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CRISPR/Cas9-Targeted Mutagenesis of Arabidopsis *DMR6* Orthologs in Tobacco Confers Resistance to *Phytophthora nicotianae*

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Abstract

Phytophthora nicotianae is an oomycete pathogen that severely threatens tobacco production worldwide. Though several dominant resistance (R) genes against *P. nicotianae* are used in tobacco breeding, they often fail due to rapid emergence of new virulent strains. Instead, targeting plant susceptibility (S) genes offers a promising approach for durable and broad resistance. Evidence from various plant species demonstrates that loss of the S gene DMR6 enhances disease resistance without compromising yield, emphasizing its value for resistance breeding. In this study, we identified and functionally characterized two DMR6 orthologs in tobacco (*Nicotianan tobaccum*), NtDMR6T and NtDMR6S, which were both induced upon *P. nicotianae* infection. Targeted mutagenesis of NtDMR6T and NtDMR6S using CRISPR/Cas9 demonstrated that single-gene knockouts conferred enhanced resistance to *P. nicotianae*, while double mutants exhibited an additive resistance effect. Notably, all mutant lines showed no obvious growth or developmental defects under greenhouse or field conditions. RT-qPCR analysis indicated that NtDMR6s negatively regulate tobacco resistance by modulating multiple defense pathways, including the MAPK signaling cascade. Collectively, our findings demonstrate that NtDMR6T and NtDMR6S act as negative regulators of resistance in allotetraploid tobacco and represent promising S gene targets for the development of *P. nicotianae* resistant cultivars, thereby providing a new strategy for tobacco disease resistance breeding.

Keywords: tobacco; *DMR6*; *Phytophthora nicotianae*; CRISPR/Cas9 technologys

1. Introduction

Phytophthora nicotianae is a soil-borne bi-flagellated oomycete, responsible for significant losses on a number of economically important species including fruit, oilseed, vegetables, and ornamental plants [1,2]. In tobacco, *P. nicotianae* can infect all parts of the plant at any growth stage, causing severe damage under both greenhouse and field conditions [3]. The infection typically begins in the roots, which become water-soaked and rapidly turn necrotic. As the disease progresses, leaves develop large circular lesions or turn brown to black. Because one of the most characteristic symptoms is blackening of the stalk base or shank, the disease is called black shank in tobacco [4].

Based on the ability to infect various cultivars carrying different resistance genes, four physiological races (0-3) of *P. nicotianaea* were reported [5]. In tobacco resistance breeding programs, two commonly mentioned resistant germplasms, Florida 301 and Beinhart 1000 (BH), have played important roles in the development of black shank-resistant cultivars [6–8]. However, both

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germplasms exhibit limitations in terms of resistance durability and breeding efficiency. In addition to these sources, single dominant resistance (*R*) genes, *Php* and *Phl*, have been successfully introgressed into cultivated tobacco through distant hybridization [4]. While these genes confer immunity to race 0 of *P. nicotianaea*, they are ineffective against other physiological races [9–11]. Similarly, another *R* gene, *NpPP2-B10*, has been shown to provide resistance that is also race-specific [12].

As *R* gene-mediated resistance relies on the recognition of specific pathogen effectors and the activation of downstream defense responses, which are often race-specific and prone to being overcome by rapidly evolving pathogens, an alternative strategy involves loss-of-function mutations in susceptibility (*S*) genes. All plant genes that facilitate infection can be considered as *S* genes [13] and disrupting these genes may interfere with the compatibility between the host and the pathogens, which can provide broad-spectrum and potentially more durable disease resistance [14–17]. One typical *S* gene is *MLO* (Mildew Locus O), which has been used in a wide range of plant crops such as apples, barley, cucumbers, grapevines, melons, peas, tomatoes, and wheat [18]. Other *S* genes, like *StDND1* and *StDMR6* in potato, showed enhanced late blight resistance upon gene knockdown [19]. Although disruption of *S* genes seems a faster and simpler way to achieve broad-spectrum and durable disease resistance, pleiotropic effects caused by their inactivation need to be evaluated and prevented [20].

Arabidopsis *DMR6,* a member of the 2-oxoglutarate Fe (II) dependent oxygenases (2OG oxygenases) superfamily, has been identified as an *S* gene as the over-expression of *AtDMR6* increases disease susceptibility. The study also shows that this loss-of-function mutants of *dmr6* exhibit enhanced resistance to multiple pathogens, but slightly affects plant development, which is associated with increased levels of salicylic acid (SA) content [21]. Subsequent research disclosed that *AtDMR6* encodes a salicylic acid 5-hydroxylase that hydroxylates SA, further establishing a mechanistic link between *dmr6*-mediated resistance and SA accumulation [22]. Comparable to Arabidopsis, knocking-out the homologs of *DMR6* in potato and tomato resulted in enhanced disease resistance, without any negative pleiotropic or growth retardation effect [16,18]. These findings indicate that *DMR6* represents a promising target for the genetic engineering of broad-spectrum and durable disease resistance in different crop species.

In this study, we identified two DMR6 orthologs in tobacco (*Nicotiana tabacum*), NtDMR6T and NtDMR6S, and generated targeted mutants using CRISPR/Cas9 to assess their breeding potential and investigate their downstream regulatory networks. Loss of either gene conferred enhanced resistance, and RT-qPCR analyses revealed that genes associated with mitogen-activated protein kinase (MAPK) signaling, energy metabolism, peroxidase activity, and phenylpropanoid biosynthesis were significantly affected. These results suggest that NtDMR6s modulate host immunity through multiple pathways, thereby broadening our understanding of *S* gene functions and underscoring the regulatory role of DMR6 in plant–pathogen interactions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Wild type tobacco (*Nicotiana tabacum* L. cv. Honghuadajinyuan, HD) was provided by the Tobacco Research Institute of Chinese Academy of Agricultural Sciences (Accession number 00000540). For greenhouse cultivation, tobacco seedlings of the *NtDMR6T* and *NtDMR6S* mutants and the wild type HD were transplanted into plastic pots filled with potting soil two weeks after sowing. Plants were grown in a controlled growth chamber under a 16 h light/8 h dark photoperiod at 25 °C. After an additional two weeks of growth, plants were used for subsequent pathogen inoculation experiments. For field trials, sixteen-week-old seedlings were transplanted into plots at the Jimo Experimental Station, Tobacco Research Institute, Chinese Academy of Agricultural Sciences (35.4°N, 119.3°E, 15 m altitude). The field layout included 10 m row lengths, with a row spacing of 1.2 m and a plant spacing of 0.5 m.

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2.2. Construction of Phylogenetic Tree

Protein sequences containing the 2-oxoglutarate Fe(II)-dependent oxygenase domain (Pfam accession: PF03171) were retrieved from InterPro [23]. Candidate 2OG oxygenase proteins in the *Nicotiana tabacum* ZY300 reference genome (NCBI: PRJNA940510) [24] were subsequently identified using HMMER 3.0 [25] and further validated via the NCBI Batch CD-Search tool. Functionally characterized 2OG oxygenase protein sequences in *Arabidopsis thaliana* were obtained from TAIR (https://www.arabidopsis.org/). Homologous sequences of DMR6 in *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potato) were retrieved from NCBI. The phylogenetic trees (Figure 1; Figure 2a) were constructed using MEGA 7.0 with the neighbor-joining (NJ) method and then displayed with the online iTOL tool (https://itol.embl.de/).

