Generation of Tomato Bacterial Wilt Resistance Gene Slmlo1/6 Mutants Using CRISPR/Cas9 Technology (Postprint)

Authors: Shi Jianlei, Xiong Zili, Su Shiwen, Fu Cunnian, ZAI Wenshan, Zai Wenshan

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Abstract

Bacterial wilt is a devastating soil-borne disease in tomato (Solanum lycopersicum) production, characterized by complex and readily variable physiological races of the pathogen. The recessive mlo mutation of the MLO gene confers broad-spectrum resistance, and previous studies have suggested that Slmlo1/6 may be involved in the resistance response to bacterial wilt in tomato. To further investigate the function of the tomato Slmlo1/6 bacterial wilt resistance gene, this study employed CRISPR/Cas9 technology to generate Slmlo1/6 gene mutant materials and performed phenotypic characterization. The results demonstrated that: (1) The SlMLO1/6 target sequence gRNA was first designed and assembled with the U6 promoter, and the U6-gRNA1/6 fragment containing the efficient target was then ligated into the CRISPR vector pBGK via Bsa I digestion to construct the dual-gene fusion knockout vector pBGK-SlMLO1/6. The recombinant plasmid was transformed into Escherichia coli competent DH5α cells, cultured on plates, and positive single colonies were selected. Following verification, genetic transformation mediated by Agrobacterium tumefaciens GV3101 and hygromycin resistance screening were conducted, ultimately yielding 9 edited tomato seedlings. (2) Target region sequencing revealed that plants M2 and M8 harbored deletions of 177 bp and 7 bp fragments in SlMLO1, respectively, M7 contained a 12 bp deletion in the SlMLO6 fragment, and M9 exhibited a single T base insertion in SlMLO6, totaling 4 single-gene homozygous mutants, while the remaining lines were heterozygous mutants. (3) RT-qPCR analysis showed that, compared with the wild type, the expression levels of SlMLO1/6 were significantly reduced in the mutant lines, particularly in M2, M7, and M8. (4) Phenotypic identification indicated that SlMLO1/6 may function as susceptibility genes for bacterial wilt in tomato. In conclusion, this study successfully constructed MLO gene editing vectors and achieved tomato transformation, with homozygous mutants acquiring resistance to bacterial wilt. Amino acid

loss and frameshift mutations may represent the primary mechanisms underlying the functional transition to resistance in Slmlo1/6. These findings provide a theoretical foundation and genetic engineering materials for functional studies of bacterial wilt resistance genes and disease-resistant breeding applications in tomato.

Full Text

Preamble

Bacterial Wilt Resistance Gene *Slmlo1/6* Mutants in Tomato Created by CRISPR/Cas9 Technology

SHI Jianlei[1],[2], XIONG Zili[1], SU Shiwen[1], FU Cunnian[1], ZAI Wenshan[1],*

1. Southern Zhejiang Key Laboratory of Crop Breeding, Wenzhou Vocational College of Science and Technology, Wenzhou 325006, Zhejiang, China

2. College of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, China

Abstract: Bacterial wilt is a devastating soil-borne disease in tomato (*Solanum lycopersicum*) production. The pathogenic species are complex and prone to variation, while the recessive *mlo* mutation of MLO genes confers broad-spectrum resistance. Previous studies have suggested that *Slmlo1/6* may be involved in the resistance response to bacterial wilt in tomato. To further investigate the function of the tomato bacterial wilt resistance gene *Slmlo1/6*, this study employed CRISPR/Cas9 technology to generate *Slmlo1/6* mutants and conducted phenotypic characterization. The results were as follows: (1) gRNA target sequences for *SlMLO1/6* were designed and assembled with U6 promoters. The U6-gRNA1/6 fragments containing efficient target sites were then ligated into the CRISPR vector pBGK via *Bsa*I digestion to construct the dual-gene fusion knockout vector pBGK-SlMLO1/6. The recombinant plasmid was transformed into *Escherichia coli* DH5α competent cells, and positive monoclonal colonies were selected through plate cultivation. After validation, *Agrobacterium tumefaciens* GV3101-mediated genetic transformation and hygromycin resistance screening were performed, ultimately yielding nine edited tomato plants. (2) Sequencing of the target regions revealed that plants M2 and M8 had deletions of 177 bp and 7 bp in the *SlMLO1* fragment, respectively, while M7 had a 12 bp deletion in the *SlMLO6* fragment, and M9 exhibited a single-base T insertion in *SlMLO6*. In total, four single-gene homozygous mutants were obtained, with the remaining plants being heterozygous mutants. (3) RT-qPCR analysis demonstrated that the expression levels of *SlMLO1/6* genes in the mutant lines were significantly reduced compared to the wild type, particularly in M2, M7, and M8. (4) Phenotypic identification indicated that *SlMLO1/6* may be suscep-

