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Establishment of a Novel CRISPR/Cas9-Based Multiplex Editing System in the Citrus Postharvest Pathogen *Penicillium digitatum*

Jing Zhang, Yujiao Liu, Wenqian Cao, Ruoxin Ruan, and Mingshuang Wang*

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ABSTRACT: *Penicillium digitatum*, the causal fungus of the citrus green mold, leads to substantial postharvest losses in the citrus industry. In this study, we engineered a versatile CRISPR/Cas9-mediated gene editing platform capable of generating multiple sgRNAs from a single polycistronic transcript. By incorporating endogenous tRNAs and the strong promoter PdMLE1 into the CRISPR/Cas9 system, the efficiency of single gene editing can reach up to 94.2%. By taking advantage of the efficient shearing and processing capabilities of tRNA, the platform enabled multiplex editing with efficiencies of 44.4% for two-gene and 33.3% for three-gene modifications, respectively. After two rounds of three-gene editing, we were able to successfully obtain hextuple-gene mutants. Finally, functional characterization revealed that the target polysaccharide-lyase-encoding genes play limited roles in pathogenicity in *P. digitatum*. Taken together, our results represent a powerful tool for genome engineering in *P. digitatum*, facilitating research into its pathogenesis.

KEYWORDS: *citrus green mold, genome engineering, tRNA processing, PdMLE1, polysaccharide lyases*

1. INTRODUCTION

The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated) system plays a crucial role in defending against phage invasion in bacteria and archaea.[1] Of all the known Cas proteins, the so-called "genetic scissors" Cas endonuclease 9 (Cas9) was the most studied, and it can directly cut foreign DNA.[2,3] In the CRISPR/Cas9 system, the mature short guide CRISPR RNA (crRNA) forms a double-stranded structure with the trans-activating crRNA (tracrRNA) via base complementarity; this complex guides the Cas9 protein to induce double-strand breaks (DSBs) at target DNA adjacent to the protospacer adjacent motif (PAM). When the dual tracrRNA−crRNA was fused into a chimeric single guide RNA (sgRNA), it also directed sequence-specific DSB.[4] The Cas9-induced DSB within the target site would then be repaired by two different mechanisms: error-prone nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ can introduce small insertions or deletions within the target site, causing a dysfunctional open reading frame, while HDR can repair DSB with high fidelity but low incidence when a donor template that has homology to flanking sequences of DSB exists.[5,6] These scientific breakthroughs laid significant foundations for the development of genome editing technology by the CRISPR/Cas9 system. Since then, CRISPR/Cas9 has been successfully applied in various organisms, for example, humans, zebrafish, rice, and yeast.[7−11]

The initial use of the CRISPR/Cas9 system in filamentous fungi occurred in the industrially important *Trichoderma reesei* and seven *Aspergillus* species.[12−14] Recently, the CRISPR/Cas9-based genome manipulation has been documented in a variety of fungal species, including *Alternaria alternata*, *Ustilago maydis*, *Botrytis cinerea*, and many *Penicillium* species.[15−20] The extensive application of CRISPR/Cas9 technology in filamentous fungi paves a new avenue to accelerate studies on functional genomics and strain improvement.

One of the major advantages of CRISPR/Cas9 lies in its capacity for multiplex genome editing. Current strategies for achieving multiplex genome editing primarily involve integrating multiple sgRNA expression cassettes into a single vector. This can be accomplished through several common methods, notably by either coexpressing RNA-processing enzymes (e.g., Csy4) or by flanking individual gRNAs with self-cleaving ribozymes or tRNAs.[21] tRNAs are highly abundant in cells, and they play critical roles in cellular function; their syntheses are precisely and universally regulated across diverse organisms. Following this principle, Xie et al. first established a general strategy that harnesses the endogenous tRNA-processing system for the precise processing and efficient production of numerous gRNAs from a synthetic polycistronic gene in rice.[22] Subsequently, this strategy was broadly used to generate multiplex gene editing in various species, such as drosophila,[23] tomato,[24] corn, and yeast.[23−26]

Penicillium digitatum, the causative agent of citrus green mold, poses severe postharvest losses due to its rapid tissue maceration and aggressive pathogenicity.[27,28] Current control measures predominantly rely on chemical fungicides; however, the emergence of resistant strains has drastically compromised their efficacy.[29,30] Besides, the molecular basis of *P. digitatum*'s

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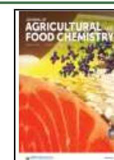

