring specialized assembly software and additional considerations for the

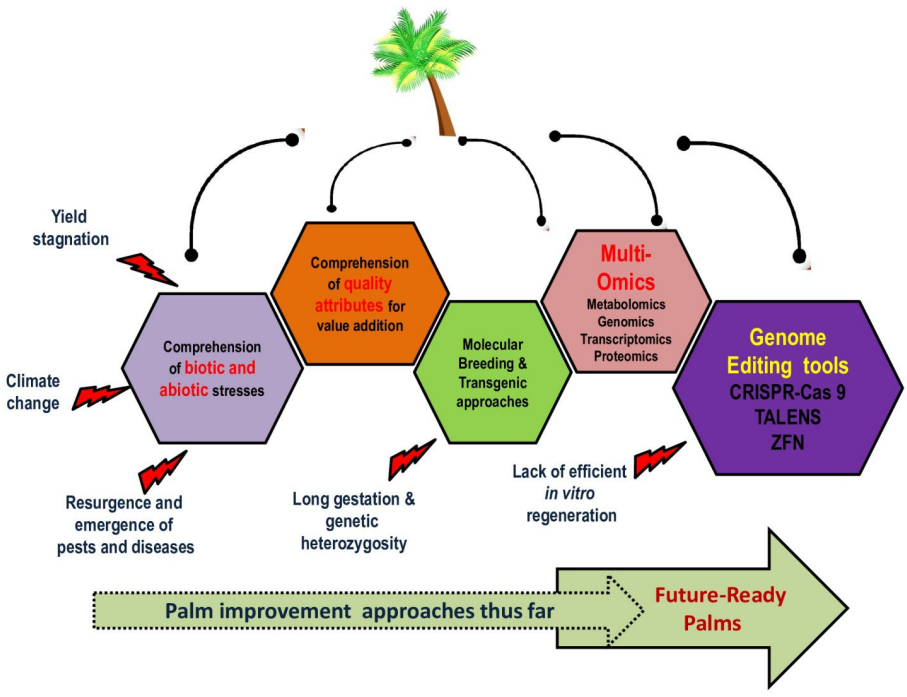


FIGURE 1
General considerations of research in palms and the defining role of genome editing in developing future-ready palms.

development of effective genomic resources (Hazzouri et al., 2020). Nonetheless, the successful demonstration of the proof-of-concept of CRISPR/Cas9 application in various fruit tree crops, including apple, cacao, coffee, citrus, grape, pomegranate, pear, and walnut (Savadi et al., 2021), as well as in some forest tree crops (Cao et al., 2022) and in palms (Yeap et al., 2021), suggests that this approach may become a mainstay in the long run.

4 Potential applications of CRISPR-Cas9 system in palms

In the context of coconut, the identification of disease susceptibility factors represents a crucial area of research. This is essential to identify genomic regions or genes responsible for disease susceptibility, with the aim of manipulating them through the adoption of gene-editing technologies. Some of the potential gene targets include PR1, PR4, the pathogenesis-related genes transcriptional activator PT15-like gene, thaumatin-like protein, HSP70, and glutathione S-transferase. These genes have been identified as susceptibility factors in the case of root (wilt) disease in coconut (Rajesh et al., 2015; Verma et al., 2017; Arumugam and Hatta, 2022). Considering the diversity of pests affecting palms, adoption of multi-pronged strategies such as CRISPR-Cas9 based sterile insect technique, and targeting female insect reproductive fitness (eg., egg-specific protein encoding gene), or incorporating ovary targeting molecular signals in CRISPR-Cas9 system for heritable genome editing are warranted. These strategies aim to enhance the resilience of palm crops against insect pest damage.

In certain plants characterized by a low transformation efficiency, such as maize, the issue of limited transformation success has been successfully addressed through the over-expression of key regulators of somatic embryogenesis, such as Baby Boom (*Bbm*) and Wuschel2 (*Wus2*). While this particular approach has not yet been explored in the context of coconut, there is potential value in enhancing the regeneration process by adopting this method.