tibility genes for bacterial wilt in tomato. In summary, this study successfully constructed an MLO gene editing vector and achieved tomato transformation, with homozygous mutants acquiring bacterial wilt resistance. Amino acid loss and frameshift mutations may be the primary causes of the functional shift in *Slmlo1/6* resistance. These findings provide a theoretical foundation and genetic engineering materials for functional studies of bacterial wilt resistance genes and disease resistance breeding in tomato.

Keywords: tomato, *Slmlo1/6*, gene editing, genetic transformation, mutant

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First Author: SHI Jianlei (1982—), Ph.D., Associate Professor, specializing in crop genetics, breeding, and biotechnology research (E-mail: sjlhebau@163.com).

Corresponding Author: ZAI Wenshan, M.S., Researcher, specializing in vegetable genetics and breeding (E-mail: 273493129@qq.com).

Introduction

As a powerful tool for plant gene function research and crop genetic improvement, the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated 9 (CRISPR/Cas9) system is currently the most widely used gene editing technology. It primarily consists of single guide RNA (sgRNA) and Cas9 protein (Mali et al., 2013). The sgRNA recognizes the protospacer adjacent motif (PAM) in the target gene and guides Cas9 to cleave the target sequence, generating double-strand breaks that introduce mutations during the repair process, thereby producing phenotypic variations.

MLO (Mildew resistance locus O) was first identified and cloned as a powdery mildew susceptibility gene in barley (*Hordeum vulgare*) (Buschges et al., 1997). The recessive *mlo* mutation confers efficient and durable resistance to nearly all physiological races of powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Reinstädler et al., 2010). Since then, homologous genes have been discovered in numerous higher plants, with mutations similarly conferring powdery mildew resistance (Kusch & Panstruga, 2017). Using gene editing technology, multiple *mlo* genes have been confirmed as powdery mildew resistance genes, including *Slmlo1* in tomato (*Solanum lycopersicum*), *Camlo1/2* in pepper (*Capsicum annuum*), *Smmlo1* in eggplant (*Solanum melongena*), *Ntmlo1* in tobacco (*Nicotiana tabacum*), and *Csmlo1* in cucumber (*Cucumis sativus*) (Zheng et al., 2013; Appiano et al., 2015; Nie et al., 2015; Bracuto et al., 2017). Beyond powdery mildew, *mlo* also participates in plant disease responses caused by various

pathogens, including *Pseudomonas*, *Xanthomonas*, oomycetes, *Fusarium oxysporum*, *Colletotrichum*, and *Magnaporthe oryzae* (Kim & Hwang, 2012; Kim et al., 2014; Acevedo-Garcia et al., 2017).

Tomato is a globally important vegetable crop and an ideal model for gene editing research, yet its production frequently suffers from various environmental stresses. Bacterial wilt caused by *Ralstonia solanacearum* is particularly devastating to tomato, necessitating the establishment of rapid and efficient methods for breeding resistant varieties. Previous studies have shown that both *SlMLO1* and *SlMLO6* contain seven transmembrane domains and are localized to the plasma membrane, with the former containing a calmodulin-binding domain (CaMBD) (Shi et al., 2020). Additionally, *SlMLO1* is a known broad-spectrum powdery mildew resistance gene, while *SlMLO6* is homologous to the pepper bacterial wilt susceptibility gene *CaMLO6* (Bai et al., 2008; Yang et al., 2020). RT-qPCR analysis has demonstrated that both genes respond to tomato bacterial wilt at the transcriptional level (Shi et al., 2020). This study focuses on creating tomato bacterial wilt resistance mutants by leveraging the broad-spectrum resistance factor *mlo* and the tomato genetic transformation system. Based on existing bioinformatics and quantitative gene expression studies of MLO genes, we employed CRISPR/Cas9 gene editing technology to construct *Slmlo1/6* knockout vectors and transform tomato. The study addresses three key questions: (1) precise knockout of MLO genes and generation of homozygous mutants in tomato; (2) mutation types at target sites and expression changes of the target genes before and after mutation; and (3) bacterial wilt resistance phenotypes of the mutant lines. The objective is to provide a theoretical foundation and genetic engineering materials for functional studies of bacterial wilt resistance genes and variety improvement.