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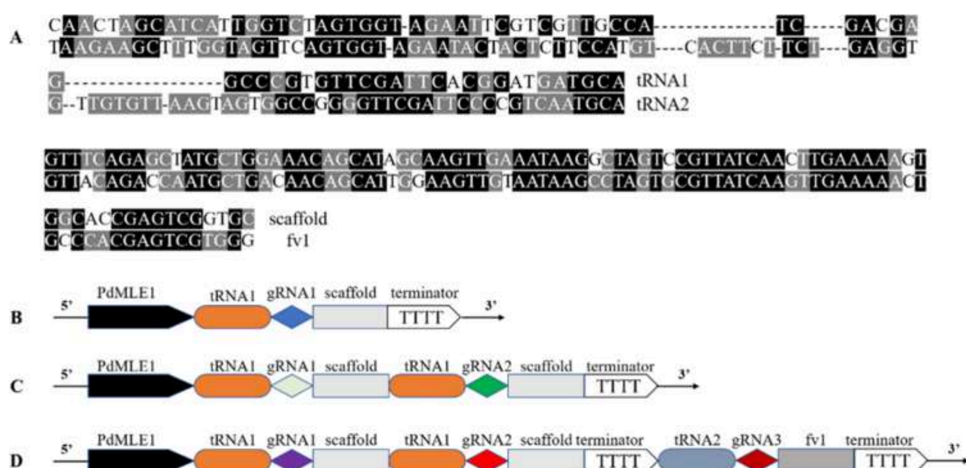

Figure 1. Strategies for Multiplex Gene Editing with CRISPR/Cas9. (A) Designed tRNA-Gly and gRNA scaffold sequences. fv1: functional gRNA scaffold variant. Schematic depiction of the method for simultaneously targeting one (B), two (C), or three gene sites (D).

pathogenicity remains poorly understood, partly due to technical challenges in genetic manipulation.[20,28,31] Genetic modification of *P. digitatum* can be traced back to the year 2008 when the *Agrobacterium*-mediated transformation (ATMT) and homologous recombination methods were utilized to achieve gene knockout mutants.[32,33] However, the efficiency rate, sometimes, is approximately 1% or even lower.[33,34] To enhance the gene targeting efficiency, Xu et al. subsequently deleted the *Ku80* gene, which is involved in ectopic DNA integration events by activating the NHEJ pathway. Although the gene disruption frequencies of test genes *PdbrlA* and *PdmpkA* were increased to 33 and 13%, respectively, using Δ*PdKu80* as a targeting strain, the process of constructing the disruption vector and transformation of *P. digitatum* spores is still very cumbersome.[35] Recently, Garrigues et al. improved the protoplasmic production methods and successfully applied the CRISPR/Cas9 technology in *P. digitatum* to edit the *WetA* gene, which mediated the color of spores, and achieved an editing efficiency of 10%.[19] Subsequently, the same group improved the gene editing efficiency to 54−83% by prolonging the culture time of protoplasts after resuscitation and increasing the number of culture generations, demonstrating the feasibility of the CRISPR/Cas9 system for gene disruption in this citrus postharvest pathogen.[20]

The existing genome editing methods in *P. digitatum*, however, still have many limitations. For example, the generation of mutants with long fragment deletion or multiple-gene disruption is hardly achieved, which hampers the in-depth study of the genetic functions of *P. digitatum*. To fill this gap, in this study, we developed a versatile CRISPR/Cas9-based gene manipulation system using a novel sgRNA construction strategy. By incorporating endogenous tRNAs and the strong promoter PdMLE1 into the CRISPR/Cas9 system,[22,31,36] the construction of vectors becomes simple, and the efficiency of gene editing can reach up to 94.2% for single-gene, 44.4% for two-gene, and 33.3% for three-gene modifications, respectively. Following two iterative rounds of triple-gene editing, we achieved the successful generation of hexa-gene knockout mutants. Our results represent a powerful tool for genome engineering in *P. digitatum*, which would remarkably contribute to research on the pathogenesis of this important postharvest pathogen.

2. MATERIALS AND METHODS

2.1. Fungal Strains and Culture Conditions

The reference *P. digitatum* strain, PDW03, was isolated from a diseased citrus fruit in Quzhou, Zhejiang Province, China. PDW03 and its derived mutants were cultured on solid potato dextrose agar (PDA) at 25 °C, and conidia were gathered following a five-day incubation period. Mycelia were grown by incubating fungal spores in liquid potato dextrose broth (PDB) on a rotary shaker at 160 rpm at 25 °C for 2 days.