Palm products, especially those derived from the minimal processing of tender coconut nuts, require suitable anti-browning agents to prevent enzymatic browning reactions, ensuring visually appealing end products. In addition to the manipulation of agronomic traits, the application of the CRISPR/Cas9 system has been explored to mitigate enzymatic browning. This is achieved by creating mutations in the *StPPO2* gene, resulting in a significant reduction in enzymatic browning by up to 75% (González et al., 2020). Similarly, Maioli et al. (2020) discussed the potential application of genome editing technologies in the development of eggplant berries, which exhibited a 52% reduction in PPO activity in edited lines compared to wild types, achieved by knocking out three PPO genes (*SmelPPO4*, *SmelPPO5*, and *SmelPPO6*). These studies demonstrate that, in addition to reducing enzymatic browning, the nutritional potential of post-harvest produce, including their antioxidant potential and phenolic content, is effectively preserved.

Palms in field conditions face a range of abiotic stresses induced by climate change, including monsoon variability, elevated temperature stress, rising atmospheric CO_2 levels, and sea-level rise leading to salinity stress. Unlike annual crops, palms endure these abiotic challenges for an extended period during their lifespan, necessitating the adoption of genomics technologies to mitigate these stresses. Genetic mapping of genes associated with abiotic stress tolerance or related traits in mature palms is hindered due to their long juvenile phase and the costs involved in cultivating and maintaining large mapping populations in the field. Additionally, regions where palms are grown are experiencing increased soil salinity and sea-level rise, making it imperative to molecularly characterize abiotic stress response pathways.

Consequently, the adoption of CRISPR/Cas9 editing in palms involves numerous strategic considerations, including the selection of target genes, the sequence features of sgRNA, the method and tissue used for delivery, and the implementation of appropriate *in vitro* regeneration protocols. The highly heterozygous nature of palm genomes presents a challenge in designing sgRNAs that effectively match the target gene sequences near a protospacer adjacent motif (PAM) site. Traditional breeding methods, which can take 15-20 years or more, coupled with obstacles in developing genetic engineering-based high oleic acid-producing oil palm lines, have prompted the use of a multiplex CRISPR/Cas9 system to target multiple genomic sites (Bahariah et al., 2023). Given the nutritional significance of dietary oils, the application of CRISPR/Cas9 technology for targeting oil palm genes such as fatty acid desaturase 2 (*FAD2*) and palmitoyl-acyl carrier protein thioesterase (PAT) to modulate fatty acid metabolism and produce high-oleic acid oil holds significant promise for similar interventions in coconut palms (Bahariah et al., 2023).

Tree crops necessitate rapid genetic modification protocols and the development of genetically modified plants within a few generations as expediently as possible. In this context, the biallelic edits achievable through the CRISPR/Cas9 approach offer a means to swiftly generate genetically homozygous lines, circumventing the need for elaborate breeding methods to introduce homozygosity (Hazzouri et al., 2020). The successful establishment of electroporation-mediated protoplast transformation of sgRNA and genome editing components in oil palm suggests the potential for generating DNA-free genome-edited palms. However, the lack of an effective regeneration protocol for oil palm protoplasts significantly hampers the development of DNA-free genome-edited palms. Moreover, the creation of an efficient *in vitro* electro-transfection assay in oil palm for rapid assessment and evaluation of gRNA efficiency would substantially reduce the time and cost of transformation and regeneration, particularly for oil palm and other palm species (Yeap et al., 2021). In addition, development of gene editing protocol through *de novo* induction of meristems in dicots and the efficiency of cut–dip–budding (CDB) delivery system could enable rapid production of genetically modified germplasm (Maher et al., 2020; Cao et al., 2023). By implementing the PEG-mediated delivery system for Cas proteins and sgRNA, coupled with the establishment of a proficient protoplast regeneration system for palms, as

successfully demonstrated in *Hevea brasiliensis* (Fan et al., 2020), we can significantly expedite the genome-editing-mediated breeding process.

Palm genomes are characterized by high allelic heterozygosity due to their outcrossing nature. Consequently, the presence of a high number of single nucleotide polymorphisms (SNPs) in the genome makes GE technologies less efficient in these crops. Thus, resequencing cultivars of interest and incorporating features related to the multi-ploidy nature of palm genomes into web-based algorithms, for sgRNA design in plants, are recommended to enhance the existing genomic resources for homozygous crops and model plants (Sattar et al., 2017). Nevertheless, it is anticipated that the application of CRISPR/Cas9-based genome editing will greatly facilitate the exploration of gene-function relationships and their impact on phenotypic traits, expediting crop improvement programs in palm species (Table 1).

