CRISPR/Cas9-mediated targeted mutagenesis of two homoeoalleles in tobacco confers resistance to powdery mildew

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Research Article

Abstract

The CRISPR/Cas9 technology has proven to be an effective tool for crop improvement. In tobacco (*Nicotiana tabacum* L.), the *Mildew Locus O* (*MLO*) genes, *NtMLO1* and *NtMLO2*, are responsible for powdery mildew susceptibility. However, modification of multiple homoeoalleles is complex in polyploidy crops. Therefore, we employed the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9) system containing a single-guide RNA (sgRNA) to enable targeted mutagenesis of both *NtMLOs* simultaneously and enhance the powdery mildew resistance of tobacco. The approach generated different mutants, of which two-thirds had both genes mutated. In the T_1 lines, three types of mutations were identified for *NtMLO1* and two types for *NtMLO2* at the expected position based on Sanger sequencing. Meanwhile, four mutation events were identified in the mutants with both genes edited in T_1 lines. These double mutants showed enhanced resistance to powdery mildew, whereas the mutants with only one edited gene showed no significant difference compared to wild-type plants. Our results suggest that CRISPR/Cas9-mediated targeted editing of *NtMLO1* and *NtMLO2* is a practical approach for improving the powdery mildew resistance of tobacco.

Introduction

Powdery mildew, caused by the biotrophic fungal species of the Erysiphales order, is a common disease affecting a wide range of plant species worldwide (Quaglia et al. 2012). In tobacco (*Nicotiana tabacum* L.), powdery mildew caused by *Golovinomyces cichoracearum* (*Gc*) is one of the most devastating diseases (Cunnington et al. 2005). The infection changes the color of leaves from green to brown and eventually affects the yield and quality of tobacco (Darvishzadeh et al. 2010). Using cultivars containing disease resistance alleles is the most effective and eco-friendly strategy to manage powdery mildew incidence.

Wild tobacco species *N. glutinosa* and *N. tomentosiformis* harbor a dominant locus for powdery mildew resistance, which has been used in tobacco breeding (Davis and Nielsen 1999). Another strategy to achieve resistance to powdery mildew is using susceptible genes. Mildew Locus O (MLO) is a class of plant-specific proteins with seven transmembrane domains localized in the plasma membrane, which act as susceptibility factors towards powdery mildew (Devoto et al. 1999). The inactivation of *MLOs* in tomato induced papilla formation and callose deposition, preventing fungal penetration into the host cells (Li et al. 2007). Meanwhile, the loss-of-function alleles of *MLO* conferred broad-spectrum and durable resistance to various fungal species causing powdery mildew in barley (Büschges et al. 1997), *Arabidopsis thaliana* (Consonni et al. 2006), tomato (Bai et al. 2008), pea (Humphry et al. 2011; Pavan et al. 2011), pepper (Zheng et al. 2013), and bread wheat (Wang et al. 2014). In tobacco, naturally mutations in the intronic regions of the two *NtMLO* homoeologs triggered aberrant splicing of *MLO* transcripts and enhanced resistance to powdery mildew (Fujimura et al. 2016). These previous studies demonstrated the feasibility of using *MLO* mutagenesis in resistance breeding. However, in allotetraploid tobacco, the introgression of these two recessive homoeoalleles using traditional breeding methods is difficult and

time-consuming. Therefore, technologies that can targeted mutate several homoeologous gene copies is necessary to improve the resistance.

Genome editing is an effective tool widely applied to improve the agricultural traits of various plants (Xu et al. 2019). The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR/Cas9) system, which performs targeted genome editing under the guidance of an engineered sequence-specific single guide RNA (sgRNA), has revolutionized genome editing and crop improvement (Kato-Inui et al. 2018). CRISPR/Cas9 system-mediated targeted mutagenesis has been widely used to genetically modify disease resistance in rice (Wang et al. 2016; Kim et al. 2019), tomato (Nekrasov et al. 2017), citrus (Peng et al. 2017), cucumber (Chandrasekaran et al. 2016), and watermelon (Tian et al. 2018). The major advantage of this technology is its ability to edit multiple target sites simultaneously (Gao 2021). Therefore, it is ideal for genetically improving polyploid crops with traits controlled by multiple gene copies (Zaman et al. 2019; Gao 2021). Recent studies have demonstrated the success of this technique in the targeted mutagenesis of multiple genes. In bread wheat, targeted editing of three homoeoalleles of *MLO* simultaneously using this approach conferred powdery mildew resistance (Wang et al. 2014).

Therefore, the present study used the CRISPR/Cas9 system for mutating two *MLO* homoeologs simultaneously in the allotetraploid tobacco aiming at powdery mildew resistance. Results showed that the CRISPR/Cas9 system successfully edited the *NtMLO1* and *NtMLO2* simultaneously, and the double mutant lines displayed enhanced resistance to powdery mildew.

