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Enhancing tolerance to *Phytophthora* spp. in eggplant through *DMR6–1* CRISPR/Cas9 knockout

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ABSTRACT

Agricultural production is affected by the worsening effects of climate change with severe yield losses caused by rising temperatures, water scarcity and consequent modifications in the interactions between crops, pests, and pathogens. The availability of stress-tolerant plants will thus be a key point to guarantee the world food security in the next future. To this purpose, a significant contribution might be provided by the New Genomic Techniques (NGTs), such as CRISPR/Cas9, which allow to insert targeted modifications in the plants' genomes offering new opportunities for crop improvement. Susceptibility genes encode proteins that pathogens can take advantage of during the colonization process, and their disabling confers a broad-spectrum and long-lasting pathogen tolerance to the plant. Among the S-genes, *Downy Mildew Resistance 6* (*DMR6*) encodes an enzyme involved in Salicylic Acid (SA) degradation, and its inactivation in other *Solanaceae* species has proven to increase SA levels and confer tolerance to a broad spectrum of pathogens. We identified two orthologs of this gene in eggplant's genome, namely *SmDMR6–1* and *SmDMR6–2*. In the 'Black Beauty' cultivar, only *SmDMR6–1* expression significantly increased upon infection by the two oomycetes *Phytophthora infestans* and *Phytophthora capsici*, suggesting its involvement in the regulation of plant responses to biotic stresses. Here we report, for the first time in eggplant, the knockout of *SmDMR6–1* gene through CRISPR/Cas9 technology. The regenerated T_0 plants were screened by Sanger sequencing and one was selected and self-pollinated to generate T_1 and then T_2 plants. The mutant lines were subjected to pathogen assays which highlighted an increased tolerance to infection by *P. infestans* and *P. capsici*, if compared to non-edited plants.

1. Introduction

According to the Food and Agriculture Organization (FAO), eggplant (*Solanum melongena* L.) is the most important berry-producing Solanaceous crops after tomato (*Solanum lycopersicum* L.), with a global production of 58.6 megatons in 2021 (https://www.fao.org/faostat/en/#data-FAOSTAT; http://faostat3.fao.org). Its bulk of production is mainly concentrated in China, India, Iran, Egypt and Turkey, with Italy representing the most important European producer (Cericola et al., 2013). Being eggplant a vegetable contributing to nutrition in vast areas of the world, it is pivotal to develop new strategies for improving its tolerance to diseases and consequently increase its production to face the dramatic increase in world population and the effects of climate change. Traditional breeding programs have allowed to develop improved eggplant cultivars through introgression of useful traits from wild relatives, such as resistance to *Verticillium* wilt from *S. linneanum* (Liu et al., 2015) and beneficial traits for adaptation to climate change from *S. incanum* (Gramazio et al., 2017). However, introducing desirable alleles in an élite cultivar through crossing is time-consuming and requires several generations of breeding and selection to get rid of potentially detrimental effects due to linkage drag (Prohens et al., 2017).

In recent years, high-density genetic maps of eggplant have been developed and a large number of quantitative trait loci (QTLs), and related candidate genes for key morphological and metabolic traits have been identified (Sulli et al., 2021; Toppino et al., 2020), making the development of new genotypes more accessible to breeders (Gramazio

Abbreviations: NGTs, New Genomic Techniques; CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nucleases 9; DMR6, Downy Mildew Resistance 6; SA, salicylic acid; TF, transcription factor; TFBS, transcription factor binding site.

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et al., 2023). Recently some chromosome-anchored genome assemblies have been made available (Barchi et al., 2019, 2021; Li et al., 2021; Wei et al., 2020) and the first eggplant pan-genome and pan-plastome has been released (Barchi et al., 2021).

The availability of the whole genome sequence encourages the advancement of new genomic techniques (NGT) such as genome editing, and in particular the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nucleases (CRISPR/Cas) system, that allows to edit, insert, delete or replace specific genomic sequences in a targeted site (Saini and Kaushik, 2019). However, to date only few applications of the CRISPR/Cas system in eggplant have been reported (Kodackattumannil et al., 2023; Maioli et al., 2020; Phad et al., 2024) also due to its recalcitrance to *in vitro* regeneration.

Fungal diseases have a widespread incidence and relevant economic impact (Kaniyassery et al., 2023) on eggplant production. Among them, *Phytophthora* species causes systemic wilting of the plant accompanied by developments of soft spots on the fruits. In particular, the oomycete *Phytophthora capsici* L. affects many solanaceous species; no resistant cultivars have been reported so far in eggplant (Naegele et al., 2014) and its chemical management in the fields is expensive and only partially effective. *Phytophthora infestans* causes extensive damages mainly due to leaf blight (Majeed et al., 2018).

To overcome these issues the CRISPR/Cas based knockout of susceptibility genes (S-genes), which are required for successful pathogen infection, may contribute to develop tolerant varieties (Zaidi et al., 2018). In other species, the induced tolerance has indeed proven to be broad-spectrum and durable other than environmentally friendly.

In Arabidopsis the *DOWNY MILDEW RESISTANCE 6* (At*DMR6*) has been identified, it encodes a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase involved in the homeostasis of the salicylic acid (SA), in particular by hydroxylating it to an intermediate of its catabolism (Zhang et al., 2017). Its mutation leads to enhanced SA levels and subsequent reduced susceptibility to the downy mildew oomycete *Hyaloperonospora parasitica* (Van Damme et al., 2008), revealing it as an interesting S-gene.

The CRISPR/Cas9 mutants on the orthologs of this gene confirmed the reduced susceptibility to infection caused by a several pathogens in many species such as *Arabidopsis thaliana* (Zeilmaker et al., 2015), *Ocimum basilicum* (Hasley et al., 2021), *Musa* spp. (Tripathi et al., 2021), *Citrus* spp. (Parajuli et al., 2022), *Vitis vinifera* (Djennane et al., 2024; Giacomelli et al., 2023) as well as in Solanaceae species such as *Solanum lycopersicum* (Maioli et al., 2024; Thomazella et al., 2021) and *Solanum tuberosum* (Kieu et al., 2021).

In particular, two *AtDMR6* orthologs (*SlDMR6–1* and *SlDMR6–2)* were identified in tomato genome and one of them, *SlDMR6–1*, demonstrated to be activated in response to several pathogens and thus involved in the plant immunity system. The CRISPR/Cas9 knockout of this gene in tomato induced a reduction of the symptoms caused by three bacterial pathogens (*Pseudomonas syringae* pv. *tomato, Xanthomonas gardneri* and *X. perforans*), the oomycetes *P. capsici* and *P. infestans* and the tomato powdery mildew causal agent *Pseudoidium neolycopersici*. These mutants showed higher levels of SA that could be associated to an activation of SA-mediated immune responses to infection (Maioli et al., 2024; Thomazella et al., 2021). Similarly, the tetra-allelic CRISPR/Cas9 knockout of *StDMR6–1* in potato caused a significant increase in resistance to late blight caused by *P. infestans* without any detrimental effect on plant growth and development. Vice versa *StDMR6–2* mutants did not show any improvement in disease tolerance (Kieu et al., 2021).

In the present work, we analysed the structure and the expression profile of the two *DMR6* orthologs in *S. melongena*, respectively *SmDMR6–1* and *SmDMR6–2*, in different plant organs/tissues and upon pathogen infection caused by the oomycetes *P. infestans* and *P. capsici*. The CRISPR/Cas9 mediated knockout of *SmDMR6–1* provided a T_1 generation of edited plants which showed enhanced tolerance to *Phytophthora* species, thus representing an interesting resource for eggplant genetic improvement.

2. Results

2.1. DMR6 orthologs in eggplant: gene structure and promoter analysis

DMR6 was first identified in an *A. thaliana* (*AtDMR6*) and two orthologs, namely *SlDMR6–1* and *SlDMR6–2* were detected in tomato (Thomazella et al., 2021). Two homologous genes were also identified in the eggplant's genome: *SmDMR6–1* (5065 nucleotides, 337 amino acids) on the positive strand of chromosome 3, and *SmDMR6–2* (4344 nucleotides, 339 amino acids) on the negative strand of chromosome 6. Apart from the orientation, they are very similar in structure (Fig. 1, Fig. S1), consistently with the hypothesis of a duplication explaining the genesis of these two homologous genes. Also the predicted protein domains are the same (Fig. 1), with a dioxygenase domain at the N-terminal (exons 1–2, in purple) and a Fe(II) dependent 2-oxoglutarate dioxygenase domain at the C-terminal (exons 3–4, in orange).

The promoter sequences of the two *SmDMR6* genes, considering the 3 Kb regulatory sequence upstream of the ATG translation start site, were screened using the cis-element prediction tools on the PlantTFdb platform with profiles from *Solanum melongena*. For *SmDMR6–1*_3 kb (3:83,551,161–83,554,161) *versus* *SmDMR6–2*_3 kb (6:91,408,246–91,411,246), the plus/minus alignment showed 1377 identical residues, resulting in a percent identity of 45.9%. Analysis of TFBS revealed 150 TF recognition sites for *SmDMR6–1* and 170 for *SmDMR6–2*. Keeping redundancy into consideration, it was observed that 103 TFBS were specific to *SmDMR6–1*, 123 to *SmDMR6–2*, and 47 TFBS were common to both (Fig. S2).

The analysis of TFBS in the *SmDMR6–1* and *SmDMR6–2* promoter regions revealed notable differences in the regulatory pattern of the two genes (Fig. 2). *SmDMR6–1* has a remarkably higher number of G2-like (12 *vs.* 2), MIKC_MADS (11 *vs.* 3), bHLH (8 *vs.* 3), bZIP (6 *vs.* 3), AP2 (3 *vs.* 0) and NAC (9 *vs.* 6) TFBS. In contrast, *SmDMR6–2* shows a markedly higher number of ERF (18 *vs.* 8), ARF (12 *vs.* 5), C2H2 (11 *vs.* 4), WRKY (25 *vs.* 2) and GATA (7 *vs.* 0) TFBS.

2.2. Transcriptional profiling of DMR6 genes

To get an overview on the transcriptional regulation of *SmDMR6* genes in eggplant, their transcription profile was analysed through real-time qPCR in different plant tissues/organs (Fig. 3).

The levels of *SmDMR6–1* transcript resulted significantly higher in roots (8 times higher) and flowers (5 times higher) if compared to the other tissues, and an increasing trend of expression was observed in the fruit peel and flesh during the fruit ripening process, up to 3 times higher than the first ripening stage. *SmDMR6–2* instead showed a lower transcript level, apart from leaves and a progressively higher level in fruit peel during the ripening, similarly to *SmDMR6–1*.

Moreover, to prove the involvement of *SmDMR6* genes in the biotic stress response mechanisms, their transcript levels were analysed in leaf discs infected by two oomycetes, *P. infestans* and *P. capsici*. In the case of *P. capsici* infection (Fig. 4A), *SmDMR6–1* resulted strongly activated at 40 h post inoculation (hpi) with expression levels up to 50 times higher than 0 hpi; after this peak, the expression decreased. Vice versa *SmDMR6–2* levels did not appear to be influenced by *P. capsici* infection. As for *P. infestans* infection (Fig. 4B), the activation of *SmDMR6–1* was delayed (highest level recorded at 72 hpi), and weaker (maximum of 2.5-fold increase). On the contrary, the level of *SmDMR6–2* transcription seemed to decrease slightly as the infection progresses.

2.3. Generation and molecular screening of smdmr6–1 mutants in eggplant

The CRISPR vector harbouring the h*Cas9* gene, the *NptII* selective marker and the two gRNAs targeting the first exons of *SmDMR6–1* (Fig. S3) was introduced into the eggplant cultivar 'Black beauty' via *Agrobacterium tumefaciens* mediated stable transformation. In the first

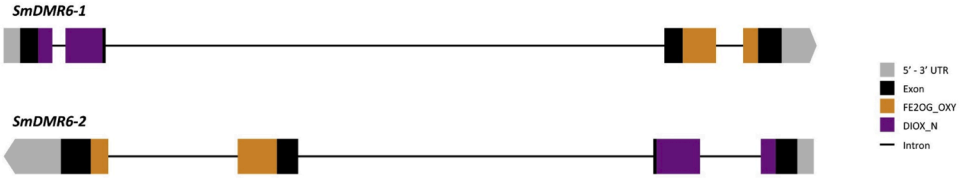

Fig. 1. Structure of eggplant *DMR6–1* and *DMR6–2* genes. Exons are represented as boxes, 5′ and 3′ UTRs as gray boxes and introns as lines. The colours show the aminoacidic regions corresponding to functional domains: in purple non-haem dioxygenase N-terminal domains, in orange Fe(II)-dependent 2-oxoglutarate dioxygenase domains.

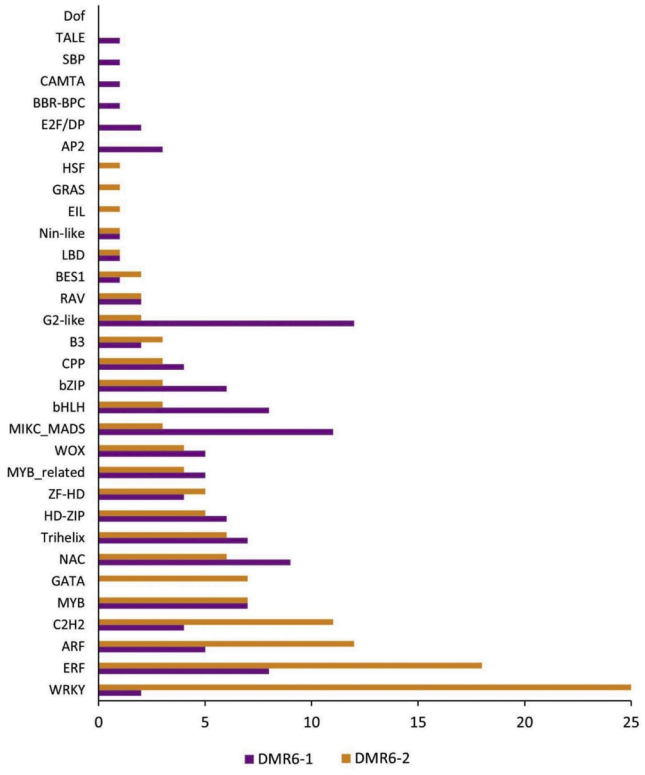

Fig. 2. Number of TFBS predicted in *SmDMR6–1* (purple) and *SmDMR6–2* (orange) promoters, considering the most representative TF families involved in stress response interacting with the TFBS.

generation of regenerated plants (T_0), the knockout score ranged from 0% to 76% (Tab. S1). The best edited plant, T_0–3, was selected for self-pollination in order to generate the T_1 lines.

Molecular screening through Sanger sequencing of the T_1 plants provided information on the inheritance pattern of the CRISPR-induced mutations and revealed a very variable knockout score, up to 100% (Fig. 5, Tab. S2). Most of the analysed individuals showed a biallelic asset, with the most frequent mutations being deletions of 1, 2 or 4 nucleotides (Table 1), all of them leading to frameshift mutations in the gene with subsequent unfunctional proteins synthesized. The presence of *hCas9* gene was investigated and plants with a higher knockout score usually had the transgene integrated in the genome, while the plants with a lower knockout score had segregated *hCas9* during the self-pollination (Table 1, Tab. S2).

Some of the plants with the best editing scores (Table 1) were chosen for further experiments in order to check their tolerance to infection caused by *Phytophthora* species.

2.4. Tolerance to *P. capsici* and *P. infestans* infection in *smdmr6–1* mutants

In eggplant, detached leaf assays with the oomycetes *P. capsici* and *P. infestans* demonstrated a reduction of the disease symptoms in *SmDMR6–1* mutants in respect to non-edited controls. In particular, leaves of four selected T_1 plants (namely, T_1–1, T_1–2, T_1–3 and T_1–4) infected with *P. capsici* mycelium showed a slower spreading of the pathogen and less rotting of the tissues. Genomic DNA extraction 4 days after inoculation and qPCR-mediated quantification of the fungal biomass confirmed a lower ratio between pathogen and plant DNA (Fig. 6A), with a maximum reduction of 54% (T_1–2), and thus a better tolerance to the oomycete's infection.

Similarly, the pathogenic assay performed on leaf discs infected with *P. infestans* zoospores revealed that 6 days after inoculation the brown/blackish necrotic lesions on the infected tissues were visibly smaller in selected edited lines (namely, T_1–5, T_1–6, T_1–7, T_1–8 and T_1–9) if compared to the control plants. Moreover, the fungal biomass was clearly lower in edited lines, with a reduction ranging from 45% (T_1–7) to 86% (T_1–8), as highlighted by qPCR analysis (Fig. 7A). As a support to these molecular data, we assigned a disease severity index to infected leaf discs and a significant reduction was observed in all *SmDMR6–1* mutants (on average −45%) compared to the control (Fig. 7B).

In vivo artificial inoculation of *P. capsici* showed a significant reduction of disease severity in T_2 *SmDMR6–1* mutants in respect to non-edited controls (−14% at 3 dpi; −23% at 6 dpi) (Fig. 6B), despite the percentage of disease incidence being 100% for both lines. These findings suggest an involvement of *SmDMR6–1* in eggplant in the plant response to biotic stress with the inactivation of this gene leading to an enhanced tolerance to infection caused by different pathogens.

3. Discussion

Climate change influences plants, pathogens and their interactions and has a great impact on losses of crop production (Roussin-Léveillée et al., 2024). It is thus imperative to generate new climate-resilient and pathogen-tolerant crops that can better adapt to the challenging growth conditions.

One of the possible strategies to improve the plants' resistance to biotic stresses is the introgression of resistance (R) genes from wild compatible species displaying tolerance to pathogen, activating a general resistance mechanism in the recipient plant. R-genes mainly encode nucleotide-binding leucine-rich repeat (NLR) proteins, intracellular immune receptors that specifically bind the pathogen's effectors conferring resistance to the plant (Ellis et al., 2000).

In eggplant the development of backcross genotypes resistant to *Verticillium* wilt has been obtained through introgression breeding with *Solanum linnaeanum* (Liu et al., 2015); although no eggplant commercial resistant cultivars have been developed so far, genotypes resistant to the fungal wilts caused by *Fusarium oxysporum* f. sp. *melongena* have been obtained as well (Gramazio et al., 2023).

However, resistance conferred by the introgression of individual resistance genes is time-consuming and often not durable, because widespread deployment of R-genes selects for pathogen strains capable

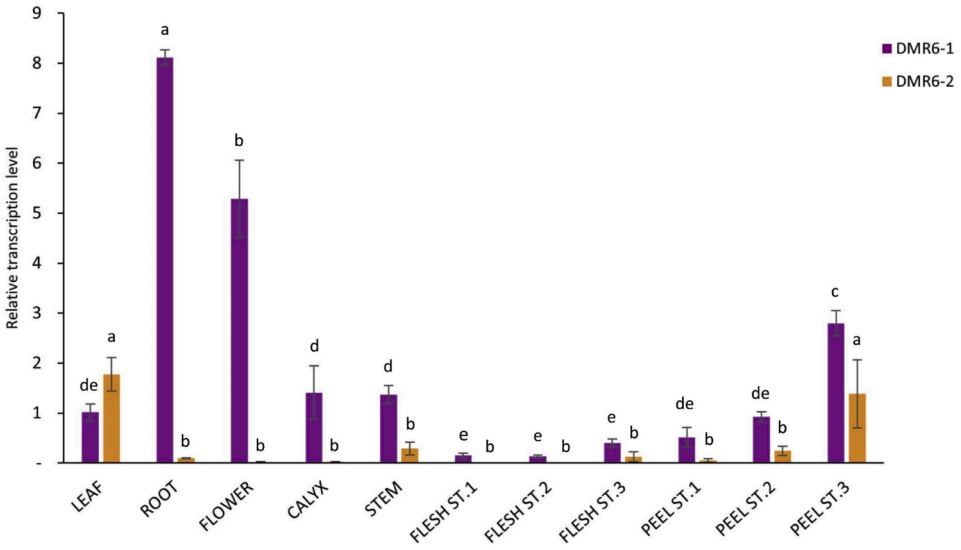

Fig. 3. Transcriptional profiling of *SmDMR6–1* (purple) and *SmDMR6–2* (orange) in different plant tissues. Statistical analysis was performed with a Duncan test on 6 biological replicates and standard error bars are shown on the histogram.

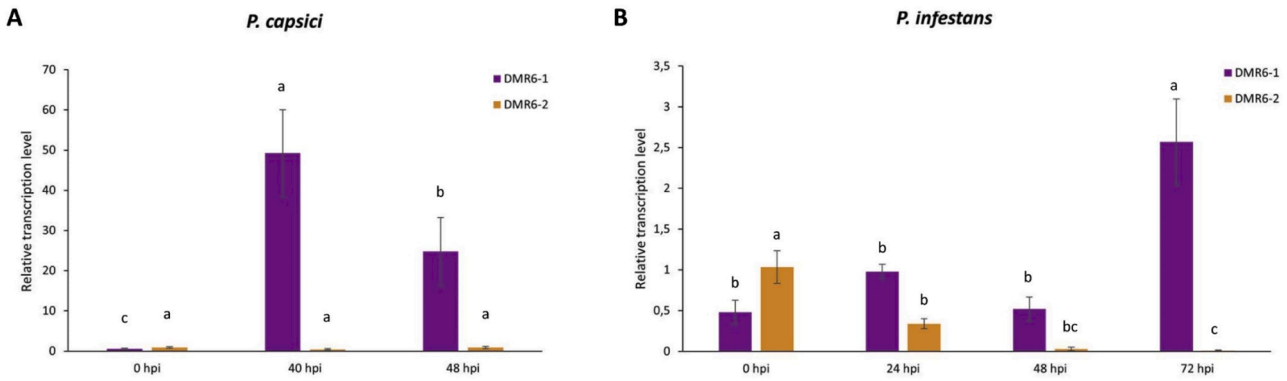

Fig. 4. Transcriptional profiling of *SmDMR6–1* (purple) and *SmDMR6–2* (orange) upon infection caused by *P. capsici* (**A**) and *P. infestans* (**B**). Statistical analysis was performed with a Duncan test on 6 biological replicates and standard error bars are shown on the histogram.

of overcoming it.

Susceptibility (S) genes, instead, are those that pathogens take advantage of during the colonization of the plant (*e.g.*, negative regulators of plant immunity or genes involved in pathogen entrance and nutrition). If these genes become dysfunctional the ability of the pathogens to cause disease is strongly limited (Schie and Takken, 2014).

In the last decades new biotechnological approaches, such as gene editing, have provided new tools for the disabling of S-genes in order to generate disease tolerant crops (Karmakar et al., 2022).

S-genes have been knocked out through CRISPR/Cas9 technology in several crops, such as tomato (Li et al., 2022; Nekrasov et al., 2017; Santillán Martínez et al., 2020), grapevine (Malnoy et al., 2016; Wan et al., 2020), rice (Zhou et al., 2022) and potato (Kieu et al., 2021), but no results have been reported in eggplant so far.

The *DMR6* gene was first identified in *Arabidopsis* (Van Damme et al., 2008), characterised in other crops and in Solanaceae species it has emerged as a promising target for genome editing because of the broad-spectrum pathogen tolerance conferred by its disabling (Karlsson et al., 2024; Kieu et al., 2021; Maioli et al., 2024; Thomazella et al., 2021). Similarly to tomato, potato and grapevine, we identified two homologs of *DMR6* gene in eggplant genome, namely *SmDMR6–1* and *SmDMR6–2*. These two genes/proteins show a very similar structure, both in terms of the introns/exons structure and the functional domains (Fig. 1). However their transcriptional profiles vary across different tissues (Fig. 3), probably due to distinct regulatory patterns (Fig. 2). Generally, *SmDMR6–1* expression is higher than *SmDMR6–2* in all the organs apart from leaves, and the remarkable level of *SmDMR6–1* transcription in roots and the activation of both *DMR6* genes during the fruit ripening, especially in the fruit peel, are consistent with the data collected in tomato (Thomazella et al., 2021). Moreover, unlike *SmDMR6–2, SmDMR6–1* is strongly activated upon infection caused by *Phytophthora* spp. (Fig. 4), similarly to what reported in tomato (Thomazella et al., 2021). This observation may be attributable to an over-representation of TFBS interacting with TFs belonging to families, such as WRKY, AP2/ERF, bZIP, bHLH and NAC (Fig. 2), that previous literature studies demonstrated to play a key role in plant defense mechanisms (Seo et al., 2015). In addition, both potato and tomato *dmr6–1* mutants demonstrated a better tolerance to pathogens infection, unlike *dmr6–2* mutants (Kieu et al., 2021; Thomazella et al., 2021), while in grapevine only double mutants for *VvDMR6–1* and *VvDMR6–2* show tolerance to downy mildew (Giacomelli et al., 2023).

Altogether, these findings suggest that, like in other *Solanaceae* species, a gene duplication presumably occurred in the eggplant genome, generating two homologous sequences which then underwent a sub-functionalisation process that left to *SmDMR6–1* alone the role of fine tuning the SA balance in response to biotic and abiotic stresses, as previously reported in tomato (Maioli et al., 2024; Thomazella et al., 2021).

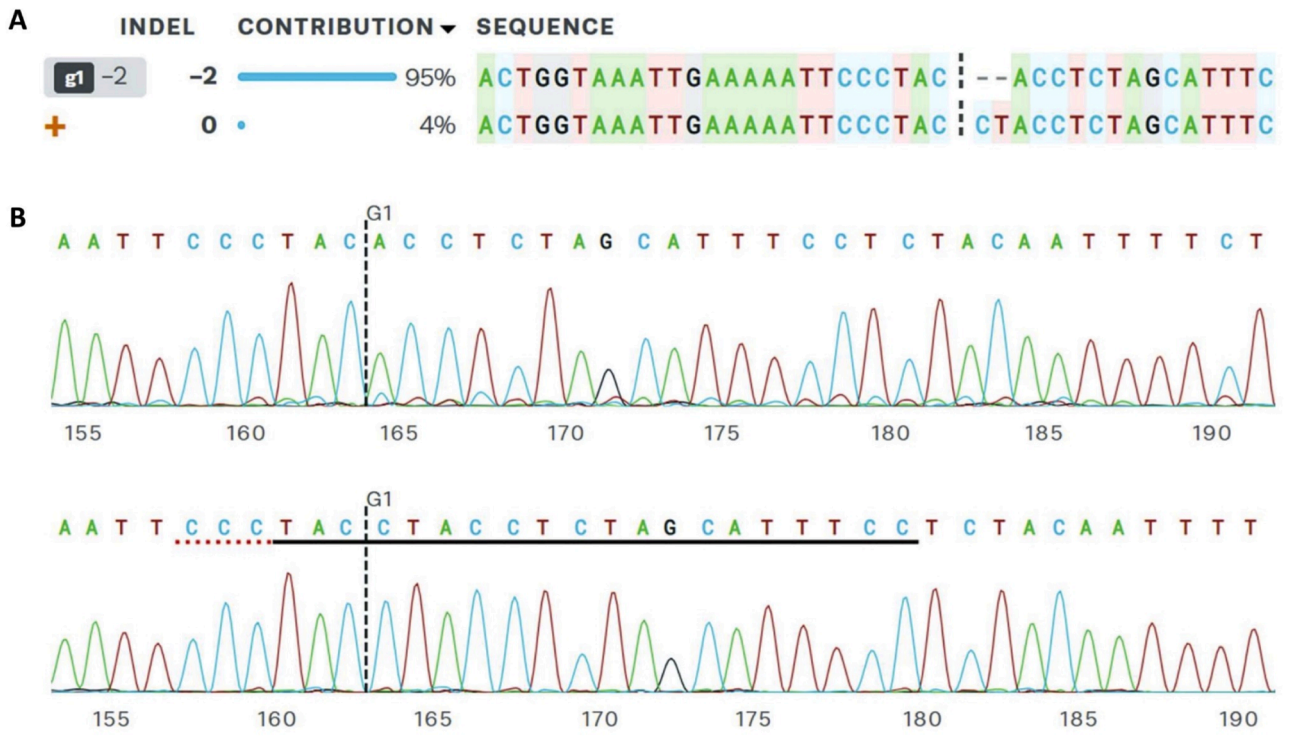

Fig. 5. Output of Sanger sequencing analysis of the T_1–3 plant analysed through Synthego software (https://ice.synthego.com/#/). (**A**) Relative contribution of each sequence present in the edited population (the wild-type sequence is marked by a "+" symbol). (**B**) Chromatograms alignment of edited (top) and wild-type (bottom) sequences. Cas9 cut sites are represented by vertical dotted lines.

Table 1
Results of the molecular screening on T_1 plants selected for further experiments. Knockout (KO) score represents the proportion of indels that cause a frameshift mutation or are 21+bp in length.

Plant	KO score	R^2	Mutation	*hCas9*
T_1–1	99	0.99	−1	+
T_1–2	97	0.96	−2/−4	+
T_1–3	95	0.99	−2	+
T_1–4	93	0.96	−1/−4	+
T_1–5	94	0.99	−2	+
T_1–6	93	0.99	−2	+
T_1–7	92	0.96	−1/−2	+
T_1–8	91	0.99	−2	+
T_1–9	90	0.97	−1/−2	+

SmDMR6–1 was therefore chosen as target for CRISPR/Cas9-mediated knockout with the aim of obtaining eggplant mutants with a better tolerance to pathogens' attacks. The regenerated T_0 plants were screened by Sanger sequencing in order to select the best edited line, which was self-pollinated for obtaining the T_1 generation. A detached leaf assay on individuals of the T_1 generation revealed that fully knocked out plants showed milder symptoms after infection by *P. capsici* and *P. infestans*. The fungal biomass developed on all the edited plants was lower than on the non-edited controls, with a reduction ranging from 34% to 54% after *P. capsici* infection (Fig. 6A) and from 46% to 87% in the case of *P. infestans* (Fig. 7A). Since in eggplant no evidence of whole plant inoculation tests with *P. infestans* can be retrieved from literature, we carried out *in vivo* artificial inoculation tests only with *P. capsici*. This experiment showed a significant reduction of disease severity in T_2 *SmDMR6–1* mutants in respect to non-edited controls (−23% at 6 dpi)

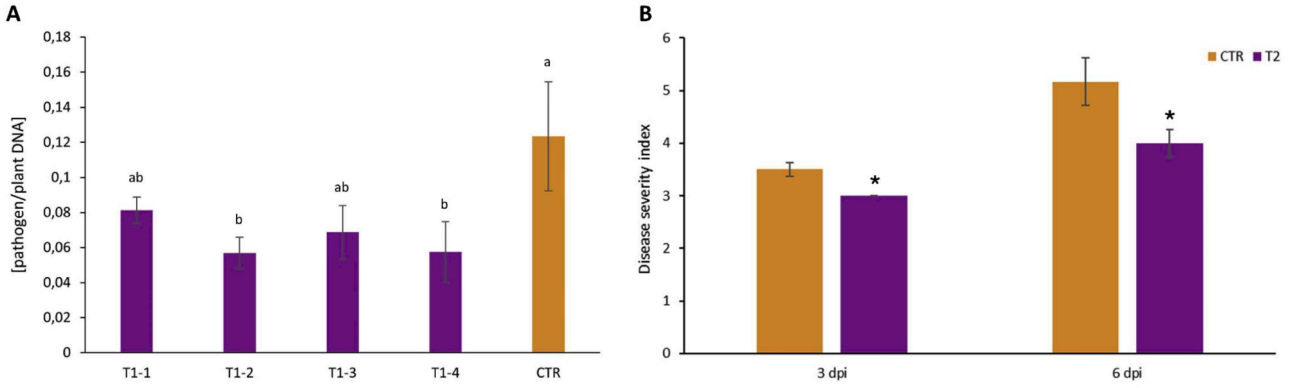

Fig. 6. Pathogen assays to test tolerance to *P. capsici*. (**A**) Detached leaf assay. Ratio between pathogen and plant DNA measured through real time qPCR. Statistical analysis was performed with a Duncan test on 6 biological replicates and standard error bars are shown on the histogram. (**B**) *In vivo* pathogen assay. Disease severity index at 3 and 6 days after *P. capsici* inoculation. Statistical analysis was performed with a Student's *t*-test with 6 biological replicates and standard error bars are shown on the histogram.

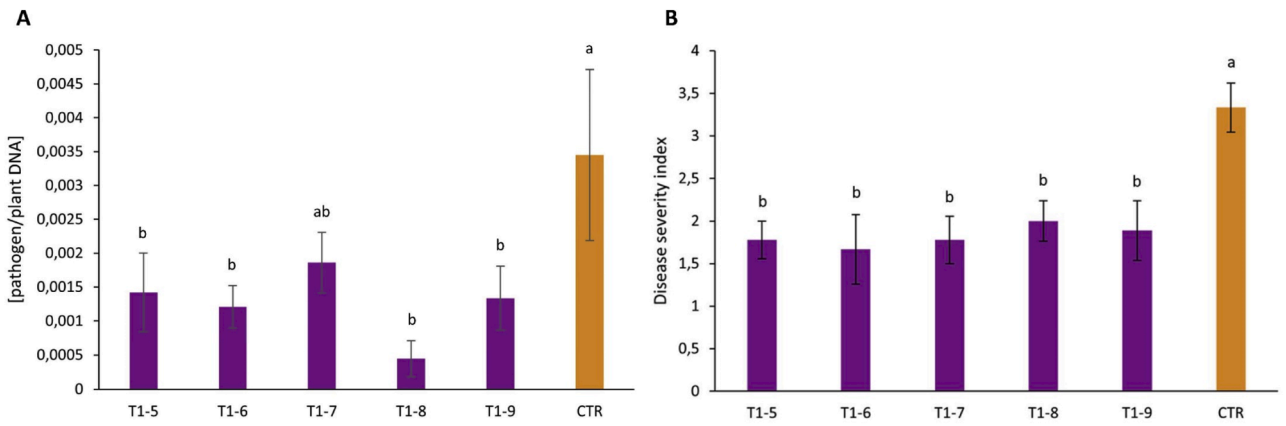

Fig. 7. Detached leaf assays to test tolerance to *P. infestans*. (**A**) Ratio between pathogen and plant DNA measured through real time qPCR. (**B**) Disease severity index at 6 days after *P. infestans* inoculation. Statistical analysis was performed with a Duncan test on 9 biological replicates and standard error bars are shown on the histogram.

(Fig. 6B). These results altogether are consistent with the increased tolerance to *Phytophthora* species recorded in other Solanaceae species after *DMR6–1* knockout. Necrotic lesions due to *P. capsici* infection developed later and were less severe in tomato edited plants (Thomazella et al., 2021) and the fungal biomass recorded in tomato mutants infected with *P. infestans* was up to 95% lower than control plants (Maioli et al., 2024). A significant increase in tolerance to *P. infestans* was detected also in potato, as measured by reduction of the number of infected leaflets (Kieu et al., 2021).

Tomato *dmr6–1* mutants demonstrated a broad-spectrum tolerance to several distinct classes of pathogens, such as bacteria, oomycetes and fungi (Thomazella et al., 2021). Our future research will be thus aimed at evaluating whether even in eggplant *SmDMR6–1* mutants show tolerance to other diseases, such as *Fusarium wilt*, caused by *Fusarium oxysporum* f. sp. *melongenae*, powdery mildews, caused by *Podosphaera xanthii* or *Leveillula Taurica*, and bacterial wilt, caused by *Pseudomonas solanacearum*.

Since the knockout of *DMR6–1* in tomato was reported to induce tolerance to water deprivation thanks to a drought avoidance mechanism (Maioli et al., 2024), and potato *Stdmr6–1* mutants showed a higher tolerance to salt/osmotic/drought stress (Karlsson et al., 2024), it would be intriguing also to test the tolerance to abiotic stresses in eggplant *Smdmr6–1* mutants, in order to achieve a multi-stress tolerance.

By the way, a deeper evaluation of stress tolerance should be carried out in field conditions in order to take into consideration all the factors affecting the plants' growth. To date, the cultivation of plants obtained through CRISPR/Cas9 is impeded by the current EU legislation that puts all the products of new genomic techniques (NGTs) under the GMO definition. Recently, the European Parliament voted in favor of a regulatory proposal that distinguishes between NGT1 category plants, which are considered equivalent to conventionally bred plants and whose cultivation will be admitted, and NGT2 category plants, that will continue to fall under current legislation (Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL on Plants Obtained by Certain New Genomic Techniques and Their Food and Feed, and Amending Regulation (EU) 2017/625, 2023). In this perspective, putting our *Smdmr6–1* mutants through subsequent cycles of self-pollination and selection would provide one individual which carries the desired indel at the target site in homozygous status and which lost the transformation cassette thanks to genetic segregation. This plant would fall into NGT1 category and would be eligible for field trials, with the future aim of bringing to the market a pathogen-tolerant eggplant line.

4. Materials and methods

4.1. Identification and in silico characterization of DMR6 orthologs

The orthologs of *SlDMR6–1* and *SlDMR6–2* were identified in the 4.1 version of eggplant genome (Barchi et al., 2021) (IDs: SMEL4.1_03g019440.1.01, SMEL4.1_06g024710.1.01) through BLASTp alignment of the tomato protein sequences collected from Sol Genomic Network (https://solgenomics.net/), using the e-value cutoff of $1e^{-5}$ and choosing the hits with the highest identity scores (tomato IDs: Solyc03g080190.3, Solyc06g073080.4).

The sequences were then analysed with the Wormweb software (http://wormweb.org/exonintron) to obtain a graphical representation of the intron/exon profile and the protein structure domains were predicted using the Pfam software (https://pfam.xfam.org/).

To compare the *SmDMR6–1* and *SmDMR6–2* promoters, 3 Kbp of sequence upstream of the ATG translation start codon were globally aligned with Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). To detect putative transcription factor binding sites (TFBSs) and corresponding transcription factors (TFs) putatively involved in the regulation of *SmDMR6–1* and *SmDMR6–2* transcription, the two genes were separately examined using the Binding Site Prediction tool present in the Plant Transcriptional Regulatory Map (PlantRegMap, https://plantregmap.gao-lab.org) suite; the analysis was conducted against the *Solanum melongena* reference database. The list of the TFBS was then annotated and GO categorised through the GO Term Enrichment function of PlantRegMap.

4.2. Oomycetes growth and inoculum preparation

Phytophtora infestans isolate (Westerdijk Fungal Biodiversity Institute strain CBS 120,920) was maintained on Rye Agar (Caten and Jinks, 1968) at 18 °C in dark and subcultured every month. 10 days before using the pathogen for an experiment, a new inoculum was prepared. To induce zoospore release, the plate was flooded with cold sterile tap water and kept for 3–4 h at 4 °C. The liquid was then collected from the plate and filtered through a cloth. The concentration of zoosporangia was assessed using a hemocytometer and was adjusted to 1×10^4 sporangia/ml for the final inoculum.

Phytophtora capsici isolate (Westerdijk Fungal Biodiversity Institute strain CBS 370.72) was maintained on V8-juice agar medium at 24 °C and subcultured every three weeks, according to provider's instructions. Mycelium discs were used as an inoculum for pathogen assays.

4.3. Transcriptional profiling

For the transcriptional profiling of *SmDMR6–1* and *SmDMR6–2* in different plant tissues/organs, samples were collected from *S. melongena* cv. 'Black Beauty' plants grown in a greenhouse (mean temperature 25 °C), in 10 litres pots containing horticultural substrate and applying standard horticultural practices. Leaves, stems, roots, flowers, calyxes, fruit peel and fruit flesh in three different ripening stages (stage 1, corresponding to small fruits enclosed in the calyx; stage 2, immature fruit; stage 3, ripe fruits) were collected, with 6 biological replicates for each tissue.

For *SmDMR6–1* and *SmDMR6–2* transcription analysis upon pathogen infection, leaf discs (2,2 cm diameter) were cut from third/fourth leaves of 2 months old *S. melongena* cv. 'Black Beauty' plants grown in soil pots in climatic chamber (25 °C, day/night cycle of 16/8 h). The leaves were first rinsed with sterile water and the discs laid on water agar in plastic trays. *P. infestans* assay was performed infecting the discs with 12 μl of a water suspension containing 14,000 zoospores/ml and incubated at 18/24 °C 16/8 h; samples were then collected 24 h, 48 h and 72 h post inoculation (6 biological replicates). For *P. capsici* assay, leaves were infected with 5 mm wide mycelium discs and incubated ad 24 °C; the discs were sampled at 40 h and 48 h post inoculation (6 biological replicates).

Total RNA was extracted from all the samples using the Spectrum plant total RNA kit (Sigma-Aldrich, St. Louis, USA), according to manufacturer's instructions, and quantified on the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific). For each sample, cDNA was synthetised through reverse transcription using 1 μg of extracted RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), following protocol's instructions. The produced cDNA was diluted 1:6 and used for the following qPCR analyses. qPCR reactions were prepared in 96-well plates with a final volume of 10 μL using a StepOnePlus Real-Time PCR system (Applied Biosystem), with technical duplicates. Each reaction contained 1,5 μl of starting cDNA, 2X Power SYBR Green PCR Master Mix (Applied Biosystem, USA) and specific primers (0.2 μM) targeting *SmDMR6–1* (forward primer: 5′-GAGCCTGGGGTTGGAGAAAG-3′; reverse primer: 5′-TCTGGTTGTGGACATGGTGG-3′), *SmDMR6–2* (forward primer: 5′-AATCTCAAAGACCCCGCCTG-3′; reverse primer: 5′-GCTTGACCAATTTGATGCACAC-3′) and two eggplant's housekeeping genes: elongation factor (*SmEF*) (forward primer: 5′-ACCAGCATCACCATTCTTCA-3′; reverse primer: 5′-ACTGCCATACTTCCCACATT-3′) and actin (*SmAct*) (forward primer: 5′-ACCACAGCTGAGCGAGAAAT-3′; reverse primer: 5′-GACCATCGGGAAGCTCATAG-3′). The following PCR protocol was used: 1 cycle of 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s. The $2^{-\Delta\Delta Ct}$ method was used for expression quantification based on Ct values of the target genes and the mean Ct value of the two housekeeping genes (*SmEF, SmAct*). Statistical analyses were carried out with the IBM SPSS statistical software to perform a one-way ANOVA test followed by a Duncan statistical test to assess significant differences between the values.

4.4. Transformation vectors design and assembly

Two gRNAs (g1: GTGGCCTATCGGATACGGGT; g2: GGAAATGCTAGAGGTAGGTA) were designed using the online CRISPOR tool (http://crispor.tefor.net/) targeting the first and second exon of *SmDMR6–1*. The assembly of the transformation vector was performed using the Golden Braid (GB) cloning system, following GB software-directed procedures (https://gbcloning.upv.es/). The final transformation vector *pDGB3_alpha1_Tnos:NptII:Pnos_P35S:hCas9:Tnos_U6–26:gRNA1:scaffold_ U6–26:gRNA2:scaffold* was inserted into *Agrobacterium tumefaciens* strain EHA105 cells through electroporation.

4.5. Plant materials and genetic transformation

S. melongena pure line 'Black beauty' seeds were sterilised by soaking them for 30 s in 70% ethanol and then for 20 min in 40% sodium hypochlorite; they were subsequently rinsed three times in sterile water and left overnight in water at RT in the dark. To enhance seed germination, the seeds were soaked for an additional day in a solution containing 750 mg/l GA3 and then placed on sterile germination medium (2.4 g/l MS + vitamins, 15 g/l sucrose, 8 g/l plant agar) in plastic boxes. The growth took place at 25 °C in the dark for a week before being transferred to a day/night cycle of 16/8 h. After 3 weeks, fully grown cotyledons were ready to be used for plant genetic transformation.

The inoculum of *A. tumefaciens* strain harbouring the final transformation vector was prepared following a three days protocol. On the first day, *A. tumefaciens* transformed strain was cultured in MGL medium (Maioli et al., 2020) supplemented with selection antibiotics (50 mg/l kanamycin and 25 mg/l rifampicin) and grown at 28 °C ON shaking. The day after, an aliquot of this culture was diluted 1:50 in TY medium (Maioli et al., 2020) containing acetosyringone 200 μM and incubated at 28 °C ON shaking. On the third day, the OD_{600} was checked and the bacterial culture was diluted to an OD_{600} of 0.12 – 0.16 in TY medium supplemented with 200 μM acetosyringone. 1 cm long explants were cut from the cotyledons and dipped in bacterial culture for 10 min, blotted dry on sterile paper and cultured for 48 h on a co-culture medium in the dark at 25 °C. *In vitro* steps of callogenesis, shoot induction, elongation and rooting were performed as previously described (García-Fortea et al., 2020) with minor modifications. *In vitro* selection of transformants was performed by adding 50 mg/l kanamycin in all the media, apart in the rooting phase. After obtaining fully developed plantlets (T_0) they were moved to soil pots and gradually acclimatised to *ex vitro* conditions. Self-pollination of a T_0 plant (the T_0–3, selected on the basis of knock-out score) provided seeds for the T_1 generation used for molecular and detached leaf assays. By selfing T_1–3 plant (Table 1) the fully knocked out (−2/−2) T_2 generation was obtained and used for *in vivo P. capsici* assay.

4.6. Molecular screenings of T_0 and T_1 mutants

Genomic DNA was extracted from cotyledons or leaves of T_0 and T_1 plants using E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, USA) following the manufacturer's instructions.

Primers were designed on the genomic regions flanking the gRNAs (forward primer: 5′- GCTGCGCCATAGATTCCATG-3′; reverse primer: 5′-GACGAGGGATTAGAAGGCCA-3′) in order to amplify the editing targets within *SmDMR6–1* locus. PCR amplification was performed using KAPA HIFI Taq (Kapa Biosystems, Boston, USA) with the following PCR program: 1 cycle of 95 °C for 3 min; 30 cycles of 98 °C for 20 s, 63 °C for 15 s, 72 °C for 45 s; 1 cycle of 72 °C for 1 min. After purification with AMPure XP Beads 0.8X (Beckman Coulter), amplicons were sequenced by Sanger method. The knock-out score and indels were estimated by analysing the chromatograms using the Synthego ICE online tool (https://ice.synthego.com/).

The screening for *hCas9* presence was performed through real-time PCR using a StepOnePlus Real-Time PCR system (Applied Biosystem), with technical duplicates. Reactions were prepared in 96-well plates with a final volume of 10 μL, each containing 1.5 μl of gDNA, 2X Power SYBR Green PCR Master Mix (Applied Biosystem, USA) and specific primers (0.2 μM) targeting *hCas9* (forward primer: 5′- CTATCCTCAGGCGGCAAGAG-3′; reverse primer: 5′- AGTCATCCACGCGAATCTGG-3′) or *SmEF* (housekeeping gene). The following PCR protocol was used: 1 cycle of 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s. The values of ΔCt were used for a relative quantification of the presence of the transgene in the genome.

4.7. Detached leaf assays

Leaf discs from selected T_1 lines and control plants (stably expressing *nptII* and *Cas9* genes) were used to set up a detached leaf assay according to the procedure described by Foolad et al. (2015) with modification to adapt to our purpose. Leaves were rinsed in sterile water, blotted dry on sterile paper and placed in plastic trays containing water agar (20 g/l).

A 10 μl drop of *P. infestans* inoculum (corresponding to about 140 zoosporangia) were placed in the center of each disk. Plastic trays were then covered with lids and incubated at 18/24 °C cycle in a growth chamber. Pictures and samples were collected 6 days post inoculation (dpi), 9 biological replicates for 5 T_1 lines were used. At 6 dpi a scale of 0–4 was used to score a disease severity index (DSI) based on the size of necrotic lesions on leaf discs. DSI data were statistically analyzed through Duncan statistical Test.

For *P. capsici* assay, 5 mm diameter mycelium discs were deposited in the center of each disk and covered trays were incubated at 24 °C. Pictures and samples were collected 4 dpi, 6 biological replicates for 4 T_1 lines were used.

4.8. Pathogens quantification through qPCR

In order to quantify pathogen infection, the ratio pathogen DNA/plant DNA was evaluated according to Pavese et al. (2021). DNA was extracted from eggplant leaf discs with E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, USA) and from oomycetes' mycelium using E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, Norcross, USA), following the manufacturer's instructions. Standard curves were prepared for the quantification of DNAs through real-time qPCR using primers on *SmEF* for egglant DNA (see above), *PiO8* for *P. infestans* DNA (Llorente et al., 2010) and *phcnlp1* for *P. capsici* DNA (Feng and Li, 2013; Saltos et al., 2021). Real-time qPCR was performed on the DNA extracted from infected leaf discs both on pathogen genes (*PiO8* or *phcnlp1*) and eggplant housekeeping gene (*SmEF*). qPCR reactions were carried out as described in the previous paragraphs. The pathogen DNA/plant DNA ratio was calculated using standard curves and the results were analysed through IBM SPSS statistical software to perform a one-way analysis of variance test (ANOVA). Each value represented the mean of the biological replicates compared using Duncan statistical Test.

4.9. In vivo pathogen assay

Six fully knocked out T_2 *SmDMR6–1* mutants together with 6 non-edited plants were used for the stem inoculation test performed as described by Thabuis et al. (2004) with minor modifications. When the plants were at the fifth-sixth leaf stage, they were decapitated, and a *P. capsici* mycelium plug was placed on the fresh section which was then covered with aluminum cap. The inoculated plants were placed in growth chamber under controlled conditions (25 °C, day/night cycle of 16/8 h). The length of stem necrosis was measured (in centimeters) 3 and 6 dpi. The Percentage of Disease Incidence (PDI) was calculated as (D/T)*100, where D = number of diseased plants, and T = total number of infected plants. The Disease Severity Index (DSI) was calculated according to following disease grading scale: <0,5 cm (1), 0,5–0,9 cm (2), 1–1,4 cm (3), 1,5–1,9 cm (4), 2–2,4 cm (5), 2,5–2,9 cm (6), 3–3,4 cm (7), 3,5–3,9 cm (8), ≥4 cm (9). DSI data were statistically analyzed by means of Student's *t*-test.

CRediT authorship contribution statement

Martina Ferrero: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Danila Valentino:** Writing – review & editing, Investigation. **Anna Maria Milani:** Writing – review & editing, Investigation. **Cinzia Comino:** Writing – review & editing. **Sergio Lanteri:** Writing – review & editing. **Alberto Acquadro:** Writing – review & editing, Funding acquisition, Data curation. **Andrea Moglia:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100691.

Data availability

Data will be made available on request.

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