2.2. Vector Construction

The powerful endogenous promoter PdMLE1 (*P. digitatum* miniature inverted-repeat transposable elements-like element), which is prevalent but unique to *P. digitatum*,[36] was utilized as an RNA polymerase III promoter to activate sgRNA expression. All *P. digitatum* glycine (Gly) tRNA sequences were retrieved from the Genomic tRNA Database,[37] and the $\text{tRNA}^{\text{Gly-GCC1-3}}$ (tRNA1) with a length of 77 bp was selected as the endogenous tRNA element for gene editing (Figure 1A). These two fragments were fused to form a PdMLE1-tRNA1 cassette (template 1, tp1) using fusion PCR. Primers used in this study are listed in Supplementary Table 1. The 20 bp protospacer sequences for the gRNA were predicted using the online CRISPOR program.[38] The summary of key design criteria is listed in Supplementary Table 2. The gRNA scaffold was first synthesized and then amplified by normal PCR.[39] To generate the PdMLE1-tRNA1-sgRNA expression cassette, the tp1 and gRNA scaffolds were fused by fusion PCR, using the 20 bp gRNA as overlap sequences (Figure 1B). To construct a cassette with 2 sgRNAs (i.e., PdMLE1-tRNA1-sgRNA1-tRNA1-sgRNA2), the gRNA scaffold and tRNA1 were first fused to form a scaffold-tRNA cassette (template 2, tp2) and then fused with tp1 and the gRNA scaffold using the 20 bp gRNAs as overlap sequences (Figure 1C). To construct a cassette with 3 sgRNAs, here we introduced a functional gRNA scaffold variant (fv1) and a different $\text{tRNA}^{\text{Gly-TCC1-1}}$(tRNA2) (Figure 1A), and combined them to form template 3 (tp3) (Figure 1D). We do this because it can reduce the difficulty of sequence fusion caused by repetitive fragments. Then, the tp1, tp2, tp3, and scaffold were fused. Finally, each cassette was inserted into the PFC332 plasmid via the ClonExpress Ultra One Step Cloning Kit (Vazyme biotech co., ltd., Nanjing, China) to generate a CRISPR/Cas9 gene editing vector (pCCv).[14] The genes used for targeting include *PdWetA* (Pdw03_g993), *PdPL1* (Pdw03_g3164), *PdPL2* (Pdw03_g5714), *PdPL3* (Pdw03_g7861), *PdPL4* (Pdw03_g744), *PdPL5* (Pdw03_g4665), and *PdPL6* (Pdw03_g6097).

2.3. Transformation

Transformation of *P. digitatum* was performed following the previously published procedures.[19,20] Transformants were selected

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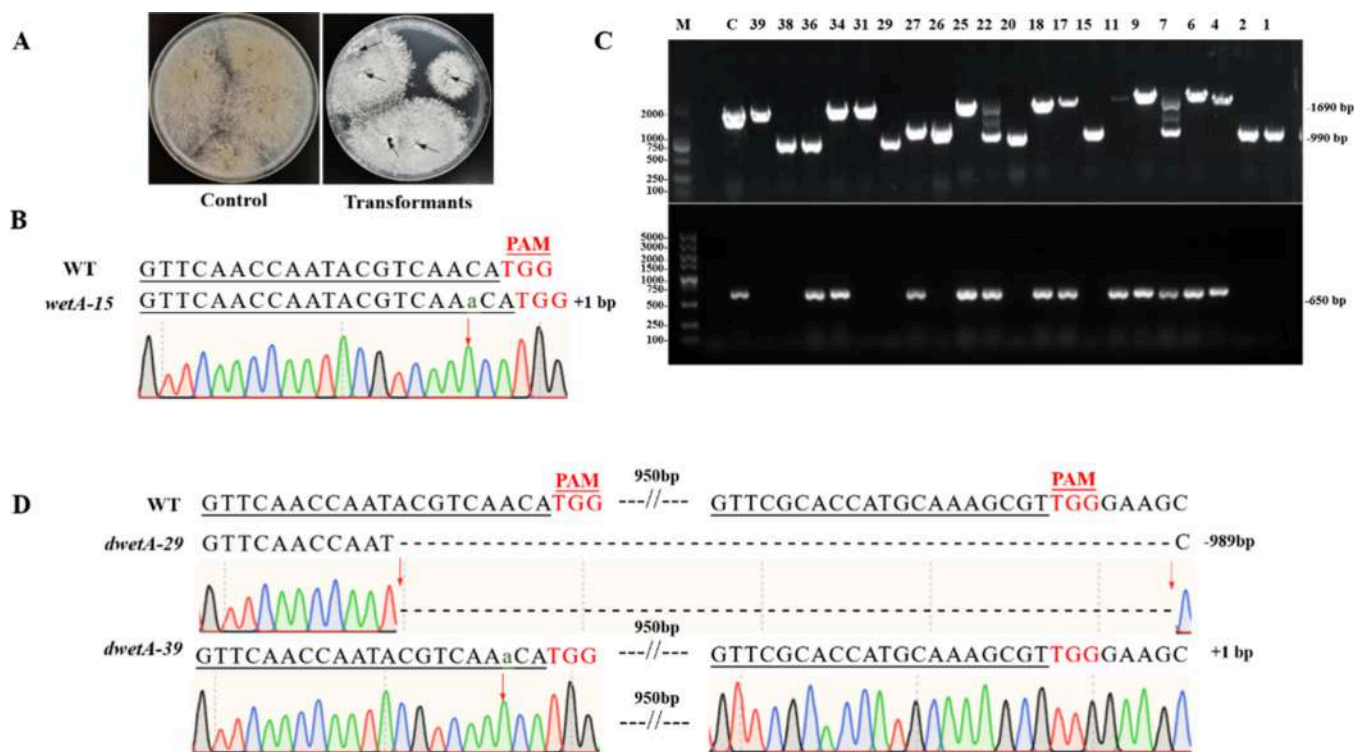