Materials And Methods

Plant materials

The *N. tabacum* cv. ZY300 susceptible to powdery mildew was obtained from the Tobacco Research Institute, Chinese Academy of Agricultural Sciences. The plants were maintained in a growth chamber at 22 °C under a 16 h light cycle with 75±15% relative humidity.

sgRNA designing and vector construction

The full-length coding sequences (CDS) of the *NtMLO1* (Genebank accession number: LC089758.1) and *NtMLO2* (Genebank accession number: LC089759.1) genes were retrieved from the National Center for Biotechnology Information database. Then, a single guide RNA (sgRNA) targeting a conserved region within exon 1 of *NtMLO1* and *NtMLO2* was designed using the online tool CRISPR MultiTargeter (Prykhozhij et al. 2015). The pORE-Cas vector (Gao et al. 2015) and the sgRNA expression cassette driven by the AtU6-26 promoter were ligated to generate the recombinant expression vector pORE-Cas-gRNA-MT. The pORE-Cas-gRNA-MT was used to transform the *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method. Primers used for constructing the plasmid are listed in Supplementary Table 1.

Generation of transgenic tobacco plants

Tobacco plants were transformed following the leaf disc method (Li et al. 2020). Leaf discs from five-week-old tobacco plants were infected with the *Agrobacterium* strain EHA105 harboring the pORE-Cas-gRNA-MT and placed on a regeneration medium (MS medium containing 0.1 mg/L 1-naphthaleneacetic acid, 1.0 mg/L 6-benzylaminopurine, 250 mg/L carbenicillin sodium, and 50 mg/L kanamycin sulfate, pH 5.8–6.0). The regenerated shoots were then transferred to the root induction medium (MS medium containing 250 mg/L carbenicillin sodium and 50 mg/L kanamycin sulfate, pH 5.8–6.0). The drug-resistant seedlings were obtained and prepared for analysis.

PCR-based identification of the targeted mutants

Genomic DNA of kanamycin resistant transgenic plants and wild type plants was extracted using DNeasy Plant Mini Kit (Qiagen, CA) to analyze targeted mutagenesis using PCR amplification and Sanger sequencing. The DNA fragment flanking the sgRNA target regions was amplified by PCR using *NtMLO1* and *NtMLO2* gene-specific primers (Supplementary Table 1) and subjected to Sanger sequencing. The study used sequence alignment to detect the homozygous mutations within the target sites and the sequencing chromatograms to identify heterozygous mutations (double peaks at the targeted sites). These T_0 plants carrying the mutations were further selected and self-pollinated to produce the T_1 progeny. The T_1 generation of plants was then screened by PCR using *NtMLO1* and *NtMLO2* gene-specific primers and the amplicons were sequenced. The plants with homozygous mutations in *NtMLO1* only, *NtMLO2* only, and both *NtMLO1* and *NtMLO2* were selected and self-pollinated to obtain the T_2 progeny; these T_2 lines were used to assess powdery mildew resistance.

Off-target analysis

The potential off-target sites of sgRNA in tobacco were identified by searching against the tobacco genome (version: AYMY01) using the Cas-OFFinder (Bae et al. 2014; http://www.rgenome.net/cas-offinder/), allowing three or fewer mismatches between the target sgRNA and aligned sequences. The presence of these potential off-target sites was checked via PCR using specific primers (Supplementary Table 1), followed by Sanger sequencing.

Pathogen inoculation and disease resistance evaluation

The resistance of T_2 mutants to powdery mildew was analyzed. The conidiospores obtained from the infected tobacco leaves were suspended in distilled water and adjusted to a concentration of about 3.0×10^5 spores mL^{-1} to prepare the inoculum. Six-week-old plants from the homozygous T_2 lines with different alleles (lines C2-8 and C8-10 for only *NtMLO1*, lines C3-1 and C1-15 for only *NtMLO2*, and lines C1-7 and C13-21 for both *NtMLO1* and *NtMLO2*) were inoculated by spraying the conidiospore suspension. A transgenic line (C4) obtained from the same CRISPR transformation experiment with the wild-type alleles was used as a control. The disease severity was assessed 20 days post-inoculation (dpi) using a scale of 0, 1, and 3 and expressed as disease index (DI) (Bai et al. 2008). Meanwhile, to measure the relative fungal biomass, the fungal and plant genomic DNAs were extracted from the infected leaves

(20 dpi) using the MiniBEST universal genomic DNA extraction kit (Takara, Dalian, China). Then, a real-time PCR was carried out using LightCycler®96 system (Roche, Switzerland). Relative fungal biomass was determined using 15 ng of the total DNA as a template and primers designed for the *Gc*-specific internal transcribed spacer sequence (*ITS*; GenBank accession number AF011292) (Zheng et al. 2016). The tobacco *Ef1a* gene was used as a reference to normalize the amount of fungal DNA in different samples. Finally, the fold change of the ratio of fungal genomic DNA to tobacco genomic DNA was calculated using the $\Delta\Delta C_T$ method (Livak et al. 2001). Primers used for real-time PCR are listed in Supplementary Table 1.

Results And Analysis

Vector construction for the CRISPR/Cas9 system

Initially, we aligned the full-length CDS of *NtMLO1* and *NtMLO2* to identify a site to design sgRNA targeting both genes simultaneously. The alignment showed 95.62% similarity between the *NtMLO1* and *NtMLO2* sequences (Supplementary Fig. 1). To increase the chances of obtaining complete loss-of-function mutations, a site targeting a conserved region, including a 20 bp nucleotide with the protospacer adjacent motif (PAM) at the 3' region, was chosen within exon 1 of these genes (Fig. 1A). The sgRNA was cloned into the pORE-Cas vector to generate the pORE-Cas-gRNA-MT construct used to transform the susceptible tobacco cultivar ZY300 (Fig. 1B).

CRISPR/Cas9-mediated mutagenesis of *NtMLO*

In total, 20 transgenic plants (T_0) were obtained from the leaf discs transformed with the *Agrobacterium* (Supplementary Fig. 2). The genomic DNA of these T_0 individuals was amplified by PCR using *NtMLO1*-specific (MJC2F/MJC2R) and *NtMLO2*-specific (MJC8F/MJC8R) primers and sequenced to characterize the mutations. Sequence analysis showed that 13 out of the 20 T_0 transgenic plants had heterozygous mutations in *NtMLO1*, 12 had heterozygous mutations in *NtMLO2* (Table 1, Supplementary Fig. 3). Among these mutants, 10 plants had heterozygous mutations in both genes. The approach achieved a mutation frequency of 65.0% in *NtMLO1* and 60.0% in *NtMLO2*. Approximately 66.7% of the mutants had both genes mutated (Table 1).

We further characterized the T_1 progenies derived from the T_0 plants to obtain the homozygous mutants. Plants homozygous for the mutant alleles were selected from the T_1 segregating lines by sequencing the specific PCR fragments. Three homozygous mutation types were identified for *NtMLO1*, including a 1 bp deletion (-T), a 2 bp deletion (-GA), and a 1 bp insertion (+A) (Fig. 2A). For *NtMLO2*, two homozygous mutation types, including a 1 bp insertion (+A) and an 11 bp deletion (-ACGGTCGATGG), were identified (Fig. 2B). We identified individual plants with simultaneous homozygous mutations in both genes from the progenies of the 10 T_0 plants (C1, C5, C7, C10, C11, C12, C13, C18, C19, C20). Four different mutation events were identified in these simultaneous homozygous mutants (Fig. 2C). Event 1 resulted in a 1 bp deletion in *NtMLO1* and a 1 bp insertion in *NtMLO2* in the T_1 plants obtained from the T_0 lines C5, C10,

C11, C12, and C20. Event 2 resulted in a 2 bp deletion in *NtMLO1* and a 1 bp insertion in *NtMLO2* in the T_1 plants from the T_0 lines C1, C7, and C18. Event 3 generated a 1 bp insertion in *NtMLO1* and an 11 bp deletion in *NtMLO2* in the T_1 plants obtained from the T_0 line C13. Event 4 created 1 bp insertions in *NtMLO1* and *NtMLO2* in the T_1 plants obtained from the T_0 line C19. We then obtained the self-pollinated seeds from the homozygous T_1 mutants for the further analysis.

Off-target analysis

Furthermore, we predicted the putative off-target mutations induced by the sgRNA in the whole genome using Cas-OFFinder (Bae et al. 2014), allowing a mismatch of three or fewer nucleotides between the sgRNA and the potential off-target regions to assess the specificity of this CRISPR/Cas9 system. Four potential off-targets with a PAM sequence were identified in the whole genome (Supplementary Table 2), of which one off-target, located on chromosome AYMY01S000835.1, had two mismatches, and the other three off-targets, located on chromosomes AYMY01S001145.1, AYMY01S001369.1, and AYMY01S024885.1, had three mismatches. We then checked for these potential off-target sites in three plants randomly selected from the homozygous T_2 lines C1-7 and C13-21 by PCR using different primer combinations (Supplementary Table 1). Sequencing showed no differences between the wild type and the mutant on these four potential sites (Supplementary Fig. 4), suggesting that no off-target mutation occurred in the potential sites.

Resistance of mutants to powdery mildew

Finally, to assess the impact of CRISPR/Cas9-induced mutations on powdery mildew resistance in tobacco, the T_2 lines with homozygous mutations in only *NtMLO1* (C2-8 and C8-10), only *NtMLO2* (C3-1 and C1-15), and both *NtMLO1* and *NtMLO2* (C1-7 and C13-21) (Supplementary Fig. 5) were inoculated with *Gc*. No apparent disease symptom was observed on the leaves of the C1-7 and C13-21 lines with homozygous mutations in both *NtMLO1* and *NtMLO2* (Fig. 3A, Supplementary Fig. 6). Meanwhile, fungal growth was obvious in the wild-type plants and the mutants with only one edited gene (Fig. 3A). Consistent with these findings, the DI and relative *Gc* biomass on the leaves of the C1-7 and C13-21 lines were significantly lower than the wild-type plants. However, no significant differences in the disease index or the relative *Gc* biomass were observed among the wild-type plants and single mutants (Fig. 3B, C). These results indicated the role of both *NtMLO1* and *NtMLO2* genes in responding to powdery mildew infection, and mutating both genes could confer resistance to powdery mildew in tobacco.

Discussion

The CRISPR/Cas9 system has significantly boosted the application of targeted genome editing in crop breeding (Hua et al. 2019). Genome editing helps eliminate the susceptible genes or negative elements and enhance disease resistance in cultivars (Chen et al. 2019). *MLO* is one of the best-studied susceptibility genes of plants; a loss-of-function mutation in *MLO* confers broad-spectrum and durable resistance against powdery mildew (Santillán Martínez et al. 2020). In common tobacco, two *MLO*

homoeologs control the resistance to powdery mildew, and these two homoeologs are functionally redundant (Appiano et al. 2015; Fujimura et al. 2016). However, the introgression of these two homoeologs into elite cultivars by traditional backcross is time-consuming. Recently, the CRISPR-Cas9-based approach edited three homoeoalleles of *MLO* simultaneously and created a bread wheat cultivar with powdery mildew resistance; this study suggested the suitability of the CRISPR-Cas9 system for polyploid crop improvement (Wang et al. 2014; Li et al. 2022). Similarly, in the present study, we used the CRISPR/Cas9 genome-editing system in allotetraploid tobacco and edited *NtMLO1* and *NtMLO2* simultaneously. Our results showed that the sgRNA, targeting a conserved region in exon 1, efficiently induced mutation. Among the 15 T_0 mutants, 10 had both genes mutated simultaneously (Table 1). Three types of homozygous mutants were obtained in T_1 generation, including *NtMLO1* edited only, *NtMLO2* edited only, and both genes edited simultaneously. In the double mutants, four different mutation events were identified (Fig. 2). Six T_1 plants (C2-8 and C8-10 *NtMLO1* edited only, C3-1 and C1-15 *NtMLO2* edited only, C1-7 and C13-21 both genes edited) were selected to further analyzed the transmission of homozygous mutations to their T_2 offspring. The homozygous mutations from six T_1 plants were all transmitted to T_2 offspring (Supplementary Fig. 5). These results suggested that CRISPR/Cas9-mediated mutations in *NtMLOs* can be stably transmitted to subsequent generations.

However, off-target activity-induced mutation at sites other than the target site is undesirable and a major concern of the CRISPR/Cas9 system, which has less happened in plant genome editing than in animals (Zhang et al. 2021). In rice and cotton, CRISPR/Cas systems did not induce sgRNA-independent off-target effects (Li et al. 2019; Tang et al. 2018). This study examined the sgRNA-dependent off-target effect of the CRISPR/Cas9 construct targeting the MLO genes in double mutants. We searched the tobacco genome for potential off-target sites allowing three or fewer mismatched nucleotides between sgRNA and the off-target regions. Four potential off-targets with a PAM sequence were found in the genome. Further verification of mutants by PCR showed no off-target mutations (Supplementary Table 2; Supplementary Fig. 4), suggesting that the target sequence was specific.

In common tobacco, natural mutations in the intronic regions of the two *NtMLO* genes, which lead to aberrant splicing of *MLO* transcripts, are responsible for the occurring broad-spectrum powdery mildew resistance (Fujimura et al. 2016). In the double mutants of the present study, both *NtMLO1* and *NtMLO2* genes had frameshift mutation, which caused abnormal protein synthesis. Finally, disease resistance evaluation showed lower powdery mildew incidence and relative *Gc* biomass in the double mutant than the control and the mutants with a single edited gene (Fig. 3). These results confirmed that both *NtMLO1* and *NtMLO2* contribute to the susceptibility of tobacco to *Gc* infection, and editing one single gene could not reduce the incidence of powdery mildew, which is consistent with the previous reports (Wang et al. 2014; Fujimura et al. 2016).

However, susceptible genes are implicated in various other biological functions, and therefore, recessive gene-mediated disease resistance is often associated with growth defects and yield losses (Li et al. 2022). In hexaploidy bread wheat, *Tamlo-aabbdd* mutants with three *TaMLO* homoeologs simultaneously

edited exhibited reduced plant height and grain yield compared to wild-type plants, although they were highly resistant to powdery mildew (Li et al. 2022). To solve this problem, an alternative breeding strategy was the use of the weak *mlo* alleles to attain a balance between disease resistance and plant growth (Acevedo-Garcia et al. 2014). Meanwhile, Li and his colleagues demonstrated a gain-of-function mutation that overcame the growth defects caused by *mlo* recessive resistance alleles in wheat postulating a promising strategy to take full advantage of the susceptible genes in crop breeding (Li et al. 2022). These observations suggest that the double mutant of tobacco generated in this study may influence growth and yield; therefore, the pleiotropic effects of *NtMLO* mutations on the yield and quality of tobacco should be further tested.

Conclusions

The present study identified a highly efficient and specific CRISPR/Cas9 system for editing two *NtMLO* homoelogs simultaneously in the allotetraploid tobacco for powdery mildew resistance. We characterized four different mutation events in mutant lines with two homoelogs mutated simultaneously and confirmed enhanced powdery mildew resistance of these lines. Our study presents an excellent approach based on the CRISPR/Cas9 system in breeding resistance against pathogens using the susceptibility genes of polyploid crops.

Declarations

Data availability

The data sets supporting the results of this article are included within the article and its additional files.

Ethics approval and consent to participate

No applicable.

Consent for publication

No applicable.

Conflict of interest

The authors declare no competing interests.