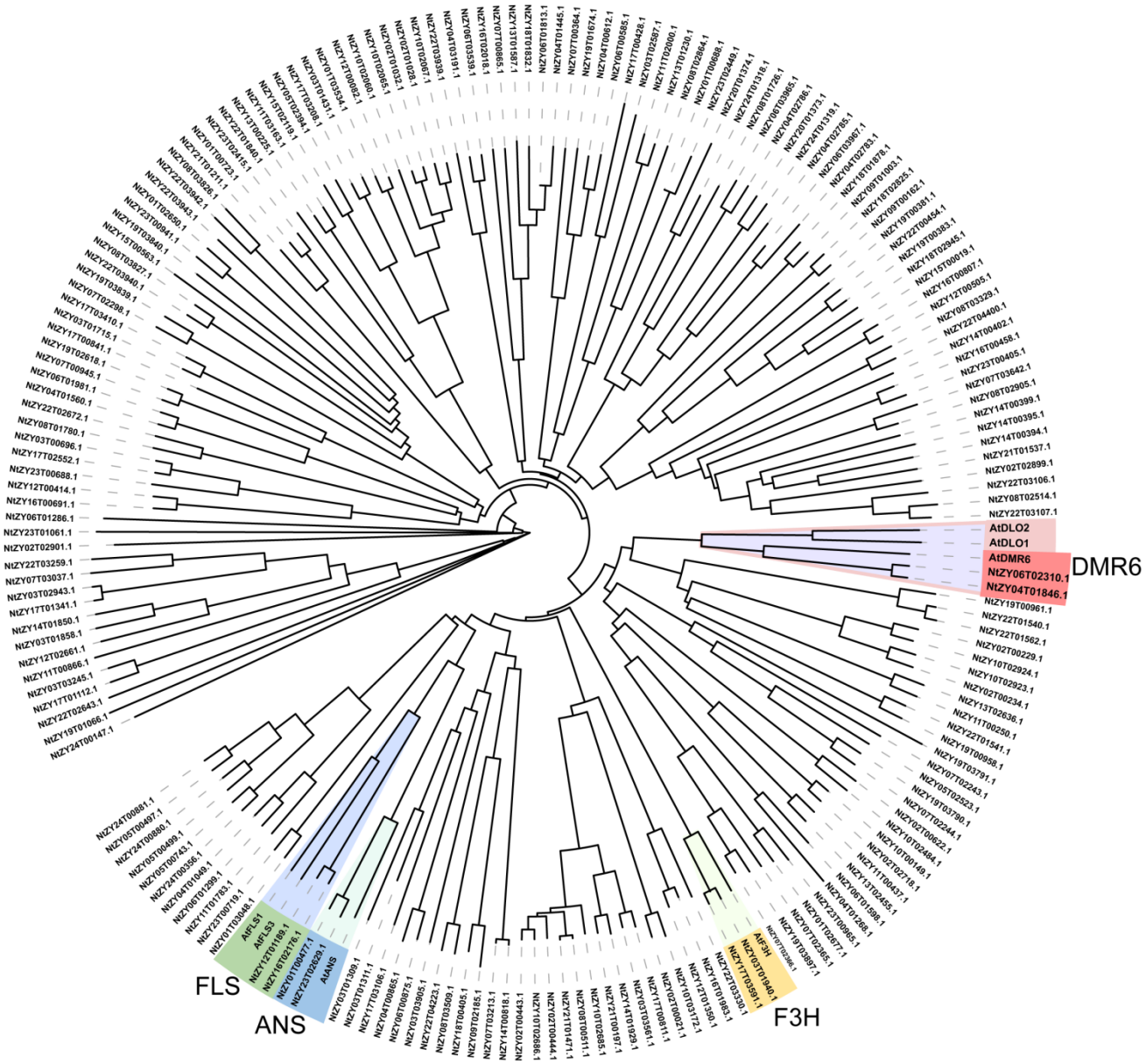

Figure 1. Identification of AtDMR6 orthologs in tobacco. Phylogenetic tree of the 2OG oxygenases (Pfam domain PF03171) of *Nicotiana tabacum* with functionally known 2-ODD members in Arabidopsis, including AtDMR6, AtDLO, AtFLS, AtANS, and AtF3H.

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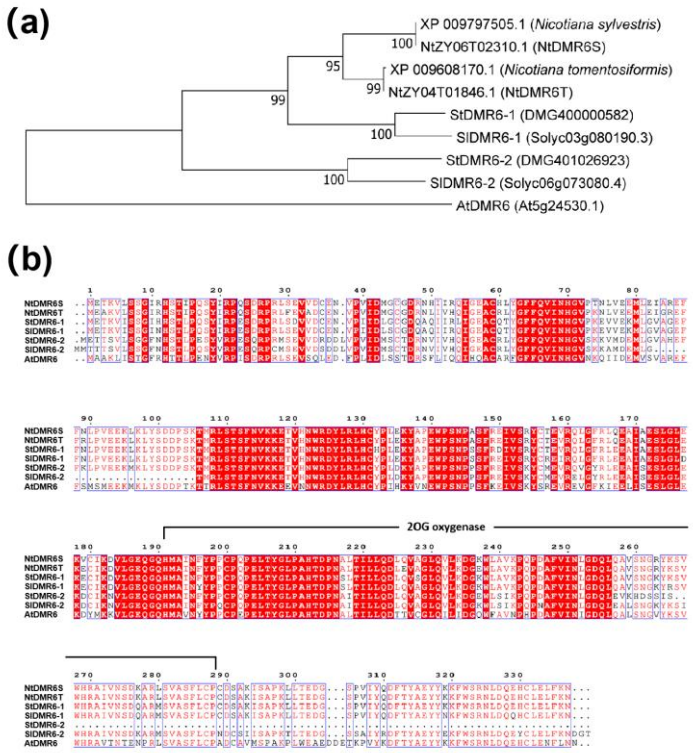

Figure 2. Phylogenetic and sequence alignment analyses of DMR6 orthologs. (**a**) Phylogenetic tree of DMR6 orthologs in different plant species, including proteins from *N. tabacum*, *N. tomentosiformis*, *N. sylvestris*, *S. lycopersicum*, *S. tuberosum*, and *A. thaliana*. The scale bar indicates amino acid substitutions per site. Bootstrap support values based on 100 replicates are shown at the nodes. (**b**) Protein sequence alignment of NtDMR6T and NtDMR6S with AtDMR6, SlDMR6-1, SlDMR6-2, StDMR6-1, and StDMR6-2. The conserved 2OG oxygenase domain (Pfam: PF03171) is indicated.