Materials and Methods

1.1 Materials and Reagents

The tomato (*S. lycopersicum*) variety Ailsa Craig (AC), a standard model for molecular functional studies, was used as the experimental material. The CRISPR system pBGK was purchased from Biogle Gene Technology (Jiangsu) Co., Ltd. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 were maintained in our laboratory.

Trizol total RNA extraction kit, gel recovery kit, and SYBR Premix Ex Taq for quantitative PCR were purchased from Sangon Biotech (Shanghai) Co., Ltd. PCR Master Mix, DNA Marker, T4 DNA ligase, and T4 polynucleotide kinase (PNK) were obtained from TaKaRa Biotechnology (Dalian) Co., Ltd. Restriction endonucleases *Bsa*I and *Sph*I were purchased from TaKaRa Company. Kanamycin and hygromycin were acquired from Dingguo Biotechnology Company. All other biochemical reagents for culture media components were of

domestic analytical grade. PCR primers were synthesized by Sangon Biotech (Shanghai).

1.2.1 Target Design and Vector Construction

Using the online tool CRISPR-P2.0 (http://cbi.hzau.edu.cn/CRISPR2/), two CRISPR target sites were designed in exons 1 and 3 of the target genes. Efficient target sites were selected and primers were designed (Table 1). After annealing the gRNA target sequences to form oligo dimers, they were ligated into the *Bsa*I-digested CRISPR vector pBGK. The reaction system consisted of 1 L gRNA-U6 fragment, 0.3 L oligo dimer, 0.3 L T4 ligase, and 0.1 L T4 PNK, incubated at 23 °C for 1 hour. Subsequently, 0.3 L T4 ligase, 4 L ddH_2O, and 1 L pBGK were added for ligation at 23 °C for 1 hour. Five microliters of the reaction mixture were added to 20 L *E. coli* DH5α competent cells, mixed, and incubated on ice for 30 minutes. The mixture was heat-shocked at 42 °C for 35 seconds, immediately placed on ice for 2 minutes, then 100 L LB medium was added and cultured at 37 °C with shaking for 1 hour. Sixty microliters of the bacterial suspension were plated on LB agar containing 50 $g \cdot mL^{-1}$ kanamycin (Kan) and incubated overnight at 37 °C. Positive monoclonal colonies were selected and sent for sequencing using the primer SR: CTGCAGAATTGGCGCACGCGCTACG.

1.2.2 *Agrobacterium*-Mediated Genetic Transformation

The correctly identified plasmid was transferred into *A. tumefaciens* GV3101 using the freeze-thaw method. Verified single colonies were used to transform tomato AC via the leaf disc method (Jian et al., 2015). The main procedures were as follows: Tomato sterile seeds were germinated on 1/2 MS medium. When cotyledons expanded but before the first true leaf emerged, cotyledons were excised for pre-cultivation. After *Agrobacterium* infection and co-cultivation, explants were transferred to hygromycin-containing differentiation and selection medium. Following three subculture passages, shoots with callus removed were transferred to rooting medium. Seedlings were acclimatized and transplanted when they reached appropriate size.

1.2.3 Target Amplification and Sequencing of Transgenic Plants

Genomic DNA was extracted from young leaves of individual transgenic plants. Primers were designed based on the target gene sequences (Table 1) for PCR detection of the target regions. The amplified products were purified and sequenced, and mutation types were analyzed by combining sequencing chromatograms with sequence alignment. The PCR amplification system contained 1 L DNA, 2 L 10×PCR buffer, 0.4 L dNTP mixture, 0.2 L each of forward and reverse primers, and 0.2 L Taq enzyme, supplemented with ddH_2O to a final volume of 20 L. The amplification conditions were: 94 °C pre-denaturation for 5 minutes; 30 cycles of 94 °C denaturation for 30 seconds, 55 °C annealing

for 30 seconds, and 72 °C extension for 30 seconds; followed by a final extension at 72 °C for 10 minutes.