References

1. Acevedo-Garcia J, Kusch S, Panstruga R (2014) Magical mystery tour: MLO proteins in plant immunity and beyond. New Phytol 204:273–281. https://doi.org/10.1111/nph.12889

2. Appiano M, Pavan S, Catalano D, Zheng Z, Bracuto V, Lotti C, Visser RG, Ricciardi L, Bai Y (2015) Identification of candidate *MLO* powdery mildew susceptibility genes in cultivated Solanaceae and functional characterization of tobacco *NtMLO1*. Transgenic Res 24:847–858. https://doi.org/10.1007/s11248-015-9878-4

3. Bae S, Park J, Kim JS (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30:1473–1475. https://doi.org/10.1093/bioinformatics/btu048

4. Bai Y, Pavan S, Zheng Z, Zappel NF, Reinstädler A, Lotti C, De Giovanni C, Ricciardi L, Lindhout P, Visser R, Theres K, Panstruga R (2008) Naturally occurring broad-spectrum powdery mildew resistance in a central American tomato accession is caused by loss of *mlo* function. Mol Plant Microbe Interact 21:30–39. https://doi.org/10.1094/MPMI-21-1-0030

5. Büschges R, Hollricher K, Panstruga R, Simons G, Wolter M, Frijters A, van Daelen R, van der Lee T, Diergaarde P, Groenendijk J, Töpsch S, Vos P, Salamini F, Schulze-Lefert P (1997) The Barley *Mlo* Gene: A novel control element of plant pathogen resistance. Cell 88:695–705. https://doi.org/10.1016/s0092-8674(00)81912-1

6. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol Plant Pathol 17:1140–1153. https://doi.org/10.1111/mpp.12375

7. Chen K, Wang Y, Zhang R, Zhang H, Gao C (2019) CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu Rev Plant Biol 70:667–697. https://doi.org/10.1146/annurev-arplant-050718-100049

8. Consonni C, Humphry ME, Hartmann HA, Livaja M, Durner J, Westphal L, Vogel J, Lipka V, Kemmerling B, Schulze-Lefert P, Somerville SC Panstruga R(2006)Conserved requirement for a plant host cell protein in powdery mildew pathogenesis.Nat Genet38:716–720. https://doi.org/10.1038/ng1806

9. Cunnington JH, Lawrie AC, Pascoe IG (2005) Molecular identification of *Golovinomyces* (Ascomycota: Erysiphales) anamorphs on the Solanaceae in Australia. Australas Plant Pathol 34:51–55. https://doi.org/10.1007/s13313-022-00868-7

10. Darvishzadeh R, Alavi R, Sarrafi A (2010) Resistance to powdery mildew (*Erysiphe cichoracearum* DC.) in oriental and semi-oriental tobacco germplasm under field conditions. J Crop Improv 24:122–130. https://doi.org/10.1080/15427520903559425

11. Davis DL, Nielsen MT (1999) Tobacco Production, Chemistry and Technology. Blackwell Science Ltd, Oxford, UK

12. Devoto A, Piffanelli P, Nilsson I, Wallin E, Panstruga R, von Heijne G, Schulze-Lefert P (1999) Topology, subcellular localization, and sequence diversity of the Mlo family in plants. J Biol Chem 274:34993–35004. https://doi.org/10.1074/jbc.274.49.34993

13. Fujimura T, Sato S, Tajima T, Arai M (2016) Powdery mildew resistance in the Japanese domestic tobacco cultivar Kokubu is associated with aberrant splicing of *MLO* orthologues. Plant Pathol 65:1358–1365. https://doi.org/10.1111/ppa.12498

14. Gao CX (2021) Genome engineering for crop improvement and future agriculture. Cell 184:1621–1635. https://doi.org/10.1016/j.cell.2021.01.005

15. Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Wu Y, Zhao P, Xia Q (2015) CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum*. Plant Mol Biol 87:99–110. https://doi.org/10.1007/s11103-014-0263-0

16. Hua K, Zhang J, Botella JR, Ma C, Kong F, Liu B, Zhu J (2019) Perspectives on the application of genome-editing technologies in crop breeding. Mol Plant 12:1047–1059. https://doi.org/10.1016/j.molp.2019.06.009

17. Humphry M, Reinstädler A, Ivanov S, Bisseling T, Panstruga R (2011) Durable broad-spectrum powdery mildew resistance in pea *er1* plants is conferred by natural loss-of-function mutations in *PsMLO1*. Mol Plant Pathol 12:866–878. https://doi.org/10.1111/j.1364-3703.2011.00718.x

18. Kato-Inui T, Takahashi G, Hsu S, Miyaoka Y (2018) Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 with improved proof-reading enhances homology-directed repair. Nucleic Acids Res 46:4677–4688. https://doi.org/10.1093/nar/gky264

19. Kim YA, Moon H, Park CJ (2019) CRISPR/Cas9-targeted mutagenesis of *Os8N-3* in rice to confer resistance to *Xanthomonas oryzae* pv *oryzae*. Rice 12:67. https://doi.org/10.1186/s12284-019-0325-7