5 Concluding remarks

Thus far, the CRISPR/Cas9 technology has demonstrated its effectiveness in genome editing of trees. Genome modification has been successfully accomplished in oil palm, and numerous fruit and nut tree crops. Several genes within these tree or perennials have manipulated through genome editing, aimed at enhancing resistance to both biotic and abiotic stressors, manipulating flowering and fruit ripening times, improving plant growth attributes, and enhancing the flavor profiles of their fruits.

TABLE 1 Major achievements in genome editing (GE) for tree crops and the prospects for its application in palms.

Sl. No	Species	Target gene(s)	Strategy	Features	Reference
1	Cocoa	*TcNPR3* (Non-Expressor of Pathogenesis-Related 3)	CRISPR/ Cas9	Knock down of defense suppressor TcNPR3 resulted in enhanced resistance to pathogen *Phytophthora tropicalis*	Fister et al., 2018
2	*Elaeis guinensis*	*EgPDS* (phytoene desaturase) and *EgBRI1* (brassinosteroid-insensitive 1)	CRISPR/ Cas9	Electroporation based transformation of protoplast showed 62.5-83.33% and 58.82-100% mutation frequency	Yeap et al., 2021
3	Sweet orange	*CsPDS* (phytoene desaturase)	CRISPR/ Cas9 sgRNA	Agrofiltration utilizing Xcc targeting carotenoids biosynthesis caused 3.2-3.9% mutation but no albinos	Jia and Wang, 2014
4	Apple	Reporter gene *uidA*	ZFN (Zinc finger nuclease)	Stable and heritable gene mutation	Peer et al., 2015
5	*Populus tomentosa*	*PtoPDS* (phytoene desaturase)	CRISPR/ Cas9	Mutation efficiency of 51.7% and albino phenotypes	Fan et al., 2015
6	*Hevea brasiliensis*	flowering time related genes (*HbFT1, HbFT2 and HbTFL1–1, HbTFL1–2, HbTFL1–3)*	CRISPR/ Cas9	Used endogenous U6 promoters in protoplasts	Dai et al., 2021
7		*HbPDS*(phytoene desaturase)	CRISPR/ Cas9	Stable transformation	Dai et al., 2021
8	*Eucalyptus grandis*	*CCR1* (cinnamoyl-CoA Reductase1), *IAA9A* (an auxin-dependent transcription factor of Aux/ IAA family)	CRISPR/ Cas9	Wood-related genes edited in *Eucalyptus* hairy root	Dai et al., 2020
9	*Elaeis guinensis*	*EgPDS* (phytoene desaturase)	CRISPR/ Cas9	Albino phenotypes with a mutation efficiency of 62.5–83.33%.	Yeap et al., 2021
		*EgBRI*1 (brassinosteroid-insensitive 1)	CRISPR/ Cas9	premature necrosis and stunted phenotype	Yeap et al., 2021
		EgWRKY, DREB1, EgRBP42, EgEREBP and EgNAC	Base editing	Abiotic stress tolerance	Yarra et al., 2020
10	*Phoenix dactylifera*	*Pdpcs* and *Pdmt*	–	Abiotic stress tolerance (Cd and Cr) resistance	Chaâbene et al., 2018
		Pdpcs and *Pdmt*	–	Abiotic stress tolerance (heavy metals)	Chaâbene et al., 2017
11	*Cocos nucifera*	**PTI5* *PR1, PR4, *pathogenesis-related genes transcriptional activator PT15-like gene, *thaumatin-like protein, HSP70, *glutathione S-transferase	–	Root (wilt) disease resistance	Verma et al., 2017

(Continued)

TABLE 1 Continued

Sl. No	Species	Target gene(s)	Strategy	Features	Reference
		NBS-LRR type resistant gene analogues	–	Root (wilt) disease resistance	Rajesh et al., 2015
12	*Elaeis guineensis*	Genes of agronomic importance (disease resistance, abiotic stress tolerance, dwarfness)	–	Ganoderma disease, abiotic stress tolerance, ease of harvesting traits such as dwarf, long stalk	Yeap et al., 2021
13	*Phoenix dactylifera*	Genetic markers for sex determination	–	Early determination of sex distinguishes dioecious palms	Sattar et al., 2017
		Resistance genes in SNP desert of date palm	–	Abiotic and biotic stress tolerance	Sattar et al., 2017

Innovations have led to the development of modified enzymes, offering increased efficiency in genome editing. Additionally, new and improved systems for gene editing and simultaneous activation of transcription have emerged, which are pertinent to the creation of novel palm varieties with wider applications.