Figure 2. Application of the developed CRISPR/Cas9-mediated genome editing platform in *P. digitatum* to target the *PdwetA* gene. (A) Colony morphology of transformants generated with a single sgRNA. (B) Sequence analysis of the *PdwetA* gene in the wetA-15 mutant strain. (C) PCR screening results of randomly selected white-spore mutants using outside and inside primers. (D) Sequence analysis of the *PdwetA* gene in the dwetA-29 and dwetA-39 mutant strains.

on a PDA medium supplemented with 100 mg/L of hygromycin. DNA of each transformant was extracted, and the mutation was verified by Sanger sequencing or PCR amplification. For generating the 6-gene disruption mutant, the hygromycin gene in PFC332 was replaced with the neomycin gene, and a 3-sgRNA expression cassette was then inserted into this plasmid. The resultant CRISPR/Cas9 gene editing vector was transformed into the protoplasts of a three-gene disruption mutant and selected on PDA medium supplemented with 100 mg/L neomycin.

2.4. Mycelial Growth Tests

To illustrate the role of pectate lyases in environmental adaptation, the *P. digitatum* wild type PDW03 and mutant strains were cultured on PDA plates containing 4 μg/mL imazalil, 0.02 μg/mL fludioxonil, 0.7 M NaCl, 0.6 M $CuSO_4$, 10 mM H_2O_2, 0.03 mM menadione, or 200 μg/mL Congo red (CR). Each plate was inoculated with a 5 mm mycelial plug, and the colony diameters were measured after incubation for 4 days at 25 °C. The experiments were repeated twice.

2.5. Virulence Tests

To know whether pectate lyases play a role in fungal virulence, infection experiments were carried out on the mandarin (*Citrus reticulata* Blanco) fruit with wild-type PDW03 and gene-disruption mutants of *P. digitatum*. The citrus peel was pierced (1−2 mm deep) with sterile needles and then infected with 10 μL of fungal spores (1×10^6 conidia/mL). Infected citrus fruit were stored at 25 °C for 4 days before being measured for lesion size. The experiments were repeated twice.

3. RESULTS

3.1. Establishment of a tRNA-sgRNA-Based CRISPR/Cas9 System

We sought to design a multifaceted and easy-to-use CRISPR/Cas9 system in the citrus postharvest pathogen *P. digitatum*. To explore whether the endogenous promoter PdMLE1 and tRNAs are usable in the *P. digitatum* CRISPR/Cas9 system, we designed a sgRNA expression cassette that contains the promoter PdMLE1, tRNA, gRNA, gRNA scaffold, and Pol III terminator (Figure 1B). The *PdwetA* was chosen as the target gene due to it mediates the development of conidiophore, and its mutant causes a visible conidial albino phenotype.[40] A total of 52 transformants were obtained, and 49 (94.2%) of them showed the phenotype of an albino colony (Figure 2A). According to the sequencing results, the coding region of the *PdwetA* gene in all 49 strains had nucleotide deletions or insertions, while that of the remaining 3 strains had no change. Taking the wetA-15 transformants as an example, there was an insertion of 'A' within the 20 bp sequence complementary to the sgRNA, causing the *PdwetA* gene frameshift and a white colony of the mutant (Figure 2B). These results suggest that the endogenous promoter PdMLE1 and tRNAs enable successful and efficient gene editing in *P. digitatum*.

3.2. Targeted Gene Deletion via CRISPR/Cas9

The conventional method for gene knockout in fungal strains involves replacement of the target gene with an exogenous antibiotic selection marker (for example, hygromycin) through homologous recombination, which is characterized by the complete inactivation of the target gene.[33,41] To achieve a similar function in gene knockout by the *P. digitatum* CRISPR/Cas9 system, we constructed a polycistronic tRNA-sgRNA gene for simultaneous production of 2 sgRNAs targeting the *PdwetA* gene. A total of 78 transformants were acquired, with 73 (93.6%) exhibiting the albino colony phenotype. The PCR amplification using flanking identification primers of the *PdwetA* gene showed that the bands (∼1 kb) of 25

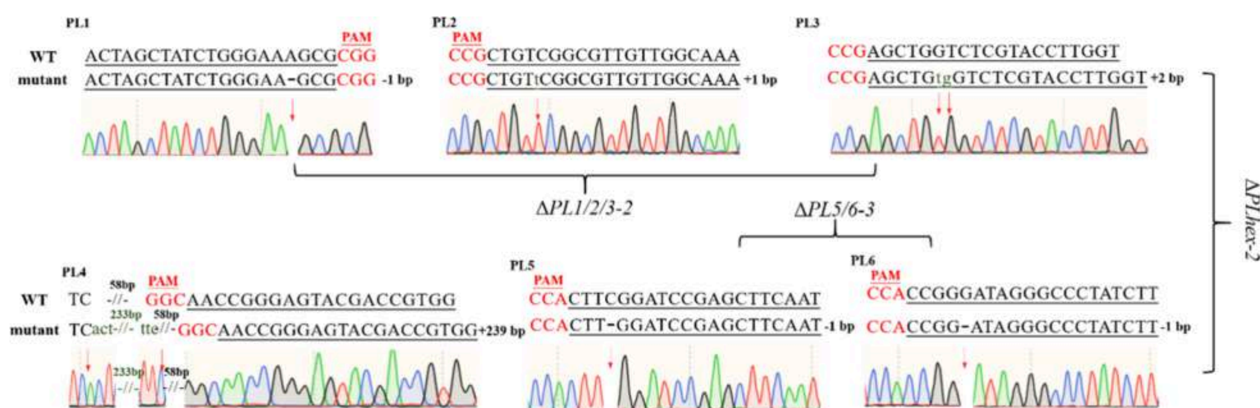

Figure 3. Sequencing results of the PL genes in the Δ*PL5/6*−3, Δ*PL1/2/3*−2, and Δ*PLhex-2* mutants. The curly brackets indicate the mutated genes contained within the mutant. The red arrows mark the locations where mutations have occurred.

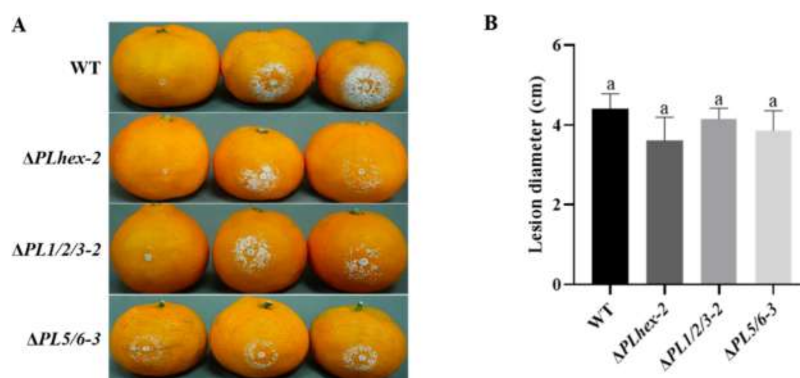

Figure 4. Infection assays of wild-type (WT) *P. digitatum* and the Δ*PL5/6*−3, Δ*PL1/2/3*−2, and Δ*PLhex-2* mutants on citrus fruit. (A) Decay symptoms of citrus fruit inoculated with the indicated strains after a 4-day incubation at 25 °C. (B) Lesion size of fruit inoculated with the wild type and gene deletion mutants. The same lowercase letters indicate no significant difference estimated by Duncan's test ($P < 0.05$).

transformants were significantly smaller than that (1.7 kb) of the wild-type PdW03, and there were no internal bands among these transformants (Figure 2C). Of the remaining 48 transformants, 31 retained wild-type band patterns, while 17 had unexpected bands (Figure 2C). To verify the results, we selected transformants dwetA-29 and dwetA-39 for Sanger sequencing. The results showed that dwetA-29 had DSBs at both sgRNA target sites, resulting in a 989 bp gene deletion mediated by the NHEJ repair, while dwetA-39 only had an "A" insertion at the gRNA1 target site, as confirmed by the PCR analysis (Figure 2C,D). These results demonstrated that our methods can result in mutants with the complete deletion of a sequence fragment between two gRNA sites.

3.3. Multiplexed Gene Disruption with CRISPR/Cas9

We then wondered if our CRISPR/Cas9 system was able to simultaneously edit 2 or more genes. In the following experiments, we chose polysaccharide lyases (PLs) encoding genes to be targeted as they are a class of carbohydrate-active enzymes that degrade uronic acid-containing polysaccharides and might be involved in virulence and nutrient acquisition in some pathogenic fungi.[42,43] We identified 6 PLs in the *P. digitatum* genome comprising 3 pectin lyases (*PdPL1*, *PdPL2*, and *PdPL3*), 1 pectate lyase (*PdPL4*), and two rhamnogalacturonan lyases (*PdPL5* and *PdPL6*). First, a polycistronic tRNA-sgRNA gene-containing vector for the simultaneous production of two sgRNAs targeting two rhamnogalacturonan lyase-encoding genes was constructed (Figure 1C). After protoplast transformation, we picked up a total of nine transformants. Sanger sequencing revealed that five transformants had mutations only in the coding sequences of *PdPL5*, while four had mutations in both genes. Taking the Δ*PL5/6*−3 double mutant as an example, it had a "C" and "G" deletion at the gRNA sites of the *PdPL5* and *PdPL6* genes, respectively (Figure 3).

Next, we aim to conduct a test on the efficiency of simultaneous three-gene editing. The occurrence of three repeated tRNA-scaffold structures will lead to the failure of fusion PCR. Here, we introduced a mutated gRNA scaffold and a different $tRNA^{Gly\text{-}TCC1-1}$ (tRNA2) (Figure 1A) and constructed a triple-gene editing vector for simultaneously targeting the *PdPL1*, *PdPL2*, and *PdPL3* genes (Figure 1D). We acquired 12 transformants, and of them, four strains with simultaneous editing of all three genes were validated by Sanger sequencing, achieving an overall triple-gene editing efficiency of 33.3%. The representative mutant strain Δ*PL1/2/3*−2 had a single "A" deletion at the gRNA sites of the *PdPL1* gene, a single "T" insertion in the *PdPL2* gene, and a "TG" insertion in the *PdPL3* gene, respectively (Figure 3).

We stopped constructing a CRISPR/Cas9 vector targeting four or more genes because combining more fragments utilizing fusion PCR will be problematic, and the efficiency will be low. To generate a hextuple-gene mutant, another triple-gene editing vector targeting the additional three polysaccharide lyase genes *PdPL4*, *PdPL5*, and *PdPL6* was designed following the method illustrated in Figure 1D and was then transformed into protoplasts of the triple-gene

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mutant strain Δ*PL1/2/3*−*2*. In this round of transformation, seven transformants were obtained, and two of them showed mutations in all three PL genes, with a triple-gene editing efficiency of 28.6%. Finally, we got two hexuple-gene mutants; the representative mutant strain Δ*PLhex-2* had a 239 bp fragment insertion at the gRNA sites of the *PdPL4* gene, a single "C" deletion in the *PdPL5* gene, and a "G" deletion in the *PdPL6* gene, respectively (Figure 3).

3.4. Phenotypic Analysis of PL Gene Mutant Strains

To assess the role of PL genes in nutrition utilization, we conducted growth arrays of the wild type and PL mutants on various carbon sources. The results showed that the growth of all mutants exhibited no significant difference from that of the wild-type strain (Figure S1), indicating that the identified PL genes are not critical for the utilization of the tested carbohydrates.

To examine the involvement of PL genes in external stress tolerance, wild-type and mutant strains were cultivated on a PDA medium supplemented with different stress inducers. No obvious change in stress tolerance was detected between the mutants and wildtype (Figure S1), indicating that PL genes are not associated with the *P. digitatum* response to osmotic, oxidative, and antifungal stresses.

To explore the effect of PL genes on *P. digitatum* virulence, infection assays were carried out on citrus fruit. Compared to the wild type, the lesion diameters of mutants Δ*PLhex-2*, Δ*PL1/2/3*−*2*, and Δ*PL5/6*−*3* were reduced by 18.1, 6.0, and 12.5%, respectively (Figure 4). However, statistical analysis indicated that the differences in pathogenicity between the mutants and the wild-type strain were not significant ($P > 0.05$).

4. DISCUSSION

The recently developed CRISPR/Cas9 technique significantly expedited genome engineering in a wide spectrum of species. One of the major advantages of CRISPR/Cas9 lies in its capacity for multiplex genome editing. Current strategies for achieving multiplex genome editing primarily contain two approaches. One is integrating multiple sgRNA expression cassettes into a single vector or using multiple plasmids carrying different sgRNAs, while the other is to employ one single promoter to transcribe all sgRNAs in one single gene, taking advantage of the endogenous tRNA processing system.[22,44] As the tRNA-processing system is more robust and precise, this method has been successfully used for multiple sgRNA expression in various organisms, including animals, plants, insects, and yeast.[23−25,45] Recently, the CRISPR/Cas9-mediated multiplex editing system was successfully developed in some filamentous fungi, including *Aspergillus oryzae*, *A. niger*, and *T. reesei*.[46−48] However, despite these advancements, the successful implementation of multiplex genome editing in *Penicillium* species remains unreported. Here in this study, we established a versatile CRISPR/Cas9 genome editing system based on the endogenous tRNA-processing system, which precisely cleaves both ends of the tRNA precursor by the RNases.[22] The arrayed tRNA−sgRNA architecture driven by the PdMLE1 promoter can produce multiple sgRNAs from a synthetic gene, resulting in simultaneously targeting 1, 2, and 3 test genes with an efficiency of around 90, 40, and 30%, respectively. Notably, the efficiency of simultaneous editing of multiple genes is not particularly high. Nevertheless, a 30% success rate can still ensure the identification of genuine mutants within a relatively small number of transformants. Besides, the editing efficiency is likely affected by the guide activity of sgRNAs. For example, the mutant dwetA-29 had DSBs at both sgRNA sites, while dwetA-39 had a mutation only at the gRNA1 site with the gRNA2 target site remaining unmodified. The cleavage efficiency of individual sgRNAs is influenced by multiple factors. A shifting balance between open and closed chromatin, driven by histone acetylation and deacetylation, may lead to differential sgRNA expression across individual cells.[49] Furthermore, the thermodynamic stability differences caused by sgRNAs' GC content of different PAM-proximal nucleotides,[50] and their distinct targeting positions have a strong, positive correlation with mutagenesis efficiency.[50,51] Therefore, optimizing sgRNA design would be helpful in minimizing off-target activity.[52]

Apart from the precise cleavage of tRNA, the selection of a proper promoter will also be critical for the sgRNA expression. Generally, there are two kinds of promoters used in the development of the CRISPR/Cas9 system in filamentous fungi: RNA polymerase II (Pol II) and III (Pol III) promoters. The Pol III can transcribe sgRNAs directly. Previous studies have reported that U6 promoters of small nuclear RNA (snRNA) genes are widely used by Pol III for the transcription of sgRNAs in filamentous fungi.[39,53,54] Besides, several tRNA promoters were also reported to be useful in transcribing sgRNAs in some fungal species.[55,56] Nonetheless, owing to the intricacies of genetic backgrounds, identification of RNA polymerase III promoters suitable for sgRNA transcription is not an easy task. In fact, we sought to locate U6 promoters that drive high expression of sgRNA, but we were ultimately unsuccessful. In the case of RNA polymerase II, the sgRNA cannot be transcribed directly, as it contains no proper cap structure or poly(A) tail. So, two self-cleavage ribozymes, the hammerhead (HH) and the hepatitis delta virus (HDV), were designed to flank the sgRNA at both ends when the RNA polymerase II was utilized.[14] The most commonly used promoter in this scenario is the strong constitutive *A. nidulans* gpdA promoter, which has an extensive application in filamentous fungi.[14,17,18,57] However, this method often requires more effort in the construction of sgRNA expression cassettes and is not suitable for multiplex gene editing. The PdMLE1 is a specific miniature inverted-repeat transposable element that acts as a promoter and confers *P. digitatum* with DMI resistance.[30,36] Due to its powerful promoter activity, the PdMLE1 was introduced in the CIRSPR/Cas9 system, and our results demonstrated its high efficiency, indicating this promoter's significant potential for application in fungal gene editing.

In the current study, we only constructed CRISPR/Cas9 vectors targeting up to three genes. This is because the fusion PCR method exhibits low efficiency in ligating repetitive fragments like tRNA and gRNA scaffolds into a long synthetic gene. However, this limitation may be overcome via a protocol refinement. For example, Wang et al. first assembled sgRNA subclusters with up to eight sgRNA units with multiple tRNAs and gRNA scaffold variants and then ligated them into a gene-editing vector with up to 24 target sites employing Golden Gate cloning.[58] Li et al. developed a multigene editing system targeting up to five *A. niger* genes utilizing different tRNAs and the Golden Gate assembly, which represents the largest number of concurrently modified genes documented in filamentous fungi to date.[48] Remarkably, the gene-editing

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efficiency decreases significantly as the number of targeted genes increases. The disruption rates of one-, two-, three-, four-, and five-target genes in *A. niger* were reported to be 89.2, 70.91, 50, 22.41, and 4.17%, respectively,[48] which shows a comparable efficiency to ours for the knockout of up to three genes. To avoid the extremely low success rate of multigene knockout, we introduced a triple-gene editing vector into a triple-gene mutant strain to obtain a hexa-gene mutant, with an efficiency of 28.6%. Generally, simultaneous knockout of three genes is sufficient for most genetic engineering requirements in filamentous fungi. In cases where disrupting more genes is necessary, researchers can adopt our approach or design a multigene editing construct using previously reported methods.[22,48,58]

In CRISPR/Cas9 technology, the Cas9 endonuclease, combined with an sgRNA, introduces a DSB in a specific genomic locus. The DSB can then be repaired by either NHEJ to allow insertions or deletions within the target sequences that typically cause a dysfunctional open reading frame or HDR, which allows precise sequence deletion with appropriate donor DNA templates.[5] In this study, we developed a highly efficient multiplex gene editing platform based only on the NHEJ repair pathway. However, we never succeeded in acquiring homologous recombination-mediated DSB repair after numerous attempts. Likewise, the pioneering team in *P. digitatum* genome editing CRISPR/Cas9 also failed to obtain HDR-mediated transformants, which indicates a potentially very low homologous recombination rate in this species.[19] Nevertheless, highly efficient gene deletions were achieved via HDR through the introduction of donor DNAs in many other fungal species, including *P. chrysogenum*, *A. fumigatus*, and *A. niger*.[39,53,59] Given the dominance of NHEJ over HDR for DSB repair in filamentous fungi, the feasibility of improving the HDR-mediated mutagenesis efficiency in *P. digitatum* by inactivating the NHEJ pathway needs to be experimentally verified.

PLs are a group of enzymes that facilitate the degradation of glycosaminoglycans and pectin, utilizing elimination chemistry to cleave sugar polymers.[60] Previous studies have shown that PL genes are related to virulence in some pathogenic fungi.[42,43,61] In the present study, disruption of PL genes resulted in mutants with slightly decreased virulence (6.0–18.1% reduction). Similarly, a previous study showed that the PL2 gene deletion mutant was less virulent than the parental strain.[61] To our surprise, the deletion of the PL genes had no effect on the strain's carbon source utilization. Also, the PL gene mutants showed no obvious change in stress tolerance. These results demonstrate that while the PL genes contribute to the pathogenicity of *P. digitatum*, they do not serve as essential virulence factors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c11144.

Functional analysis of the role of the PL genes, primers used in this study, and summary of key design criteria (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Mingshuang Wang − *College of Life and Environmental Sciences, Zhejiang Provincial Modern Biology and Medicine Industry College, Hangzhou Normal University, Hangzhou, Zhejiang 311121, China;* orcid.org/0000-0001-8565-3729; Email: mswang@hznu.edu.cn

Authors

Jing Zhang − *College of Life and Environmental Sciences, Zhejiang Provincial Modern Biology and Medicine Industry College, Hangzhou Normal University, Hangzhou, Zhejiang 311121, China*

Yujiao Liu − *College of Life and Environmental Sciences, Zhejiang Provincial Modern Biology and Medicine Industry College, Hangzhou Normal University, Hangzhou, Zhejiang 311121, China*

Wenqian Cao − *College of Life and Environmental Sciences, Zhejiang Provincial Modern Biology and Medicine Industry College, Hangzhou Normal University, Hangzhou, Zhejiang 311121, China*

Ruoxin Ruan − *Hangzhou Academy of Agricultural Sciences, Hangzhou, Zhejiang 310024, China*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.jafc.5c11144

Notes

The authors declare no competing financial interest.

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