20. Li C, Bonnema G, Che D, Dong L, Lindhout P, Visser R, Bai Y (2007) Biochemical and molecular mechanisms involved in monogenic resistance responses to tomato powdery mildew. Mol Plant-Microbe Interact 20:1161–1172. https://doi.org/10.1094/MPMI-20-9-1161

21. Li J, Manghwar H, Sun L, Wang P, Wang G, Sheng H, Zhang J, Liu H, Qin L, Rui H, Li B, Lindsey K, Daniell H, Jin S, Zhang X (2019) Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. Plant Biotechnol J 17:858–868. https://doi.org/10.1111/pbi.13020

22. Li S, Lin D, Zhang Y, Deng M, Chen Y, Lv B, Li B, Lei Y, Wang Y, Zhao L, Liang Y, Liu J, Chen K, Liu Z, Xiao J, Qiu JL, Gao C (2022) Genome-edited powdery mildew resistance in wheat without growth penalties. Nature 602:455–460. https://doi.org/10.1038/s41586-022-04395-9

23. Li YY, Sui XY, Yang JS, Xiang XH, Li ZQ, Wang YY, Zhou ZC, Hu RS, Li-u D (2020) A novel bHLH transcription factor, *NtbHLH1*, modulates iron homeostasis in tobacco (*Nicotiana tabacum* L.). Biochem Biophys Res Commun 522:233–239. https://doi.org/10.1016/j.bbrc.2019.11.063

24. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

25. Nekrasov V, Wang C, Win J, Lanz C, Weigel D, Kamoun S (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. Sci Rep 7:482. https://doi.org/10.1038/s41598-017-00578-x

26. Pavan S, Schiavulli A, Appiano M, Marcotrigiano AR, Cillo F, Visser RG, Bai Y, Lotti C, Ricciardi L (2011) Pea powdery mildew *er1* resistance is associated to loss-of-function mutations at a *MLO* homologous locus. Theor Appl Genet 123:1425–1431. https://doi.org/10.1007/s00122-011-1677-6

27. Peng AH, Chen SC, Lei TG, Xu LZ, He YR, Wu L, Yao LX, Zou XP (2017) Engineering canker resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus. Plant Biotechnol J 15:1509–1519. https://doi.org/10.1111/pbi.12733

28. Prykhozhij SV, Rajan V, Gaston D, Berman JN (2015) CRISPR MultiTargeter: A web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. PLoS ONE 10:e0138634. https://doi.org/10.1371/journal.pone.0119372

29. Quaglia M, Fabrizi M, Zazzerini A, Zadra C (2012) Role of pathogen-induced volatiles in the *Nicotiana tabacum-Golovinomyces cichoracearum* interaction. Plant Physiol Biochem 52:9–20. https://doi.org/10.1016/j.plaphy.2011.11.006

30. Santillán Martínez MI, Bracuto V, Koseoglou E, Appiano M, Jacobsen E, Visser RGF, Wolters AMA, Bai Y (2020) CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene *PMR4* for resistance against powdery mildew. BMC Plant Biol 20:284. https://doi.org/10.1186/s12870-020-02497-y

31. Tang X, Liu G, Zhou J, Ren Q, You Q, Tian L, Xin X, Zhong Z, Liu B, Zheng X, Zhang D, Malzahn A, Gong Z, Qi Y, Zhang T, Zhang Y (2018) A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. Genome Biol 19:84. https://doi.org/10.1186/s13059-018-1458-5

32. Tian SW, Jiang LJ, Cui XX, Zhang J, Guo SG, Li MY, Zhang HY, Ren Y, Gong GY, Zong M, Liu F, Chen QJ, Xu Y (2018) Engineering herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing. Plant Cell Rep 37:1353–1356. https://doi.org/10.1007/s00299-018-2299-0

33. Wang F, Wang C, Liu P, Lei C, Hao W, Gao Y, Liu YG, Zhao K (2016) Enhanced Rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene *OsERF922*. PLoS ONE 11:e0154027. https://doi.org/10.1371/journal.pone.0154027

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

35. Xu J, Hua K, Lang Z (2019) Genome editing for horticultural crop improvement. Hortic Res 6:113. https://doi.org/10.1038/s41438-019-0196-5

36. Zaman QU, Li C, Cheng HT, Hu Q (2019) Genome editing opens a new era of genetic improvement in polyploid crops. Crop J 7:141–150. https://doi.org/10.1016/j.cj.2018.07.004

37. Zhang D, Zhang Z, Unver T, Zhang B (2021) CRISPR/Cas: A powerful tool for gene function study and crop improvement. J Adv Res 29:207–221. https://doi.org/10.1016/j.jare.2020.10.003

38. Zheng Z, Appiano M, Pavan S, Bracuto V, Ricciardi L, Visser RG, Wolters AM, Bai Y (2016) Genome-wide study of the tomato *SlMLO* gene family and its functional characterization in response to the powdery mildew fungus *Oidium neolycopersici*. Front Plant Sci 7:380. https://doi.org/10.3389/fpls.2016.00380

39. Zheng Z, Nonomura T, Appiano M, Pavan S, Matsuda Y, Toyoda H, Wolters AM, Visser RG, Bai Y (2013) Loss of function in *Mlo* orthologs reduces susceptibility of pepper and tomato to powdery mildew disease caused by *Leveillula taurica*. PLoS ONE 8:e70723. https://doi.org/10.1371/journal.pone.0070723

Tables

Table 1

Frequencies of CRISPR/Cas9-induced mutation in the T_0 transgenic tobacco plants

Variety	Total number of plants[a] tested	Number of *NtMLO1* mutated plants	Number of *NtMLO2* mutated plants	Number of *NtMLO1* and *NtMLO2* mutated plants	Number of plants without mutation
ZY300	20	13 (65.0%)[b]	12 (60.0%)	10 (50.0%)	5 (25.0%)

[a] Referred to transgene-positive plants

[b] Frequencies based on the number of mutated plants over the total number of transgene plants tested.
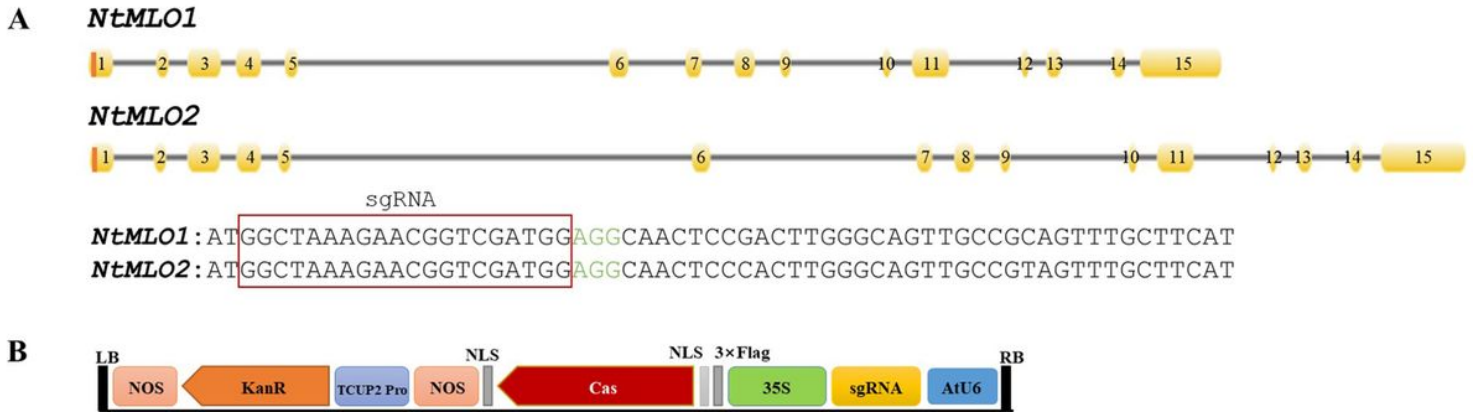
Figures

Figure 1

Schematic representation of CRISPR/Ca9-mediated mutagenesis of *NtMLO1* and *NtMLO2* in common tobacco. A. CRISPR/Cas9-targeted site selected within a conserved region of exon 1 of tobacco *NtMLO* homoeologs. The target site is shown in a box, and the protospacer adjacent motif (PAM) sequence is highlighted in green. B. The pORE-Cas-gRNA-MT construct for tobacco transformation.
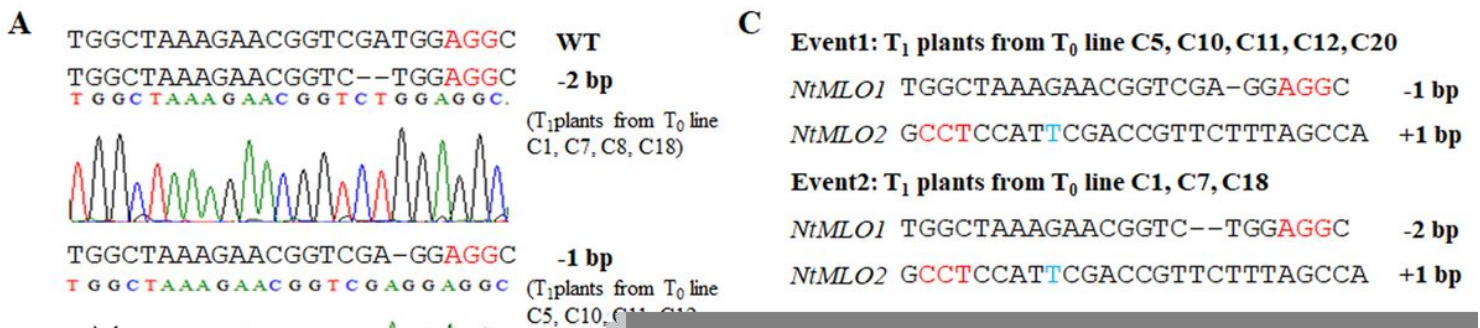

Figure 2

Targeted mutagenesis of *NtMLO* genes using the CRISPR/Cas9 system. A. The sequences and Sanger sequencing chromatograms show the homozygous *NtMLO1* mutations in T_1 transgenic plants. B. The sequences and Sanger sequencing chromatograms show the homozygous *NtMLO2* mutations in T_1 transgenic plants. C. Two *NtMLO* genes with simultaneous homozygous mutation events identified by Sanger sequencing in the T_1 transgenic plants. Each dashed line represents a deleted nucleotide. The letter in blue represents an inserted nucleotide. The numbers on the right side indicate the involved; "-" and "+" indicate deletion and insertion, respectively. The numbers in the bracket represent corresponding T_0 transgenic lines. PAM motif is marked in red. WT: Wild type.
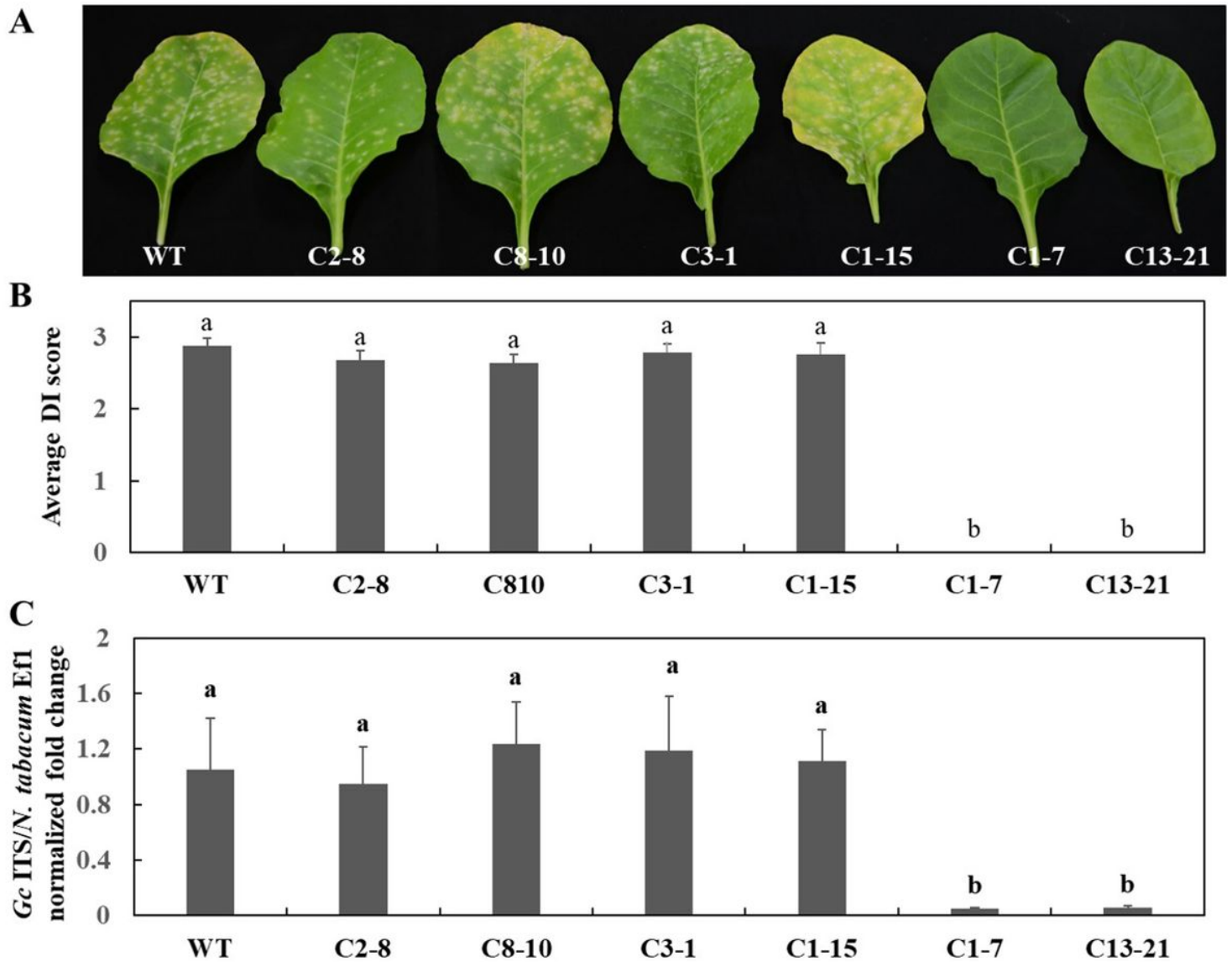

Figure 3

Phenotype of the *NtMLO* mutants after *Golovinomyces cichoracearum* infection. A. Symptoms on the representative leaves of a wild-type plant (WT) and the indicated mutants 20 days after inoculation. B. Average disease index score of the mutant lines. C. Relative fungal biomass calculated as the ratio of fungal *ITS* gene to the tobacco *Ef1a* and normalized with the WT values. WT, transgenic line C4 obtained

from the same CRISPR transformation experiment with the wild-type alleles; C2-8 and C8-10, T_2 lines with homozygous mutations in *NtMLO1* only; C3-1 and C1-15, T2 lines with homozygous mutations in *NtMLO2* only; C1-7 and C13-21, T2 lines with homozygous mutations in *NtMLO1* and *NtMLO2*. Values shown are the mean ± SD of at least ten individual plants per line. Means followed by the same letter are not significantly different at P ≤ 0.05, according to Tukey's test.

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