Author contributions

SR: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. MR: Formal analysis, Writing – review & editing. KH: Formal analysis, Resources, Writing – review & editing. AD: Formal analysis, Funding acquisition, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Authors acknowledge the funding for this work from ICAR-Central Plantation Crops Research Institute (ICAR-CPCRI Grant No: 1000766014) and ICAR-NEH fund of ICAR-CPCRI, Kahikuchi.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Ali, M. A., Al-Hattab, T. A., and Al-Hydary, I. A. (2015). Extraction of date palm seed oil (Phoenix dactylifera) by soxhlet apparatus. *Int. J. Adv. Eng. Technol.* 8 (3), 261–271.

Aljohi, H. A., Liu, W., Lin, Q., Zhao, Y., Zeng, J., Alamer, A., et al. (2016). Complete sequence and analysis of coconut palm (Cocos nucifera) mitochondrial genome. *PloS One* 11 (10), e0163990. doi: 10.1371/journal.pone.0163990

Al-Mssallem, I. S., Hu, S., Zhang, X., Lin, Q., Liu, W., Tan, J., et al. (2013). Genome sequence of the date palm phoenix dactylifera l. *Nat. Commun.* 4 (1), 2274. doi: 10.1038/ncomms3274

Arumugam, T., and Hatta, M. A. D. (2022). Improving coconut using modern breeding technologies: Challenges and Opportunities. *Plants* 11, 3414. doi: 10.3390/plants11243414

Bahariah, B., Masani, M. Y. A., Fizree, M.P.M.A.A., Rasid, O. A., and Parveez, G. K. A. (2023). Multiplex CRISPR/Cas9 gene-editing platform in oil palm targeting mutations in EgFAD2 and EgPAT genes. *J. Genet. Eng. Biotechnol.* 21, 3. doi: 10.1186/s43141-022-00459-5

Cao, X., Xie, H., Song, M., Lu, J., Ma, P., Huang, B., et al. (2023). Cut–dip–budding delivery system enables genetic modifications in plants without tissue culture. *Innovation* 4 (1), 100345. doi: 10.1016/j.xinn.2022.100345

Cao, X. H., Vu, G. T. H., and Gailing, O. (2022). From genome sequencing to CRISPR-based genome editing for climate-resilient forest. *Int. J. Mol. Sci.* 23, 966. doi: 10.3390/ijms23020966

Chaâbene, Z., Hakim, I. R., Rorat, A., Elleuch, A., Mejdoub, H., and Vandenbulcke, F. (2018). Copper toxicity and date palm (Phoenix dactylifera) seedling tolerance: monitoring of related biomarkers. *Environ. Toxicol. Chem.* 37 (3), 797–806.

Chaâbene, Z., Imen, R. H., Walid, K., Grubb, C. D., Bassem, K., Franck, V., et al. (2017). The phytochelatin synthase gene in date palm (Phoenix dactylifera l.): Phylogeny, evolution and expression. *Ecotoxicol. Environ. Saf.* 140, 7–17.

Chinnusamy, V., Schepler-Luu, V., Mangrauthia, S. K., and Ramesh, S. V. (2023). Genome editing in plants: a tool for precision breeding and functional genomics. *J. Plant Biochem. Biotechnol.* 32, 657–660. doi: 10.1007/s13562-023-00867-9

Dai, Y., Hu, G., Dupas, A., Medina, L., Blandels, N., San Clemente, H., et al. (2020). Implementing the CRISPR/cas9 technology in *eucalyptus* hairy roots using wood-related genes. *Int. J. Mol. Sci.* 21, 3408. doi: 10.3390/ijms21103408

Dai, X., Yang, X., Wang, C., Fan, Y., Xin, S., Hua, Y., et al. (2021). CRISPR/Cas9-mediated genome editing in hevea brasiliensis. *Ind. Crops And Products* 164, 113418. doi: 10.1016/j.indcrop.2021.113418