2.3. Promoter Analysis

The 2-kb promoter regions upstream of *NtDMR6T* and *NtDMR6S* were retrieved from the *Nicotiana tabacum* ZY300 reference genome (NCBI: PRJNA940510) [24]. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze *cis*-acting elements within these promoter sequences [26].

2.4. Construction of the CRISPR/Cas9 Plasmids and Mutant Construction

Codon optimized hSpCas9 was linked to the maize ubiquitin promoter (UBI) in an intermediate plasmid, and then this expression cassette was inserted into binary pCAMBIA1300 (Cambia, Australia) which contains the *HPT* (hygromycin B phosphotransferase) gene. The originally existing *Bsa*I site in the pCAMBIA1300 backbone was removed using a point mutation kit (Transgen, China). A fragment comprised of a OsU6 promoter, a negative selection marker gene ccdB flanked by two *Bsa*I sites and a sgRNA derived from pX260 was inserted into this vector using the In-fusion cloning kit (Takara, Japan) to produce the CRISPR/Cas9 binary vector pBGK032. *E. coli* strain DB3.1 was used for maintaining this binary vector.

The 23-bp targeting sequences (Table S1) were selected within the target genes and their targeting specificity was confirmed using a Blast search against the tobacco genome (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The designed targeting sequences were synthesized and annealed to form the oligo adaptors. Vector pBGK032 was digested by *Bsa*I and purified using DNA purification kit (Tiangen, China). A ligation reaction (10 μL) containing 10 ng of the digested

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pBGK032 vector and 0.05 mM oligo adaptor was carried out and directly transformed to *E. coli* competent cells to produce CRISPR/Cas9 plasmids.

The constructed CRISPR/Cas9 plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105. Tobacco transformation was performed following the protocol described by Sparkes et al. [27], using HD as the recipient genotype. Genomic DNA was extracted from the regenerated transformants, and the regions flanking the target sites were amplified by PCR using specific primer pairs (Table S1). The resulting PCR products were sequenced to confirm genome editing events.

2.5. Pathogen Inoculation and Resistance Evaluation

Phytophthora nicotianae (race 0), originally isolated from infected tobacco stems in Jimo, Qingdao, Shandong Province in 2011, was cultured on oatmeal medium for 14 days. To induce zoospore release, 15 mL of 0.1% KNO_3 solution was added to each plate, followed by chilling at 4 °C for 20 min and incubation at 25 °C for 25 min. The resulting zoospore suspension was collected and adjusted to 1×10^5 cfu·mL^{-1}. One-month-old tobacco seedlings were immersed in the zoospore suspension in the dark at 25 °C for 3 h, then transferred to new Petri dishes containing 10 mL of sterile water to immerse the roots. Two layers of filter paper were placed over the roots to maintain a good moisture content. Plants were grown at 25 °C under a 16 h light/8 h dark photoperiod. Disease severity was scored on a 0-9 scale, and the disease index was calculated using a standard formula. Meanwhile, root samples were independently collected at 0 h, 12h, 24h, 48 h, 72h post-inoculation, and these samples were subsequently stored at –80 °C. The experiment was independently repeated three times with consistent results.

$$\text{Disease index} = \frac{(a \times 0) + (b \times 1) + (c \times 3) + (d \times 5) + (e \times 7) + (f \times 9)}{a + b + c + d + e + f} \times \frac{100}{9}$$

2.6. cDNA Synthesis and RT-qPCR

Total RNA (1 μg) was reverse-transcribed using the HiScript III Reverse Transcriptase kit (Vazyme, China). Quantitative real-time PCR was performed with the SYBR Premix Ex Taq kit (TaKaRa, Japan) on a LightCycler 96 system (Roche Diagnostics, Switzerland) in 20-μL reactions. The composition of each PCR reaction was as follows: 10 μL master mix, 7 μL ddH2O, 1 μL cDNA, and 1 μL of both forward and reverse primers (5 μM). The RT-qPCR assay was performed three times with the following conditions: 95 °C for 30 s initially, then 40 cycles consisting of 95 °C for 10 s and 60 °C for 15 s. Relative expression levels were computed via the $2^{-\Delta\Delta Ct}$ method. *NtActin* (*NtZY23T00337.1*) was used as an internal reference for normalization. Each assay included three biological replicates. Primer sequences are listed in Table S1.

2.7. Measurement of Tobacco Agronomic Traits

To determine the effects of *NtDMR6* impairment on plant growth in the greenhouse, the plant height (calculated as the height of the stem from the soil surface to stem apex) of 15 plants of each mutant (DC6198L, DC6527L, DC6229L) and 15 wild type (HD) plants were measured using a tape measure. To assess the effects of *NtDMR6* impairment on plant growth under field conditions, four agronomic traits—plant height, leaf number, leaf length, and leaf width—were measured at maturity, approximately sixteen weeks after sowing.

2.8. Statistical Analysis

GraphPad Prism (GraphPad Software, USA) was used to perform Student's *t*-test (Table S2) and to generate histograms and other relevant graphical representations. A *P*-value less than 0.05 ($P < 0.05$) was considered statistically significant.

2.9. Gene Accession Numbers

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NtDMR6T (NtZY04T01846.1), NtDMR6S (NtZY06T02310.1), AtDMR6 (AT5G24530), AtDLO (AT4G10490, AT4G10500), AtFLS1 (AT5G08640), AtFLS3 (AT5G63590), AtANS (AT4G22880), AtF3H (AT3G51240), WRKY6 (NtZY03T00604.1), WRKY46 (NtZY21T01503.1), PR1 (NtZY07T00599.1), NPR1 (NtZY10T03125.1), RRS1 (NtZY21T01803.1), PSAF (NtZY03T03735.1), PNSL4 (NtZY11T01075.1), ARFA (NtZY13T01719.1), TCP4 (NtZY02T01123.1), MKK4 (NtZY22T03510.1), MPK3 (NtZY17T00471.1), MPK7 (NtZY17T03798.1), PCK1 (NtZY12T02043.1), PDHC (NtZY23T00063.1), PER1 (NtZY24T00949.1), PER2 (NtZY05T00437.1), CAMT3 (NtZY17T01647.1), CAS1 (NtZY01T02870.1).

3. Results

3.1. Identification of Tobacco DMR6 Orthologs

To identify the DMR6 orthologs in tobacco, we performed a phylogenetic analysis of the 2-oxoglutarate (2OG) oxygenase protein family (Pfam domain PF03171) in *Nicotiana tabacum* [28], in comparison with functionally characterized members in *Arabidopsis thaliana*. A total of 235 members of the tobacco 2OG superfamily, along with several well-characterized 2-ODD members from Arabidopsis, including AtDMR6, AtDLO, AtFLS, AtANS, and AtF3H, were selected to construct the phylogenetic tree with the protein sequences (Table S3). Based on the resulting tree, two tobacco homologs, NtZY06T02310.1 and NtZY04T01846.1, were identified as putative orthologs of AtDMR6, as they clustered within the same clade (Figure 1). No DLO orthologs were identified in tobacco, which is consistent with previous work in tomato [29].

3.2. Phylogenetic and Sequence Alignment Analysis of NtDMR6 Orthologs

Tobacco is a natural allotetraploid species derived from the hybridization of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. To trace the evolutionary origin of the two identified orthologous proteins, we performed phylogenetic analyses using their corresponding homologs from *N. sylvestris* (XP_009797505.1) and *N. tomentosiformis* (XP_009608170.1). Meanwhile, considering that in potato and tomato only DMR6-1 is responsive to pathogen infection, whereas DMR6-2 lacks this function [18,29], we incorporated these homologs into the phylogenetic tree to clarify their evolutionary relationships and potential functional divergence. The results indicated that NtZY06T02310.1 (NtDMR6S) originated from *N. sylvestris*, while NtZY04T01846.1 (NtDMR6T) was derived from *N. tomentosiformis*. Both NtDMR6S and NtDMR6T clustered more closely with DMR6-1 homologs than with DMR6-2 homologs in other species (Figure 2a). Sequence alignment further showed that NtDMR6T shares 96.14% identity with NtDMR6S, and both homologs exhibit approximately 67% identity to AtDMR6. In addition, the 2OG-Fe(II) oxygenase domain was found to be highly conserved across all four species (Figure 2b).

3.3. NtDMR6T and NtDMR6S Are Up-Regulated upon Pathogen Inoculation

Based on the possibility of homologous gene silencing in allotetraploid plant [30,31], we further tested whether both NtDMR6T and NtDMR6S were expressed in tobacco tissues. RT-qPCR analysis showed that transcripts of both genes were detectable in major tissues, with relatively higher expression levels in stems (Figure 3a). We next examined whether NtDMR6T and NtDMR6S respond to pathogen infection. The expression levels of both genes were up-regulated following inoculation with P. nicotianae (Figure 3b), suggesting that they may play functional roles in the plant's defense response. To understand the regulation patterns of NtDMR6S and NtDMR6T, cis-acting elements located on the sequences of 2000 bp upstream regions from the translation start site of each NtDMR6 were determined using PlantCARE. A total of 31 types of elements were found in the promoter regions, and the most enriched were the core and common promoter elements, CAAT and TATA boxes (Figure 3c; Table S4). Based on previous study in cucumber [32], we categorized the cis-acting elements into five functional groups: plant growth, stress response, hormone response, light

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response, and other elements. Interestingly, the two tobacco genes exhibit different patterns of cis-element enrichment. NtDMR6T is primarily enriched with elements related to stress and hormone responses, including MYB, MYC, CGTCA-motif, ERE, and TGACG-motifs. In contrast, NtDMR6S is mainly enriched with light-responsive elements, such as Box 4 and GATA-motifs. This suggests that although both genes respond to pathogen infection, they may be regulated by different upstream signaling pathways.

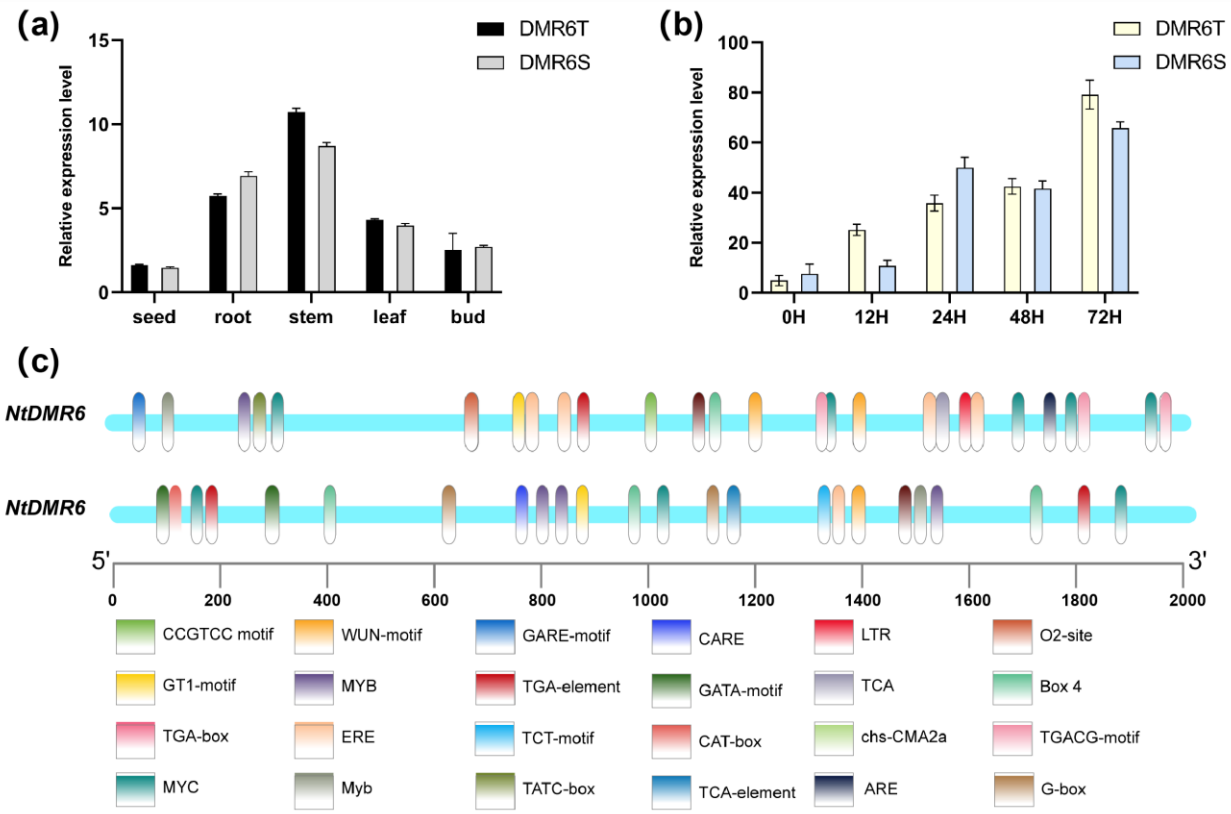

Figure 3. Gene expression patterns of *NtDMR6T* and *NtDMR6S*. (**a**) Expression pattern of *NtDMR6T* and *NtDMR6S* in different tissues, including seed, root, stem, leaf, and bud. (**b**) Time-course expression of *NtDMR6T* and *NtDMR6S* following inoculation with *P. nicotianae* (race 0) at 0, 12, 24, 48, and 72 h post-infection. (**c**) Distribution of *cis*-acting elements in the promoter regions of the two *NtDMR6* genes. Colored rectangles represent different *cis*-elements.

3.4. CRISPR/Cas-9-Targeted Mutagenesis of NtDMR6s

Given that both *NtDMR6s* respond to pathogen invasion, we employed the CRISPR/Cas9 system to induce mutations in the *NtDMR6s*. Based on the high DNA sequence similarity within their conserved domains, we designed two specific guide RNAs (gRNAs) targeting the exon 3 of each *NtDMR6s* and transformed them into the susceptible tobacco cultivar HD (Figure 4a). A total of 25 T_1 transgenic plants were obtained; however, no homozygous mutants were identified in this generation. To obtain homozygous lines, we analyzed T_2 plants derived from self-pollinated T_1 transgenics. Mutation events were identified by sequencing PCR fragments amplified with specific primers (Table S1). Three representative events, covering both single- and double-gene mutations (Table 1), were selected, and their self-pollinated T_3 progeny were used for subsequent experiments. Event 1 is a single mutant of *NtDMR6T*, designated DC6198L (Figure 4b); Event 2 is a single mutant of *NtDMR6S*, designated DC6527L (Figure 4c); and Event 3 is a double knockout mutant of *NtDMR6T* and *NtDMR6S*, designated DC6229L (Figure 4d). All mutations were predicted to result in premature termination codons in the respective transcripts.

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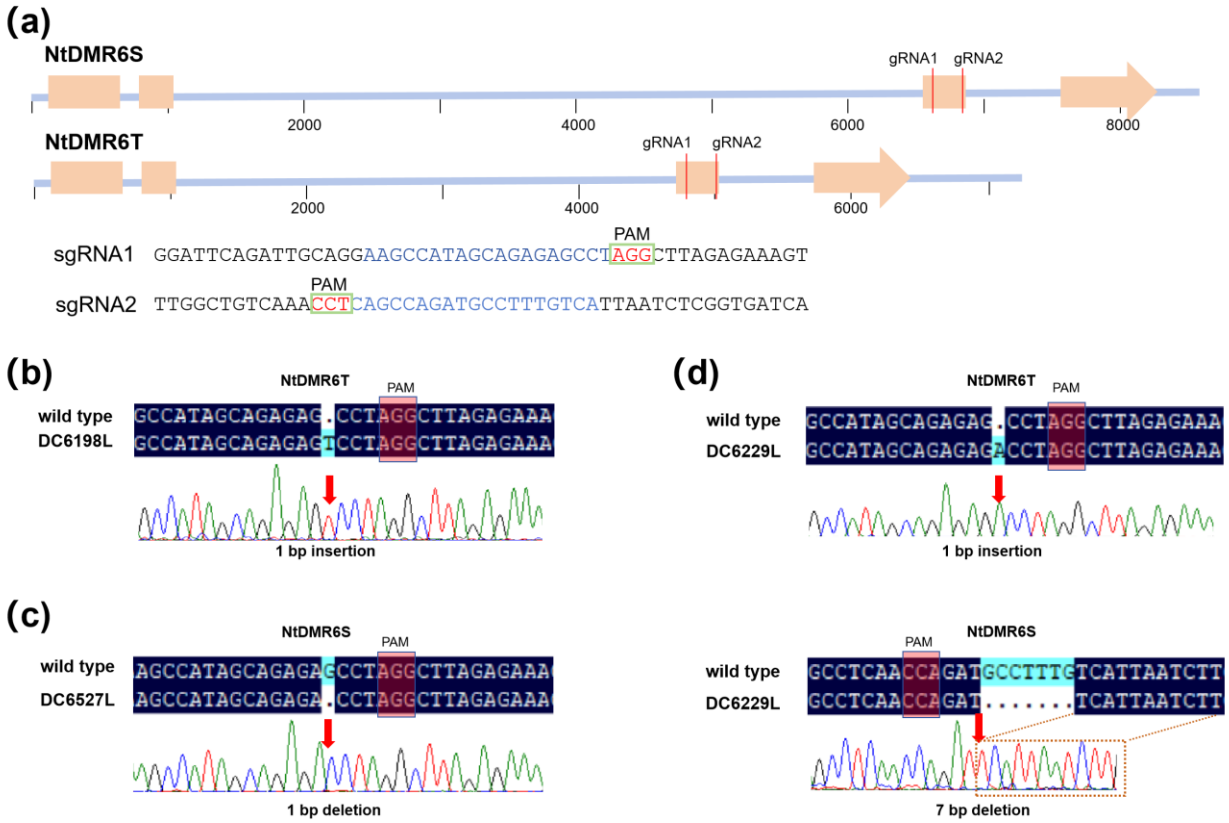

Figure 4. Schematic representation of *NtDMR6*-specific gRNAs and mutant alleles. (**a**) Genomic structure of *NtDMR6S* and *NtDMR6T* showing the positions of two gRNAs designed to generate knockout mutants. The target sequences of gRNA1 and gRNA2 are shown, with the PAM sequence highlighted. (**b**) DC6198L, a homozygous line carrying a 1-bp insertion in *NtDMR6T*, while *NtDMR6S* remains unchanged. (**c**) DC6527L, a homozygous line carrying a 1-bp deletion in *NtDMR6S*, while *NtDMR6T* remains unchanged. (**d**) DC6229L, a homozygous line carrying a 1-bp insertion in *NtDMR6T* and a 7-bp deletion in *NtDMR6S*. The arrows indicate the positions of insertions or deletions that result in frame-shift mutations in the protein sequence.

Table 1. Overview of the mutation events in the tobacco NtDMR6 CRISPR mutants.

Event	*NtDMR6T*	*NtDMR6S*	T_2-Plants	T_3-Lines
1	1-bp (T) insertion at gRNA1	no mutation	DC6198	DC6198L
2	no mutation	1-bp (G) deletion at gRNA1	DC6527	DC6527L
3	1-bp (A) insertion at gRNA1	7-bp deletion at gRNA2	DC6229	DC6229L

3.5. Characterization of Disease Phenotypes of Ntdmr6 Mutants

To evaluate whether *Ntdmr6* mutant lines showed reduced susceptibility against *P. nicotianae*, wild type HD and all three type of *Ntdmr6* mutants (DC6198L, DC6527L, DC6229L) were infected with *P. nicotianae* in the greenhouse conditions. Inoculation was performed using the suspension of *P. nicotianae* zoospores at 1×10^5 zoospores/mL. At 5 days post inoculation, HD displayed more intense symptoms, the basal part of stem was severely necrotic and became black (Figure 5a). Relative to HD, the mutants DC6198L, DC6527L, and DC6229L displayed significantly enhanced resistance to *P. nicotianae*, as evidenced by the presence of only a few wilted leaves and limited stem necrosis (Figure 5b-d). The average disease index (DI) was significantly reduced in the *Ntdmr6* mutants compared to the HD. Moreover, double knockout mutant DC6229L showed lower DI than single gene mutants DC6198L and DC6527L (Figure 5e).

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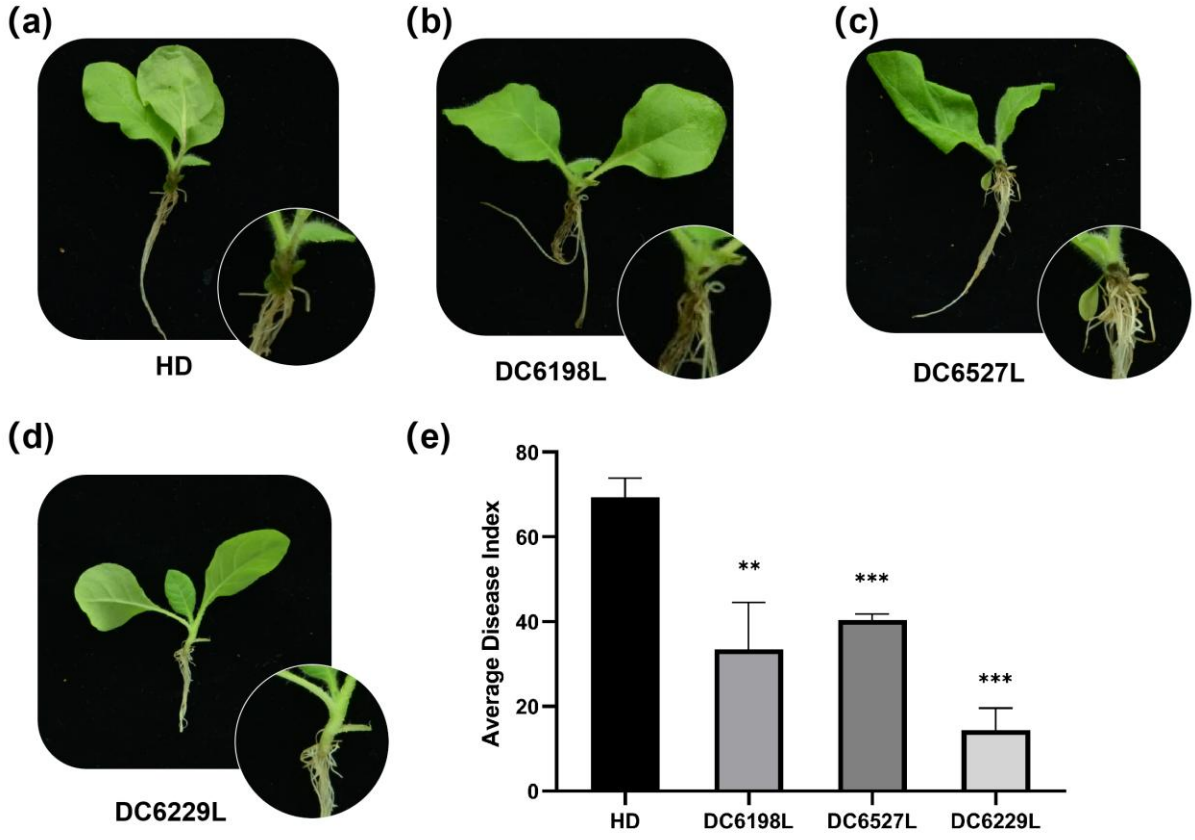

Figure 5. Wild type HD and *Ntdmr6* mutant lines infected with *P. nicotianae* at 5 days post inoculation. (**a–d**) Disease symptoms of HD and *Ntdmr6* mutant lines, DC6198L, DC6527L, and DC6229L. Inoculation was performed using the suspension of *P. nicotianae* zoospores at 10^5 zoospores/mL. (e) Average disease index of HD and *Ntdmr6* mutant lines. **, $P < 0.005$; ***, $P < 0.001$, Student's *t*-test.

3.6. Characterization of Growth Traits of Ntdmr6 Mutants

To assess the breeding potential of *Ntdmr6* mutants, we examined the effects of *Ntdmr6s* disruption on plant growth. Plant height was measured under greenhouse conditions for each mutant line and the wild type HD. No statistically significant differences in height were observed among the three *Ntdmr6* mutants compared to the wild type, indicating that loss of *NtDMR6* function does not adversely affect overall plant growth (Figure S1).

Further morphological characteristics of the *Ntdmr6* mutants were evaluated under field conditions. Compared to the wild type HD, none of the three *Ntdmr6* mutant lines exhibited adverse morphological effects (Figure 6a). Consistent with the greenhouse results, the plant heights of the *Ntdmr6* mutants in field conditions were comparable to or even greater than that of the wild type HD (Figure 6b), indicating that disruption of *NtDMR6* does not adversely affect plant growth under natural conditions. Given that the primary economic value of tobacco is derived from leaf yield, we further investigated leaf-related traits in the mutant lines. While the number and length of leaves remained largely unchanged (Figure 6c,d), all three mutants displayed wider leaves than HD (Figure 6e), suggesting a potential increase in above-ground biomass. Taken together, these results indicate that *NtDMR6* knockout has no detrimental effect on plant morphology and may even enhance tobacco leaf yield, underscoring its potential value in breeding programs aimed at improving both disease resistance and agronomic performance.

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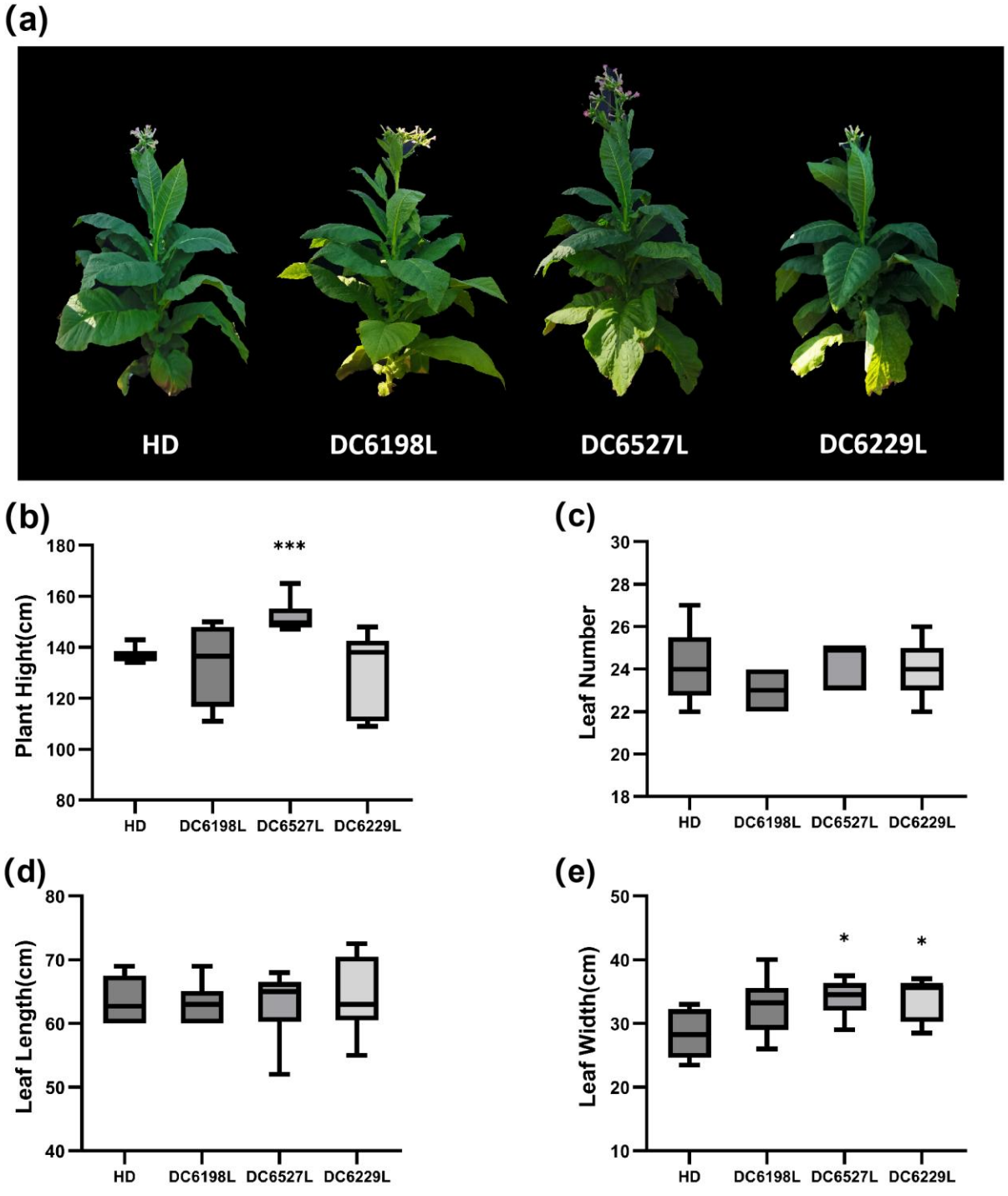

Figure 6. Morphological characteristics of wild type HD and *Ntdmr6* mutant lines in the field. (**a**) Growth phenotype of HD and *Ntdmr6* mutant lines. (**b**) Plant height, (**c**) leaf number, (**d**) leaf length, and (**e**) leaf width of HD and *Ntdmr6* mutant lines. *, $P < 0.05$; ***, $P < 0.001$, Student's *t*-test.

3.7. Ntdmr6 Mutants Display Enhanced Activation of Defense Genes upon P. nicotianae Infection

In tomato, loss of *SlDMR6-1* results in elevated SA levels and up-regulation of immune response genes [29]. To investigate whether *Ntdmr6s* have a similar role in tobacco, we compared gene expression between wild-type HD and the double knockout mutant DC6229L at 0 h post inoculation (hpi) with *P. nicotianae*. RT-qPCR data showed that in DC6229L, immunity related genes (*WRKY6*, *PR1*, *RRS1*, *NPR1*) were modestly up-regulated, indicating that plant defenses are activated under normal conditions. Meanwhile, the expression of growth-related genes, such as *PSAF*, *ARFA*, and

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TCP4, did not differ between HD and DC6229L (Figure 7a). To further assess the regulatory consequences of *NtDMR6s* disruption, the expression differences between HD and DC6229L at 48 hpi were compared. MAPK pathway genes, such as *MKK4*, *MPK3*, and *MPK7*, were strongly induced in DC6229L compared with HD. In addition, genes associated with energy metabolism (*PCK1*, *PHDC*), peroxidase activity (*PER1*, *PER2*), and phenylpropanoid biosynthesis (*CAMT3*, *CAS1*) were also up-regulated (Figure 7b). These findings indicate that *NtDMR6s* promote disease susceptibility by repressing multiple defense pathways, and that their loss primes tobacco for enhanced defense responses upon pathogen challenge.

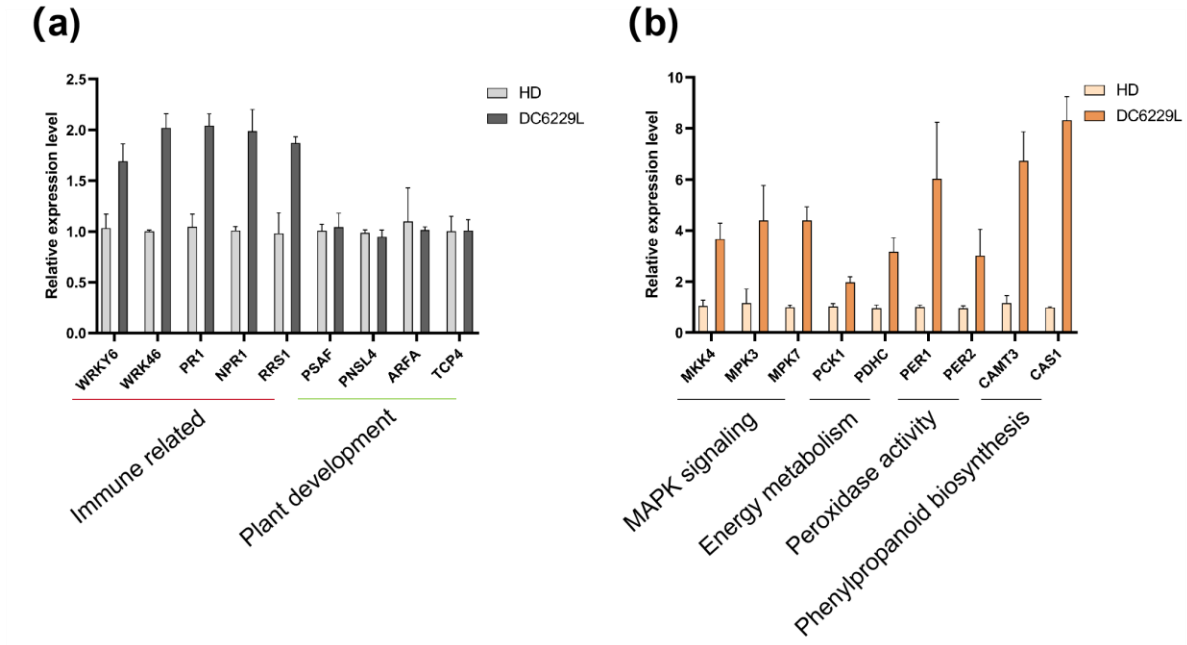

Figure 7. RT-qPCR analysis of selected genes in wild-type HD and DC6229L mutant after P. nicotianae inoculation. (**a**) Relative expression levels of immune related genes (*WRKY6*, *WRKY46*, *PR1*, *NPR1*, *RRS1*) and plant development related genes (*PSAF*, *PNSL4*, *ARFA*, *TCP4*) at 0 hpi, as determined by RT-qPCR. (**b**) Relative expression levels of genes associated with MAPK signaling (*MKK4*, *MPK3*, *MPK7*), energy metabolism (*PCK1*, *PDHC*), peroxidase activity (*PER1*, *PER2*), and phenylpropanoid biosynthesis (*CAMT3*, *CAS1*) at 48 hpi. Error bars indicate standard deviations ($n = 3$).

4. Discussion

Nicotiana tabacum (2n = 4x = 48) is a natural allotetraploid resulting from genome duplication. As a consequence, many gene copies are retained, some of which may maintain similar functions, while others may have become silenced through mutations or epigenetic modifications [33]. For example, the *NtAn1* family members *NtAn1a* (originated from *N. sylvestris*) and *NtAn1b* (originated from *N. tomentosiformis*) appeared to be functionally and spatiotemporally redundant [34]. A pair of plant natural resistance-associated macrophage protein (*NRAMP*) homologous genes, *NtNRAMP6a* (originated from *N. tomentosiformis*) and *NtNRAMP6b* (originated from *N. sylvestris*) mainly affected the transport of cadmium from roots to shoots. But *NtNRAMP6a* had manganese transport activity, while *NtNRAMP6b* did not, although the main difference was only 12 amino acids between NtNRAMP6a and NtNRAMP6b protein sequence [35]. In our study, we identified two DMR6 orthologs in tobacco, NtDMR6T, derived from *N. tomentosiformis*, and NtDMR6S, derived from *N. sylvestris*. Phylogenetic analysis revealed that NtDMR6T and NtDMR6S clustered with DMR6-1 from other species, suggesting that both proteins may be involved in disease resistance (Figure 2a). Consistent with this notion, expression analyses showed that both genes are induced by pathogen infection and exhibit similar tissue expression patterns. Nevertheless, their functional roles may have

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diverged, as promoter analysis revealed distinct profiles of *cis*-acting regulatory elements. *NtDMR6T* harbors a greater number of stress- and hormone-responsive elements, while *NtDMR6S* is enriched in light-responsive elements (Figure 3c). These differences imply that NtDMR6T is likely to be integrated more directly into stress- and hormone-mediated defense networks, while NtDMR6S may be more responsive to environmental or developmental cues. Such divergence suggests that although both genes are pathogen inducible, they may contribute to disease susceptibility through distinct upstream signaling pathways.

In tomato, the *Sldmr6*-1 mutants exhibited increased resistance to various classes of tomato pathogens [16]. In potato, *StDMR6* mutant lines can enhance the resistance to late blight [36]. In banana, mutants of the *DMR6* orthologue can enhance resistance to *Xanthomonas campestris* pv. *musacearum* [37], and in grapevine, *VviDMR6-1* is a candidate gene for susceptibility to downy mildew [38]. By generating CRISPR/Cas9 mutants of *NtDMR6* genes, we aimed to evaluate whether *NtDMR6T* or *NtDMR6S* contribute to susceptibility to *P. nicotianae* and to gain preliminary insights into their roles in tobacco. Compared to the susceptible control HD, all three mutant lines showed significantly reduced susceptibility to *P. nicotianae.* Notably, the single mutants DC6527L and DC6198L exhibited similar resistance levels, whereas the double mutant DC6229L showed a significantly lower disease index than either single mutant (Figure 5). The stronger resistance phenotype of the double mutant implies that *NtDMR6T* and *NtDMR6S* may function in partially redundant but complementary pathways. Considering the distinct *cis*-acting elements in each gene, such redundancy may represent an evolutionary strategy that safeguards susceptibility functions and ensures successful pathogen colonization.

Prior to utilizing impaired *S* gene alleles in crop breeding, it is essential to identify and minimize potential negative side effects [39]. Mutation of *DMR6* can lead to elevated SA levels, which may in turn result in dwarf phenotypes [40,41]. In our study, the *Ntdmr6* mutants—DC6527L, DC6198L, and DC6229L—did not exhibit any growth defects under field conditions (Figure 6). This observation aligns with previous reports in other species: inactivation of *StDMR6-1* in potato showed no apparent growth penalty [36], and knockout of *SlDMR6-1* in tomato did not cause detectable growth reduction under laboratory conditions [16]. RT-qPCR analysis showed that the expression of a subset of plant development-related genes was not altered in DC6229L compared with HD, consistent with the observation that loss of *NtDMR6s* did not cause developmental defects. Moreover, the leaves of DC6527L, DC6198L, and DC6229L were significantly wider than those of HD, suggesting that knocking out *NtDMR6s* not only enhances disease resistance but may also contribute to increased yield potential. These findings support the notion that *NtDMR6s* are promising *S* gene targets for improving tobacco resistance without compromising plant growth.

Existing studies have rarely investigated the downstream regulatory pathways regulated by DMR6. Our results indicate that, upon the loss of *NtDMR6s* function, SA levels likely increase, as the core regulatory gene *NPR1* was up-regulated in the DC6229L mutant. After *P. nicotianae* inoculation, genes associated with POD enzymes, MAPK cascades, and phenylpropanoid biosynthesis were markedly up-regulated in DC6229L. These responses are unlikely to act in isolation but are more plausibly downstream amplification effects driven by SA. Specifically, SA accumulation can activate SA-responsive transcription factors (such as *WRKYs*; Figure 7a), which in turn amplify MAPK signaling, induce peroxidase activity to modulate reactive oxygen species, and stimulate the biosynthesis of antimicrobial secondary metabolites, including phytoalexins (Figure 7b). Future work can focus on the direct downstream targets of *NtDMR6s*. Such studies will not only clarify the causal role of DMR6 within SA-centered immune networks but also provide a molecular framework for exploiting S-gene editing to enhance broad-spectrum resistance in tobacco. Future work can focus on identifying the direct downstream targets of *NtDMR6s*. This will not only clarify the role of DMR6 within SA-centered immune networks but also provide a molecular framework for leveraging *S* gene editing to enhance broad-spectrum resistance in tobacco.

5. Conclusions

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In conclusion, our study demonstrates that *NtDMR6s* are promising targets for susceptibility (*S*) gene–based resistance breeding in tobacco. Tobacco possesses two *DMR6* homologs, *NtDMR6T* and *NtDMR6S*, both of which are induced upon *Phytophthora nicotianae* infection. Knocking out these genes significantly enhances resistance to *P. nicotianae* without compromising agronomic performance. Although *NtDMR6T* and *NtDMR6S* display functional redundancy, their promoter regions harbor distinct *cis*-acting elements, pointing to possible differences in their regulatory mechanisms. RT-qPCR analysis comparing wild-type HD with *Ntdmr6* double knockout mutants indicates that *NtDMR6s* modulate resistance through multiple defense pathways, including the MAPK signaling cascade. Together, these findings deepen our understanding of *NtDMR6s* function in tobacco immunity and highlight the need for future studies to clarify its molecular basis and evaluate its potential in resistance breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1, Figure S1: Comparison of plant height between wild-type HD and *Ntdmr6* mutants in greenhouse; Table S1: Primers used in this study; Table S2: Statistical analysis in this study; Table S3: Amino acid sequences used in phylogenetic tree construction in Figure 1; Table S4: *Cis*-acting elements of *NtDMR6s* in promoter region.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, Y.B. and H.M.; methodology, H.W.; software, Z.L. and L.W; validation, L.C., A.Y. and R.V.; formal analysis, R.W.; investigation, H.W.; resources, Z.L.; data curation, B.M.; writing—original draft preparation, B.M. and H.M; writing—review and editing, L.W., L.C., and R.V.; visualization, Y.W.; supervision, H.M.; project administration, A.Y.; funding acquisition, Z.L. and L.C.; All authors have read and agreed to the published version of the manuscript.

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