1.2.4 RT-qPCR Detection of Target Genes

Young leaves from 4-6 leaf-stage tomato seedlings were sampled, wrapped in aluminum foil, and placed in liquid nitrogen, with two biological replicates. RNA was extracted from wild-type and mutant plants and reverse-transcribed into cDNA. Using *Slactin* and *SlRPL2* as reference genes, quantitative primers were designed with Primer Premier 5.0 software (Table 1). The RT-qPCR reaction system included 2 L cDNA, 0.4 L PCR primer, 10 L SYBR, and 7.2 L ddH_2O. The amplification program was 95 °C for 3 minutes, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. The entire reaction was performed on a StepOne Plus Real-Time PCR System (ABI, USA), and relative gene expression levels were calculated using the 2^(-ΔΔCt) method.

1.2.5 Bacterial Wilt Inoculation Assay at Seedling Stage

When tomato seedlings developed 4-6 true leaves, partial root systems were wounded and immersed in *Ralstonia solanacearum* suspension (OD_{600} = 1.0) for 20 minutes. Plants were then cultivated at 30 °C, and resistance phenotypes were observed on day 3.

Results

2.1 Fusion Vector Construction and Transformation

The *SlMLO1* target 1 sequence was CCACAGCAATTGCCCACGTAGGG, and target 2 was ATGGCATCCTTGTATGGCAAAGG. The *SlMLO6* target 1 sequence was ACACCAACTTGGGCTGTGGCTGG, and target 2 was CAAAGGAGGAGGAACACCGTAGG, with each target being 20 bp in length (Figure 1 [Figure 1: see original paper]). The corresponding off-target site numbers were 5/31 and 26/33, respectively, so the first target site was selected for both genes.

The fusion vector was named pBGK-SlMLO1/6, containing a dual-gene target gRNA cassette driven by the U6 promoter, a Cas9 gene driven by the 35S promoter, and a hygromycin resistance marker gene (HYG) (Figure 2 [Figure 2: see original paper]). The product recovered after *Sph*I digestion showed two bands of approximately 5,500 bp and 10,000 bp on 0.8% agarose gel electrophoresis (Figure 3 [Figure 3: see original paper]), consistent with the 15,386 bp size of the recombinant plasmid. Sequencing confirmed that the target sites matched the designed sequences, indicating successful insertion of the two gRNA cassettes into the vector. Subsequently, the fusion vector was used for *Agrobacterium*-mediated tomato genetic transformation (Figure 4 [Figure 4: see original paper]) and PCR sequencing (Figure 5 [Figure 5: see original paper]), ultimately yielding nine edited individual plants (M1-4, 6-10).

2.2 Mutation Types at Target Sites in Edited Plants

Sequencing results revealed that edited plant M1 was a heterozygous mutant for *SlMLO6*. Plants M2 and M8 had deletions of 177 bp (including the start codon, encoding MEATPTWAIAVVCFILLAIS) and 7 bp (GGGCAAT, encoding WAI) in the *SlMLO1* gene, respectively, while *SlMLO6* remained heterozygous. M3 and M10 were heterozygous mutants for *SlMLO1*. M4 and M6 were heterozygous mutants for both genes. M7 had a 12 bp deletion (CAACTTGGGCTG, encoding PTWAV) in *SlMLO6*, while *SlMLO1* was heterozygous. M9 exhibited a single-base T insertion between positions 382-383 in *SlMLO6* (encoding V), while *SlMLO1* was heterozygous (Figure 5).

2.3 Quantitative Expression Analysis of Target Genes

Quantitative expression analysis showed that under both reference genes, the expression levels of *SlMLO1* in homozygous mutants M2 and M8 were extremely significantly ($P < 0.01$) lower than in the wild type (WT), with M2 showing lower expression than M8, though the difference was not significant (Figure 6 [Figure 6: see original paper]: A, C). Using *Slactin* as the reference gene, the expression levels of *SlMLO6* in homozygous mutants M7 and M9 were extremely significantly ($P < 0.01$) lower than in WT, with M7 showing significantly lower expression than M9 (Figure 6: B). Using *SlRPL2* as the reference gene, both M7 and M9 showed lower *SlMLO6* expression than WT, but with different significance levels: M7 was extremely significant ($P < 0.01$) while M9 was significant ($P < 0.05$) (Figure 6: D). Overall, *Slactin* was more suitable as a reference gene, and the mutation effects were more pronounced in M2, M7, and M8.

2.4 Bacterial Wilt Resistance Phenotypes of Edited Plants

Based on the sequencing and quantitative expression results, three homozygous mutants with obvious knockout effects (M2, M7, and M8) were selected for bacterial wilt resistance phenotyping. After inoculation with *R. solanacearum*, these plants showed significantly better growth than wild-type tomatoes and essentially remained symptom-free during the observation period (Figure 7 [Figure 7: see original paper]), suggesting that *SlMLO1/6* may be involved in negative regulation of bacterial wilt resistance in tomato.

Discussion and Conclusion

Ralstonia solanacearum exhibits broad environmental and ecological adaptability. To date, using forward genetics, only one bacterial wilt resistance gene (*RRS1*) has been cloned in *Arabidopsis* (Deslandes et al., 1998), and research on related gene identification, functional studies, and practical applications remains very limited. Consequently, mining for broad-spectrum resistance and inactivating susceptibility genes have become research hotspots. Compared with other

gene function study technologies, gene editing offers advantages such as relatively simple cloning strategies, multi-target knockout capability, low off-target rates, and wide applicability (Ma et al., 2015). Directed knockout of unfavorable genes using CRISPR/Cas9 technology enables genetic improvement of target traits, and transgene-free mutant lines can be obtained through subsequent genetic segregation.

The *mlo* mutation represents a novel broad-spectrum resistance mechanism controlled by host gene mutations that can participate in responses to various biotic and abiotic stresses (Nguyen et al., 2016). Building upon this, our study successfully constructed *Slmlo1/6* gene editing vectors using the CRISPR/Cas9 system. Through tomato transformation and sequencing identification, we obtained nine mutant lines, approximately 50% of which were homozygous mutants.

The editing efficiency and mutation types varied at different sites in the *Slmlo1/6* mutants. The homozygous mutations included fragment deletions and single-base insertions, demonstrating that gene editing in diploid plants primarily generates simple mutations (Ma et al., 2015). The *SlMLO1* encoded proteins in M2 and M8 lost 20 and 3 amino acids, respectively, and underwent frameshift mutations. The M7 *SlMLO6* protein lost 5 amino acids but gained a new leucine, with downstream sequences remaining unchanged. In M9, the *SlMLO6* codon GTG mutated to GTT, both encoding valine, but subsequent amino acids underwent frameshift mutations. We hypothesize that the functional shift in resistance primarily stems from amino acid loss and frameshift mutations. Studies have shown that multiple active U6 promoters can drive multiple sgRNAs, causing large chromosomal fragment deletions. Meanwhile, stable transgenic plants predominantly exhibit base deletions and insertions, possibly because continuous expression of sgRNA and Cas9 leads to further editing of base substitutions (Pu et al., 2018). Errors frequently occur during non-homologous end joining (NHEJ) repair, resulting in small fragment deletions or insertions at DNA break sites, whereas homology-directed repair (HDR) can achieve targeted gene repair or insertion (Hsu et al., 2014). The dual-gene target editing in this study supports these conclusions regarding stable transformation mutations and likely proceeds primarily through NHEJ repair. Quantitative analysis revealed that target gene expression levels decreased in homozygous mutants, with more pronounced effects in *SlMLO1* than in *SlMLO6*. Heterozygous lines require further segregation and purification. The lack of mutations in M1 *SlMLO1*, M3, and M10 *SlMLO6* may be related to off-target effects.

MLO negatively regulates plant resistance and mesophyll cell death through papilla formation, facilitating pathogen infection and suppressing defense responses (Kim et al., 2002a). Additionally, MLO can enhance its functional activity by binding calmodulin (CaM) to reduce plant disease resistance (Kim et al., 2002b). Previous studies have shown that *SlMLO1* contains a CaMBD, suggesting it may promote disease susceptibility in tomato through CaM binding. Pepper *CaMLO6* negatively regulates bacterial wilt resistance by interact-

ing with *CaWRKY40* (Yang et al., 2020). Given the phylogenetic relationship between tomato and pepper, we speculate that *SlMLO6* may have similar functions. Preliminary resistance phenotyping suggests that *SlMLO1/6* may be bacterial wilt susceptibility genes. However, bacterial wilt resistance is genetically complex and controlled by multiple genes. Due to potential off-target effects, how non-target gene editing (in coding and non-coding regions) affects editing efficiency and plant phenotype requires further evaluation through whole-genome sequencing or off-target site analysis. Additionally, whether functional redundancy and multiple allele effects exist among MLO family genes needs further verification. In conclusion, comprehensive phenotypic, physiological, and molecular characterization of *Slmlo1/6* mutants and their progeny across different tomato varieties is necessary to obtain pure, stable breeding materials with practical application value.

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Note: Figure translations are in progress. See original paper for figures.