Fan, D., Liu, T., Li, C., Jiao, B., Li, S., Hou, Y., et al. (2015). Efficient CRISPR/Cas9-mediated targeted mutagenesis in Populus in the first generation. *Sci. Rep.* 5, 12217. doi: 10.1038/srep12217

Fan, Y., Xin, S., Dai, X., Yang, X., Huang, H., and Hua, Y. (2020). Efficient genome editing of rubber tree (*Hevea brasiliensis*) protoplasts using CRISPR/Cas9 ribonucleoproteins. *Ind. Crop Prod.* 146, 112146. doi: 10.1016/j.indcrop.2020.112146

Fister, A. S., Landherr, L., Maximova, S. N., and Guiltinan, M. J. (2018). Transient expression of CRISPR/Cas9 machinery targeting TcNPR3 enhances defense response in *Theobroma cacao*. *Front. Plant Sci.* 2;9. doi: 10.3389/fpls.2018.00268

González, M. N., Massa, G. A., Andersson, M., Turesson, H., Olsson, N., Fält, A. S., et al. (2020). Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 System. *Front. Plant Sci.* 10. doi: 10.3389/fpls.2019.01649

Hazzouri, K. M., Flowers, J. M., Nelson, D., Lemansour, A., Masmoudi, K., and Amiri, K. M. A. (2020). Prospects for the study and improvement of abiotic stress tolerance in date palms in the post-genomics era. *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.00293

Hebbar, K. B., Ramesh, S. V., Ghosh, D. K., Beegum, S., Pandiselvam, R., Manikantan, M. R., et al. (2022). Coconut sugar-a potential storehouse of nutritive metabolites, novel bio-products and prospects. *Sugar Tech* 24 (3), 841–856.

Jia, H., and Wang, N. (2014). Xcc-facilitated agroinfiltration of citrus leaves: A tool for rapid functional analysis of transgenes in citrus leaves. *Plant Cell Rep.* 33, 1993–2001. doi: 10.1007/s00299-014-1673-9

Maher, M. F., Nasti, R. A., Vollbrecht, M., Starker, C. G., Clark, M. D., and Voytas, D. F. (2020). Plant gene editing through *de novo* induction of meristems. *Nat. Biotechnol.* 38, 84–89. doi: 10.1038/s41587-019-0337-2

Maioli, A., Gianoglio, S., Moglia, A., Acquadro, A., Valentino, D., Milani, A. M., et al. (2020). Simultaneous CRISPR/Cas9 editing of three PPO genes reduces fruit flesh browning in *Solanum melongena* L. *Front. Plant Sci.* 11. doi: 10.3389/fpls.2020.607161

Peer, R., Rivlin, G., Golobovitch, G., Lapidot, M., Gal-On, A., Vainstein, A., et al. (2015). Targeted mutagenesis using zinc-finger nucleases in perennial fruit trees. *Planta* 241, 941–951. doi: 10.1007/s00425-014-2224-x

Rajesh, M. K., Rachana, K. E., Naganeeswaran, S. A., Shafeeq, R., Thomas, R. J., Shareefa, M., et al. (2015). Identification of expressed resistance gene analog sequences in coconut leaf transcriptome and their evolutionary analysis. *Turkish J. Agric. Forestry* 39 (3), 489–502.

Ramesh, S. V., Pandiselvam, R., Shameena Beegum, P. P., Saravana Kumar, R. M., Manikantan, M. R., and Hebbar, K. B. (2021). Review of *Cocos nucifera* L. testa- derived phytonutrients with special reference to phenolics and its potential for encapsulation. *J. Food Sci. Technol.* 60, 1–10. doi: 10.1007/s13197-021-05310-2

Saini, H., Thakur, R., Gill, R., Tyagi, K., and Goswami, M. (2023). CRISPR/Cas9-gene editing approaches in plant breeding. *GM Crops Food* 14, pp.1–pp17. doi: 10.1080/21645698.2023.2256930

Sattar, M. N., Iqbal, Z., Tahir, M. N., Shahid, M. S., Khurshid, M., Al-Khateeb, A. A., et al. (2017). CRISPR/Cas9: a practical approach in date palm genome editing. *Front. Plant Sci.* 8. doi: 10.3389/fpls.2017.01469

Savadi, S., Mangalassery, S., and Sandesh, M. S. (2021). Advances in genomics and genome editing for breeding next generation of fruit and nut crops. *Genomics* 113, 3718–3734. doi: 10.1016/j.ygeno.2021.09.001

Singh, R., Ong-Abdullah, M., Low, E. T. L., Manaf, M. A. A., Rosli, R., Nookiah, R., et al. (2013). Oil palm genome sequence reveals divergence of interfertile species in old and new worlds. *Nature* 500 (7462), 335–339.

Verma, S. K., Jasrotia, R. S., Iquebal, M., Jaiswal, S., Angadi, U., Rai, A., et al. (2017). Deciphering genes associated with root wilt disease of coconut and development of its transcriptomic database (CnTDB). *Physiol. Mol. Plant Pathol.* 100, 255–263. doi: 10.1016/j.pmpp.2017.03.011

Wang, S., Xiao, Y., Zhou, Z. W., Yuan, J., Guo, H., Yang, Z., et al. (2021). High-quality reference genome sequences of two coconut cultivars provide insights into evolution of monocot chromosomes and differentiation of fiber content and plant height. *Genome Biol.* 22, 304. doi: 10.1186/s13059-021-02522-9

Yang, Z., Liu, Z., Xu, H., Li, Y., Huang, S., Cao, G., et al. (2023). ArecaceaeMDB: a comprehensive multi-omics database for Arecaceae breeding and functional genomics studies. *Plant Biotechnol. J.* 21, 11. doi: 10.1111/pbi.13945

Yang, Y., Huang, L., Xu, C., Qi, L., Wu, Z., Li, J., et al. (2021). Chromosome-scale genome assembly of areca palm (Areca catechu). *Mol. Ecol. Resour.* 21 (7), 2504–2519.

Yarra, R., Cao, H., Jin, L., Mengdi, Y., and Zhou, L. (2020). CRISPR/Cas mediated base editing: A practical approach for genome editing in oil palm. *3 Biotech.* 10, 306. doi: 10.1007/s13205-020-02302-5

Yeap, W.-C., Norkhairunnisa, K. C. M., Norfadzilah, J., Muad, M. R., Appleton, D., and Harikrishna, K. (2021). An efficient clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 mutagenesis system for oil palm (*Elaeis guineensis*). *Front. Plant Sci.* 12. doi: 10.3389/fpls.2021.773656

Zhao, Z., Yu, S., Li, M., Gui, X., and Li, P. (2018). Isolation of exosome-like nanoparticles and analysis of microRNAs derived from coconut water based on small RNA high-throughput sequencing. *J. Agri Food Chem.* 66 (11), 2749–2757.

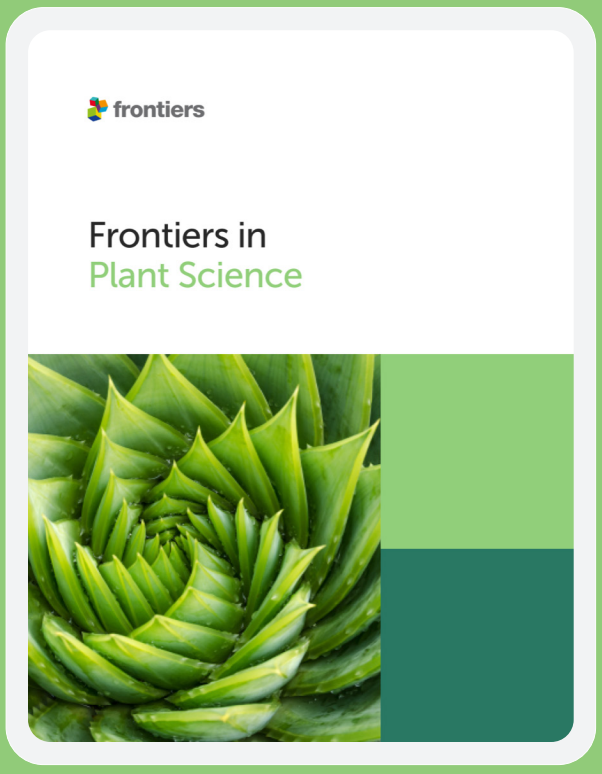
Frontiers in Plant Science

Cultivates the science of plant biology and its applications

The most cited plant science journal, which advances our understanding of plant biology for sustainable food security, functional ecosystems and human health.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact