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Knock-out of *SlDMR6-1* in tomato promotes a drought-avoidance strategy and increases tolerance to Late Blight

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ARTICLE INFO

ABSTRACT

The DOWNY MILDEW RESISTANCE 6 (DMR6) protein is a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, involved in salicylic acid (SA) metabolism. SA is recognized as an abiotic stress-tolerance enhancer, and in tomato the inactivation of DMR6 was found increase its level and induce disease-resistance against several pathogens. By applying the CRISPR/Cas9 technique, we generated *Sldmr6-1* tomato mutants and tested their tolerance to drought as well as to Late Blight. Wild-type tomato cultivar 'San Marzano' and its *Sldmr6-1* mutants were subjected to water deprivation for 7 days. WT plants exhibited severe wilting, while T_2 *Sldmr6-1* mutants showed turgid leaves and maintained higher Soil Relative Water Content. Eco-physiological measurements highlighted that *Sldmr6-1* mutants adopted a water saving behavior reducing transpiration rate by decreasing stomatal conductance. Under drought stress the assimilation rate decreased as well, resulting in no alteration of the CO_2 concentration in the sub-stomatal chamber and increasing the Water Use Efficiency. Furthermore, in *Sldmr6-1* mutants the drought stress induced up-regulation of the anti-oxidant related genes *SlAPX* and *SlGST* as well as down-regulation of *SlCYP707A2* gene, which is involved in ABA catabolism. At last we highlighted, for the first time in tomato, that *Sldmr6-1* mutants exhibited a reduced susceptibility to *Phytophthora infestans*, the causal agent of Late Blight.

1. Introduction

The lack of water, due to the rising temperatures and changes in precipitation patterns, represents a limiting factor to plant growth and concurs with plant pathogen or pest attacks to cause severe plant yield reductions (Cappetta et al., 2020). World food security in the coming years will hence largely depend on the availability of biotic and abiotic stress-tolerant plants.

Tomato (*Solanum lycopersicum* L.) suffers severe yield losses due to both abiotic and biotic stresses (Kissoudis et al., 2016), thus the development of élite genotypes endowed of tolerance towards them is a major objective for tomato breeders (Egea et al., 2022).

To this end, a significant contribution can be provided by the emergent CRISPR/Cas9 technology for genome editing, which may greatly contribute to precision breeding in respect to complex, imprecise and lengthy conventional breeding strategies (Lassoued et al., 2019; Zhu et al., 2020). Genome editing has been applied to tomato since 2014 (Brooks et al., 2014; Lor et al., 2014) and has greatly facilitated the functional characterization of genes involved in many processes, including stress response (Vu et al., 2020; Lobato-Gómez et al., 2021; Salava et al., 2021). Indeed, negative regulators of abiotic stress response pathways have been targeted in tomato through CRISPR/Cas9, and key genes involved in drought, salinity and chilling stress response have been identified (Salava et al., 2021; Bouzroud et al., 2020; Tran et al., 2021; Yin et al., 2018).

Regarding the biotic stress, one of the mechanisms of plant tolerance to pathogen is due to the loss-of-function of genes required for their onset and referred to as plant susceptibility (S) genes (Pavan et al.,

2010). Among these a classic example is the Mildew resistance locus O (*Mlo1*) (Büschgeset al, 1997), whose loss-of-function natural mutants have been exploited for over 70 years in barley breeding programs (Piffanelli et al., 2002). The disabling of S genes though CRISPR/Cas9 thus represents a key target for genome editing (Engelhardt et al., 2018; Chaudary et al., 2022) and in tomato, successful examples have been reported with the goal to confer resistance against distinct classes of pathogens such as viruses (Atarashi et al., 2020; Yoon et al., 2020; Kuroiwa et al., 2022), bacteria (Ortigosa et al., 2019; Thomazella et al., 2021), fungi (Nekrasov er al, 2017; Santillán Martínez et al., 2020) and oomycetes (Thomazella et al., 2021; Li et al., 2022).

Among the S-genes, DOWNY MILDEW RESISTANCE 6 (*DMR6*) encodes a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase, which reduces the active salicylic acid (SA) pool acting as SA 5-hydroxylase (Van Damme et al., 2008; Zhang et al., 2017). CRISPR/Cas9 knock-out mutants of *DMR6* have been generated in different species such as *Arabidopsis thaliana* (Zeilmaker et al. 2015), *Vitis vinifera* (Giacomelli et al., 2022), *Ocimum basilicum* (Hasley et al., 2021), *Musa* spp. (Tripathi et al., 2021), *Solanum tuberosum* (Kieu et al., 2021), and *Citrus* spp. (Parajuli et al., 2022).

In tomato two AtDMR6 orthologs have been identified: Solyc03g080190 (*SlDMR6-1*) and Solyc06g073080 (*SlDMR6-2*). The impairment of *SlDMR6-1* resulted in resistance to bacteria (*Pseudomonas syringae, Xanthomonas gardneri, Xanthomonas perforans*), oomycetes (*Phytophthora capsici*) and fungi (*Pseudoidium neolycopersici*) (Thomazella et al., 2021). Likewise, according to Kieu et al. (2021), the disabling of the potato *DMR6-1* gene resulted in plants with increased resistance to Late Blight (LB) caused by *Phytophthora infestans*. Vice versa *SlDMR6-2* knocked-out mutants did not show improved tolerance against pathogens. It has thus been proposed that *SlDMR6* duplication in tomato resulted in subsequent sub-functionalization, causing the specialization of SlDMR6-2 in balancing the SA levels during flowering/fruit development, while the ability of SlDMR6-1 to fine-tune the SA levels during pathogen infection (Thomazella et al., 2021). SA is a natural phenolic compound which has been shown to improve plant tolerance to major abiotic stresses such as salinity, metal, osmotic, drought and heat stresses (Khan et al., 2015; Liu et al., 2022). Furthermore, it has been observed that exogenous application of SA in low concentration in tomato could mitigate the oxidative stress generated by the water stress (Chakma et al., 2021; Aires et al., 2022). Interestingly, tomato plants pre-treated with SA and experiencing water deficit had an improved water-use efficiency and net photosynthetic rate (Lobato-Gómez et al., 2021).

In this study, CRISPR/Cas9 editing technology was used for disabling the *SlDMR6-1* gene in the tomato 'San Marzano', an Italian well known cultivar used in the canning industry. Through Illumina Whole Genome Sequencing (WGS) we assessed potential off-target effects and mutational status of one selected T_1 mutant, characterized by *Cas9* absence. Due to the link between SA and drought stress response, we tested the potential drought resistance of *Sldmr6-1* tomato mutants. In addition, we also characterized for the first time their tolerance to Late blight, a potentially devastating disease of tomato.

2. Results

2.1. Molecular screening of SlDMR6-1 mutants

A CRISPR-Cas9 vector containing the h*Cas9* gene, the selective marker (*NptII*) and the polycistronic tRNA–gRNA structure with the 3 gRNAs targeting the *SlDMR6-1* gene was introduced via *Agrobacterium tumefaciens*-mediated transformation into the tomato cultivar 'San Marzano'. These gRNAs target the first three exons of *SlDMR6-1* in order to disrupt the protein's catalytic site (Fig. S1). Editing efficiency spanned greatly between the targets in T_0 plants: it was higher for gRNA1 and gRNA3, while no editing was detectable for gRNA2 (Data S1).

T_1 plants were sequenced by Sanger approach at the target loci to

evaluate editing efficiency and transmission pattern of CRISPR/Cas9-induced mutations using the web tool TIDE (Brinkman et al., 2018). Out of the 14 analysed individuals, 6 showed homozygous, heterozygous and biallelic mutations at the target sites of gRNA1 and gRNA3 (Fig. 1; Data S1). The T_1_6 plant showed homozygous mutations for gRNA1 ($-3/-3$) and gRNA3 ($+1/+1$), and the presence of h*Cas9*. The T_1_7 plant was then selected for further molecular analyses due to both its homozygous mutations for gRNA1 ($+1/+1$) and gRNA3 ($+1/+1$), and the absence of h*Cas9*.

2.2. Whole genome resequencing of a SlDMR6-1 mutant

T_1_7 and WT plants were sequenced through Illumina WGS. Genome sequencing of T_1_7 yielded 196.4 million raw paired-end reads (29.5 Gb), with an average length of 150 bp. These were reduced to 196.1 million after filtering and trimming high-quality reads. The sequence depth of coverage ranged from 37.7X (T_1_7) to 42.8X (WT) (Data S1).

A *de novo* genome assembly of T_1_7 was produced and the integration of T-DNA was inspected through the scanning of the scaffolds with Blast analysis, which did not identify any T-DNA insertions. These results clearly demonstrated h*Cas9* segregation.

Scanning of *SlDMR6-1* in the gRNA1 region revealed a 100% editing effect with homozygous mutations (a 1 bp insertion at position SL4.0ch03:46628534, Fig. 2) and no reference alleles, supporting the analysis performed with TIDE (Fig. 1a and b). Scanning of *SlDMR6-1* in the gRNA3 region highlighted an editing efficiency of 100% with homozygous mutations (a 1 bp insertion at position SL4.0ch03:46624776, Fig. 2) and no reference alleles, in agreement with the TIDE analysis (Fig. 1a and b). The mutations impacting *SlDMR6-1* result in a premature stop codon in exon 1 leading to a truncated protein.

2.3. SNP and off-target analyses in SlDMR6-1 mutant

Leveraging resequencing data, we identified polymorphisms in both T_1_7 and WT, employing the Heinz tomato genome as reference. In T_1_7, we detected 42,196 SNPs, with 88.5% of them in a heterozygous state, while in WT a total of 40,998 SNPs were identified, of which 91.3% were in a heterozygous state. Observed SNPs appeared uniformly distributed along the genome (Data S1). The average number of SNPs in both not edited (52.4 SNPs per Mb) and edited (53.9 SNPs per Mb) plants proved comparable, as did the average mutation rate (0.0052% for non edited and 0.0054% for edited plants), as documented in Table 1.

To confirm that T_1_7 displayed mutations only in *SlDMR6-1* locus and to get a deep insight into possible nonspecific editing activity, we analysed the candidate off-target loci by using the resequencing data. At first we generated a list of 53 potential off-targets for the gRNA1, gRNA2 and gRNA3 used to target the *SlDMR6-1* locus (Data S1). All the 53 candidate off-target regions showed a number of mismatches higher than 1 bp with respect to the gRNAs (Table 2). They fell in both non-coding (46) and coding (7) regions (Table 2).

An off-target analysis was conducted by aligning Illumina reads from both the WT and T_1_7 genomes to the tomato reference genome (Heinz 1706). This examination encompassed 53 potential off-target regions, ensuring a thorough evaluation to rule out the possibility of substantial deletions. Through a side-by-side comparison of DNA alignments between the WT and the mutant (*Sldmr6-1*), we ascertained that none of the candidate off-target regions exhibited any SNPs, indels, or significant deletions (Table 2). Even if minor indels or SNPs were present in the surrounding areas, they did not signify off-target effects for two critical reasons: i) these variations were conserved between mutants and WT, while they displayed polymorphism compared to the Heinz 1706 genome; ii) variations were located beyond the 20-base pair window associated with the gRNA-like sequence. The absence of off-target effects unequivocally established the specificity of Cas9-mediated *SlDMR6-1* gene editing.
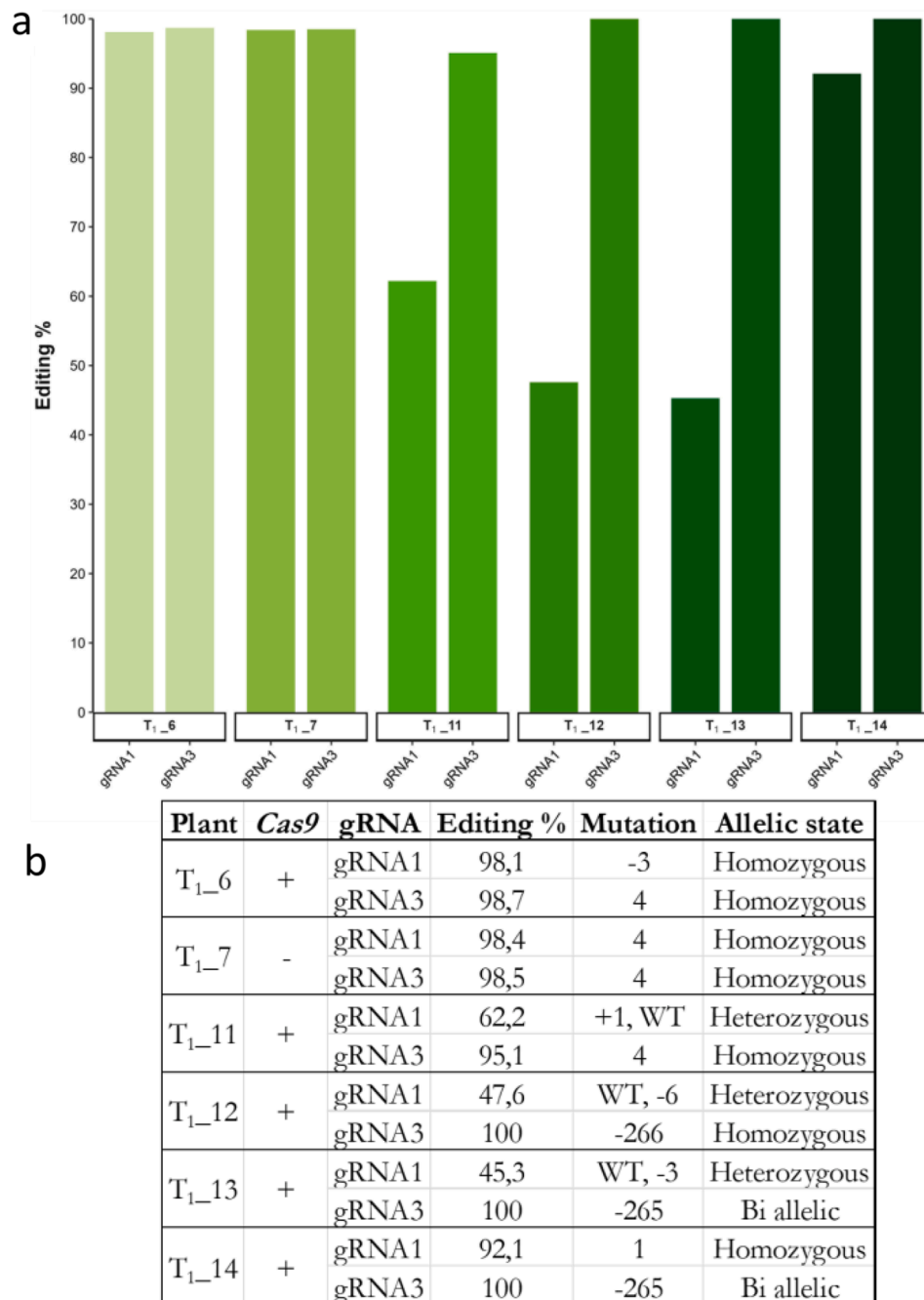
Fig. 1. Genotyping of targeted gene mutations induced by CRISPR/Cas9 in selected T_1 plants. (a) Mutagenesis frequencies (%) at gRNA1 and gRNA3 targets in six plants of the T_1 progenies. (b) h*Cas9* presence (+) or absence (-), editing efficiency, observed mutations (insertions and deletions are indicated as + and -, respectively) and allelic forms. Data were retrieved through TIDE analysis of Sanger sequences.

Plant	*Cas9*	gRNA	Editing %	Mutation	Allelic state
T_1_6	+	gRNA1	98,1	-3	Homozygous
		gRNA3	98,7	4	Homozygous
T_1_7	-	gRNA1	98,4	4	Homozygous
		gRNA3	98,5	4	Homozygous
T_1_11	+	gRNA1	62,2	+1, WT	Heterozygous
		gRNA3	95,1	4	Homozygous
T_1_12	+	gRNA1	47,6	WT, -6	Heterozygous
		gRNA3	100	-266	Homozygous
T_1_13	+	gRNA1	45,3	WT, -3	Heterozygous
		gRNA3	100	-265	Bi allelic
T_1_14	+	gRNA1	92,1	1	Homozygous
		gRNA3	100	-265	Bi allelic

2.4. Agronomic performance of SlDMR6-1 mutants

Different parameters on WT and T_2_7 plants (obtained by self-pollination of the T_1_7 mutant) grown under greenhouse conditions were measured to highlight potential differences in agronomic traits. Two groups of variables were analysed: growth variables (plant height) and yield component variables (fruit weight; number of fruits per plant). No statistically significant differences emerged by analysing these traits (Fig. S2).

2.5. Effects of SlDMR6-1 knock-out on drought resistance

To investigate the role of *SlDMR6-1* in drought stress resistance, six-

week-old WT and T_2_6 (obtained by self-pollination of the T_1_6 mutant) and T_2_7 were subjected to drought stress conditions by withholding water during a further week. At the end of the 7 day–stress period WT plants exhibited severe wilting whereas T_1_7 and T_2_6 plants remained turgid whereas (Figs. 3 and S3, respectively).

As shown in Fig. 4, the Soil Relative Water Content (SRWC) of both the WT and the T_2_7 plants decreased during drought treatments, as expected. However, every day the rate of water loss in the T_2_7 plants was lower than that of WT, indicating that edited plants transpired less. Different lines of edited plants behaved similarly: although T_2_7 was slightly superior to T_2_6, no statistically significant differences between the two T_2 lines were observed (Fig. S4).

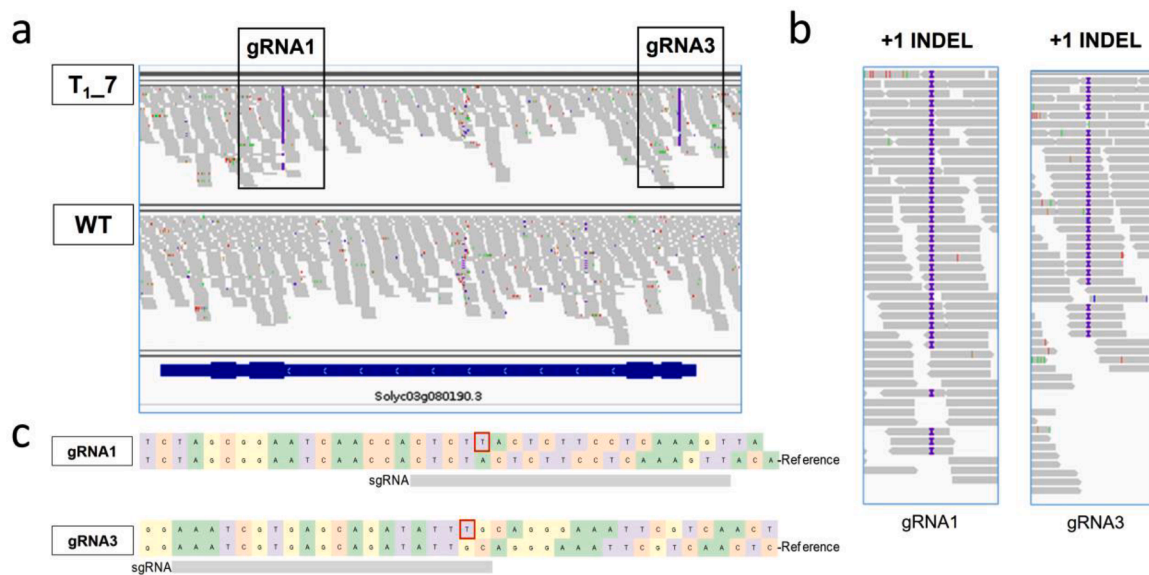Also, leaf area and dry weight (leaves, stems, roots) were evaluated

Fig. 2. Whole Genome Sequencing of T$_1$_7 *Sldmr6-1* mutant and WT. (a) Genomic sequence alignment depicting differences in the gRNA 1 and gRNA3 regions between the T$_1$_7 mutants and WT. Black boxes indicate the gRNA 1 and gRNA3 regions in the mutant detailed on the right-side; (b) Aligned reads showing the insertion of one nucleotide in both the gRNA regions in the T$_1$_7 mutant. (c) Genotyping analysis illustrating the targeted gene mutations in the T$_1$_7 plant obtained with CRISPR/Cas9.

Table 1
SNPs statistics of WGS. WT and T$_1$_7 plants were compared at genomic level with reference genome Heinz.

Genotype	Plant type	SNPs	Homozygous	Heterozygous	SNP (%)	SNP per Mb
T$_1$_7	edited	42,196	4860	37,336	0.0054	53.93
WT	*in vitro*	40,998	3566	37,432	0.0052	52.40

Table 2
Analysis of *SlDMR6-1* off targets in plant T$_1$_7. For each sgRNA-related off-target, the number of "type of mismatch" is reported. For example, "A22" means the PAM level is A (-NGG), the counts of mismatches in the seed and non-seed regions are 2 and 2, respectively.

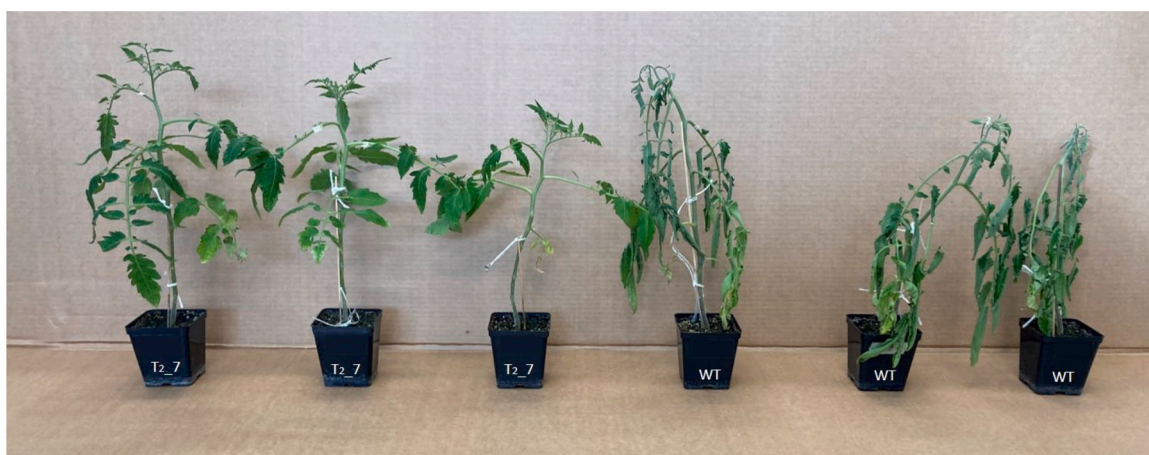| gRNA	Number of off target in the genome	Type of Mismatch							In coding	Non coding	SNP/Indel
		A22	A21	A20	A12	A11	A10	A00			
gRNA1	21	16	5	0	0	0	0	0	2	19	0
gRNA2	22	13	7	0	1	1	0	0	4	18	0
gRNA3	10	6	3	0	1	0	0	0	1	9	0
Total	53	35	15	0	2	1	0	0	7	46	0

Fig. 3. Drought stress analysis. T$_2$_7 *Sldmr6-1* and WT plants growing in a greenhouse after 7 days of withholding water.
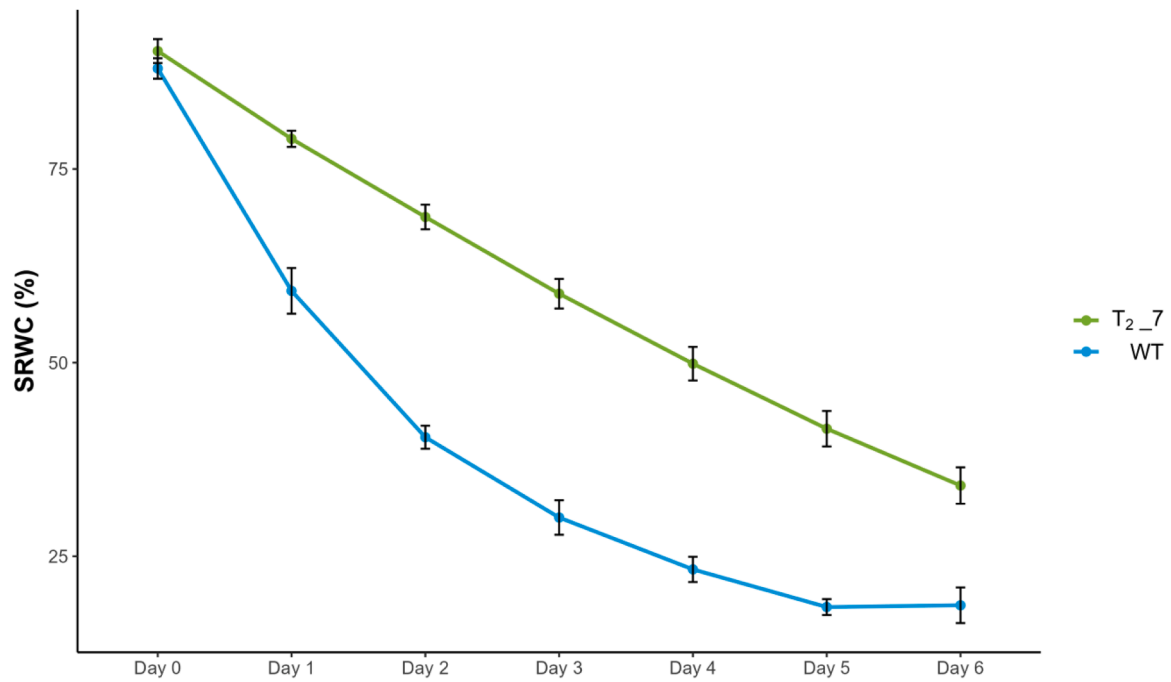
Fig. 4. Soil relative water content (SRWC) of WT and *Sldmr6-1* lines (T$_2$_7) during the drought period. Each value represents the mean of six biological replicates ± SE.
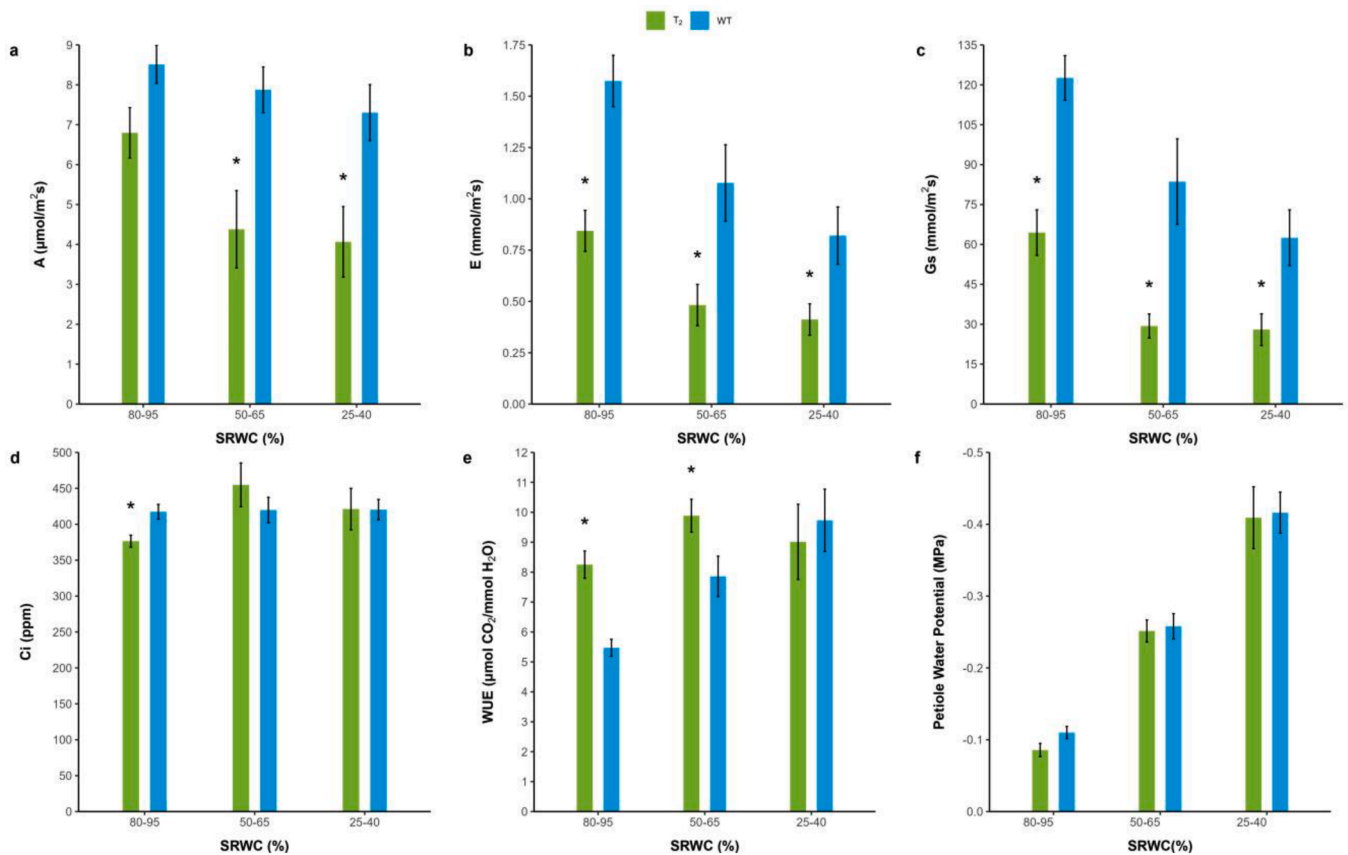

Fig. 5. Leaf gas exchange of WT and *Sldmr6-1* lines (T$_2$_7) during the drought period, according to the decreasing trend of soil relative water content (SRWC). Plants under water stress were analysed to determine different eco-physiological traits: (a) assimilation rate (A), (b) transpiration rate (E), (c) stomatal conductance (Gs), (d) CO_2 concentration in the sub-stomatal chamber (Ci), (e) Water Use Efficiency (WUE) and (f) petiole water potential. The data presented are the average values from six biological replicates with the standard error (SE) indicated. An asterisk denotes a statistically significant difference as determined by an ANOVA test ($p \leq 0.05$).

between WT and T_2_7 plants highlighting no significant differences (Fig. S5).

During soil drying kinetics, assimilation rate (A), transpiration rate (E), stomatal conductance (Gs) and CO_2 concentration in the sub-stomatal chamber (Ci) were measured in both WT and T_2_7 plants, together with water use efficiency (WUE) and petiole water potential (Fig. 5).

Water Use Efficiency (WUE) was calculated as A/E. E and Gs were significantly reduced in T_2_7 plants with respect to the WT at any SRWC range. A was significantly reduced at SRWC range 50–65% (moderate stress) and 25–40% (severe stress), whereas Ci did not show significant differences. WUE significantly increased in T_2_7 at SRWC range 80–95% (no stress) and 50–65%. Ecophysiological traits of WT plants had an abrupt collapse concurrently with wilting at day 6; at this time point SRWC for WT plant was around 20% while was higher in T_2_7 plants (around 40%). No significant differences in ecophysiological traits were underlined by comparing T_2_6 and T_2_7 (Fig. S4). In *Sldmr6-1* plants, despite being under conditions of reduced stomatal conductance, no metabolic damage that affect carboxylation activity was identified and this resulted in a Ci trend similar to that measured in WT controls, and a gain in WUE till to a moderate stress condition.

The increased drought resistance of *Sldmr6-1* lines prompted us to examine whether the expression of genes involved in ABA biosynthesis (*SlNCED1, SlNCED2, SlNCED3*) and catabolism (*SlCYP707.A1, SlCYP707.A2, SlCYP707.A3*) was altered in the edited lines under drought conditions. Moreover, we examined the transcript levels of key

anti-oxidant related genes (*SlGST, SlPOD, SlSOD, SlAPX1, SlCAT1*) (Fig. 6).

Among anti-oxidant related genes, a significant up-regulation in T_2 plants was detected for *SlGST* and *SlAPX*. Among genes related to ABA biosynthesis, a strong up-regulation in T_2 plants was demonstrated for *SlNCED1* and *SlNCED3* (around 10 and 16 fold higher respectively).

Among genes related to ABA catabolism, *SlCYP707.A2* was down-regulated in the T_2 line, while *SlCYP707.A3* up-regulated in WT.

2.6. Knock-out of SlDMR6-1 improves tolerance against P. infestans

The impairment of S-genes leads to resistance or tolerance against several biotic stresses. *SlDMR6-1* knock-out in tomato is related to tolerance against a wide array of pathogens (Thomazella et al., 2021). In this work we assessed tolerance against *P. infestans* in six selected T_1 lines ($T_1\_6, T_1\_7, T_1\_11, T_1\_12, T_1\_13, T_1\_14$). A pathogenicity assay was performed by using a detached leaf assay (Foolad et al., 2015). 72 h after inoculation the edited T_1 lines showed reduced susceptibility to *P. infestans* as highlighted by smaller necrotic and chlorotic foliar lesions than the control plants (Fig. 7a). Genomic DNA was extracted from foliar disks cut around infection site and qPCR was used to quantify the fungal biomass (Fig. 7b). Edited T_1 lines showed a clear reduced fungal biomass, from 64% (T_1_6) to 95% (T_1_7, T_1_13) reduction compared to WT.

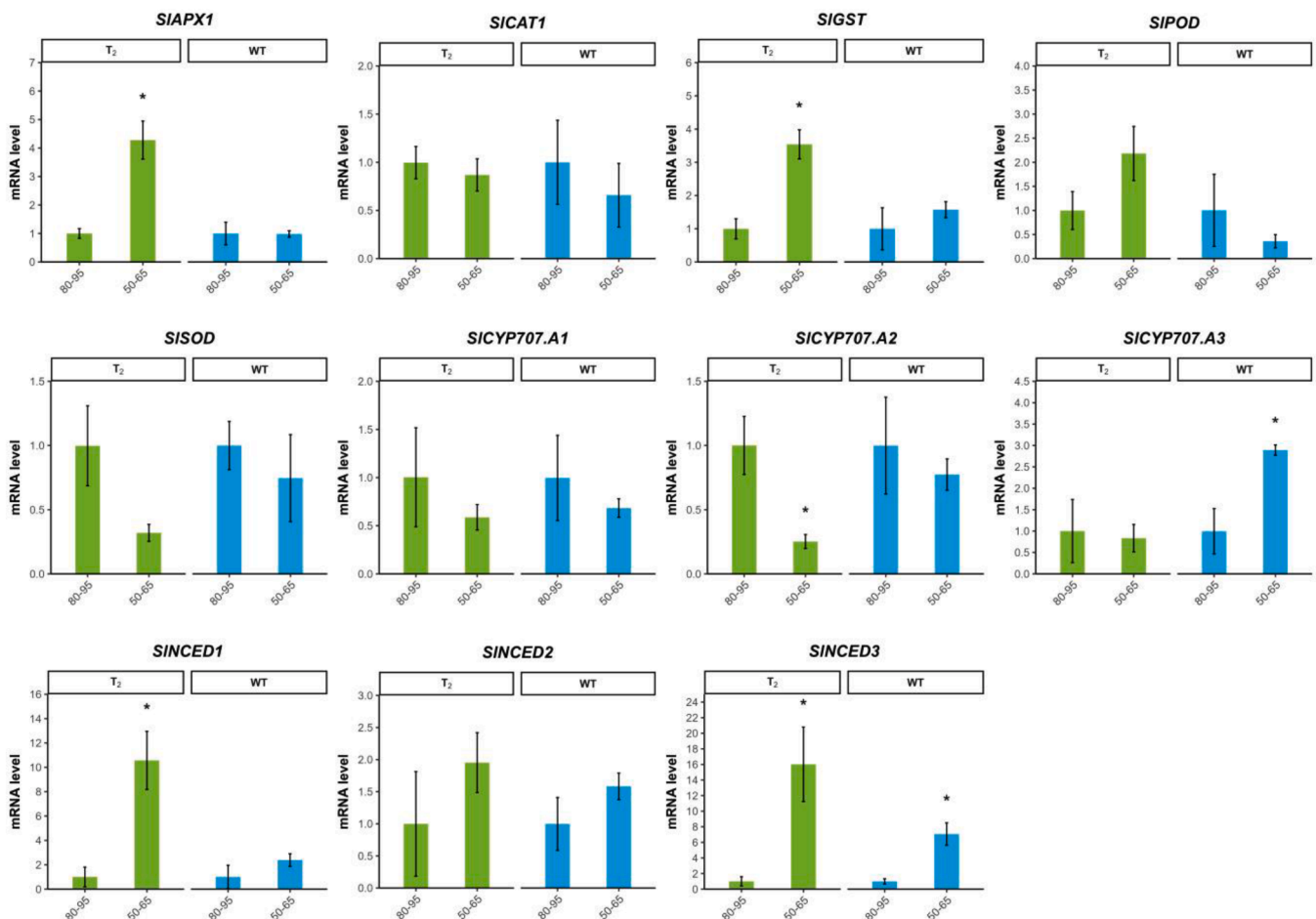**Fig. 6.** Transcriptional levels of anti-oxidant related genes (*SlAPX1, SlCAT1, SlGST, SlPOD, SlSOD*) and ABA-related genes (*SlCYP707.A1, SlCYP707.A2, SlCYP707.A3, SlNCED1, SlNCED2, SlNCED3*) during the drought assay. The values are expressed as relative mRNA abundance at SRWC range 50–65%, and compared to SRWC range 80–95%. Tomato *actin* and *β-Tubulin* genes were used as reference genes. Data are means of three biological replicates ± SE. Data refer to T_2_7 line. An asterisk denotes a statistically significant difference as determined by an ANOVA test ($p \leq 0.05$).
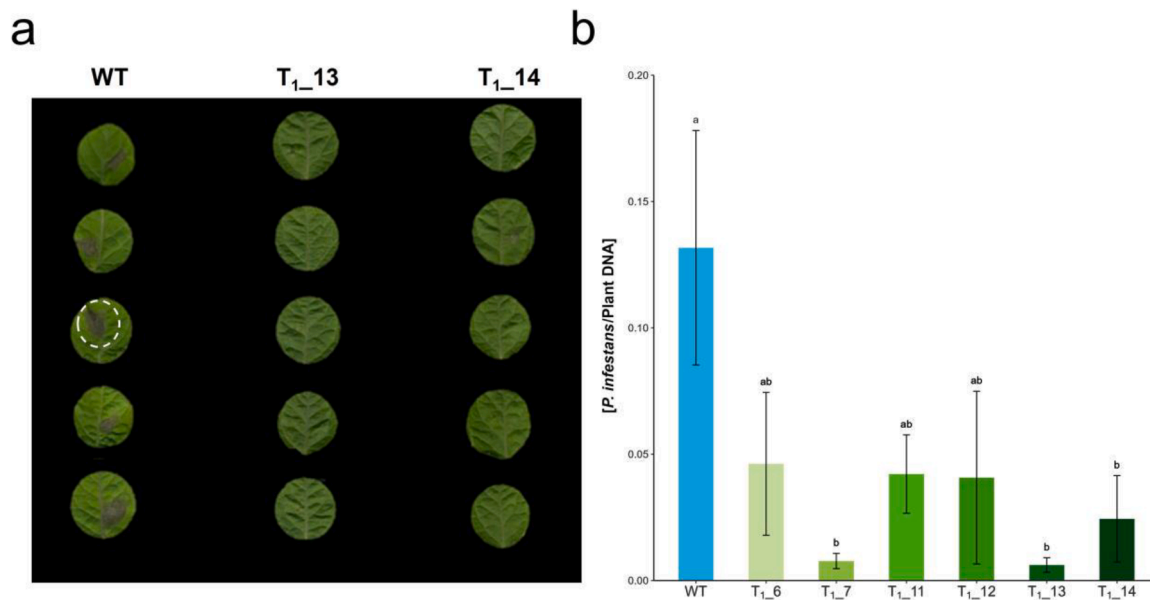
Fig. 7. Pathogen assay on WT and *Sldmr6-1* lines (a) Detached leaves assay with *Phytophthora infestans* performed on two *dmr6-1* mutants and a WT plant as a control group at three days post-inoculation. The white circle indicates the pathogen lesion (b) q-PCR pathogen DNA quantification after *Phytophthora infestans* infection. Data are the means of five biological replicates ± SE. Letters indicate significant differences based on Tuckey's HSD Test.

3. Discussion

Climate change and the resulting limited water availability represent the most limiting factors for in field tomato production. However, to date the limited genetic variation within *S. lycopersicum* has not allowed to enhance the drought tolerance of the species through traditional breeding strategies.

It has been reported that foliar application of salicylic acid (SA) to tomato plants under water deficit conditions can increase stomatal conductance, CO_2 assimilation, and water use efficiency, mitigating the oxidative stress caused by ROS over-production (Aires et al., 2022). One of the key enzyme in SA metabolism is DOWNY MILDEW RESISTANT 6 (DMR6), which catalyzes the formation of 2,5-dihydroxybenzoic acid through the hydroxylation of SA at the C5 position of its phenyl ring (Zhang et al., 2017). Indeed, the inactivation of *DMR6* resulted in increased SA acid levels (Thomazella et al., 2021; Zeilmaker et al.,2015).

Here we analysed *Sldmr6-1* tomato mutants obtained through CRISPR/Cas9. CRISPR/Cas9 editing induces random mutations which are functionally equivalent to spontaneous ones, although the functional equivalence between natural and induced mutations is not easily predictable. It has been suggested that most of the untargeted variations in edited lines are induced by somaclonal variation during in vitro culture, inheritance from the maternal plants and pre-existing variation across the germline (Sturme et al. 2022). Several studies have employed Whole Genome Sequencing (WGS) analysis of WT and CRISPR/Cas9-edited plants to investigate the specificity of genome editing, and it has been highlighted that off-target mutations occur at a much lower level than background mutations, due to pre-existing/inherent genetic or/and somaclonal variations (Liu et al., 2022; Sturme et al., 2022; Tang et al., 2018; Li et al., 2019; Wang et al., 2021).

Our edited T₁_7 line, characterized by the disabling of *SlDMR6-1* locus in homozygosity and by the absence of any transgene (Fig. 1 and 2), did not show any mutations in off-target loci nor an increased average number of SNPs when compared to not edited line (Tables 1 and 2).

We assessed the agronomic performances of T₂ *Sldmr6-1* mutants and wild-type plants growing under well-watered conditions and no phenotypic differences and pleiotropic effects were observed (Fig. S2), in agreement with results previously reported (Kieu et al., 2021;

Thomazella et al., 2021). Vice versa when we compared the impact of the T₂ *Sldmr6-1* mutants with WT plants after 7 days of water deprivation, edited plants showed turgid and green leaves while WT plants exhibited severe wilting (Fig. 3). Under water deprivation, plants can adopt the strategy of modulating gas exchange by reducing the stomatal conductance and transpiration, resulting in lower assimilation of CO_2. Ecophysiological traits measured during the period of water stress showed that the modification of *Sldmr6-1* prompted a water saving behavior reducing Gs, and in turn E and A, and supporting an efficient photosynthetic metabolism, since no difference in Ci and an increase in Water use efficiency were detected (Fig. 5).

The regulation of stomatal closure and maintenance of high soil Relative Water Content is an important strategy for water conservation under drought stress. In our study *Sldmr6-1* lines maintained higher soil SRWC than control plants during the whole imposed 7 days water stress (Fig. 4), presumably by reinforcing stomatal closure or preventing stomata opening. Drought avoidance (referred to as dehydration avoidance in recent literature) occurs when plants increase their Water Use Efficiency by reducing transpiration and avoiding dehydration during periods of drought stress (Kooyers et al., 2015). The lower transpiration detected in our *Sldmr6-1* mutants suggests that their improved performance under deficit conditions was due to the drought avoidance mechanism, as previously observed in other tomato mutants (Shohat et al., 2021).

Water deficit causes the increase of reactive oxygen species (ROS) within plant cells, which provoke oxidative damage, especially in plants adopting drought avoidance strategy by reducing transpiration, which in turn increases the dangers associated with heating the leaves (Bleau et al., 2021). It has been previously demonstrated that the increased activity of enzymes such as peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) can contribute to the enhancement of drought resistance in tomato (Chen et al., 2021; Liang et al., 2022). Our data highlight that, following drought stress, *Sldmr6-1* mutants up-regulated the transcription of *SlGST* and *SlAPX* (Fig. 6), two key anti-oxidant genes significantly upregulated when tomato plants were exposed to abiotic stress (Khan et al., 2015). This leads to the assumption that the increased antioxidant activities in *Sldmr6-1* mutants might lead to a less severe oxidative damage under drought stress. The successful coupling between the drought avoidance strategy

and an efficient ROS scavenging activity may have contributed to stomatal control of photosynthesis, avoiding or limiting metabolic imbalances and negative feedbacks on photosynthetic activity. This is evident from the overlapping of the Ci trends assessed on the basis of gas exchange data in both edited and WT plants and leading to the maintenance of high Water Use Efficiency values only in the edited plants.

In response to water stress, a crosstalk between jasmonate acid (JA), SA, and abscisic acid (ABA) in tomato has been highlighted (Muñoz-Espinoza et al., 2015). In salt-stressed tomato, SA modulated the expression of the genes involved in ABA accumulation and promoted the ABA transport to the shoot (Horváth et al., 2015). A cross-talk between ABA and SA signaling is critical for the regulation of plant reproduction and growth under combined abiotic and biotic stresses (Horváth et al., 2015).

Drought avoidance is mainly regulated by ABA, which induces stomatal closure by regulating the expression of many stress-responsive genes and whose accumulation is regulated by a balance between its biosynthesis (catalysed by 9-cisepoxycarotenoid dioxygenase enzymes) and catabolism (catalysed by 8′-hydroxylases). Three *SlNCED* genes involved in ABA biosynthesis have been characterized in tomato, while for ABA catabolism the *SlCYP707.A1, A2, A3*, and *A4* genes play a key role (Liang et al., 2022;

Our qPCR analyses demonstrated that the knock-out (KO) of *SlDMR6-1* prompted the up-regulation of *SlNCED1* and *SlNCED3* and downregulation of *SlCYP707.A2* upon stress application in contrast with WT plants. Furthermore, *SlCYP707.A3* was up-regulated in the WT lines, while no significant variations were observed for edited lines (Fig. 6). These results suggest that *Sldmr6-1* mutation might induce an increase in the endogenous ABA level by promoting ABA synthesis and suppressing its degradation, thereby positively affecting the water stress resistance mechanisms of edited plants.

Although the KO mutation of *SlDMR6-1* has been demonstrated to confer a broad-spectrum disease-resistance phenotype in tomato (Thomazella et al., 2021), the potential resistance to *Phytophthora infestans* (the causal agent of Late Blight) has never been tested. Late blight is a serious disease that may devastate an entire unprotected tomato crop within 7–10 days of infection. For the first time, our results showed an improved tolerance to Late Blight in *Sldmr6-1* tomato edited lines (Fig. 7) in agreement with what was observed in potato (Kieu et al., 2021).

In field conditions, plants can undergo different stress at the same time, thus the development of multi stress resistance cultivars represents an intriguing strategy for breeding. Different studies on the interaction between drought stress and pathogen infection have been already published, and the interaction between stress responses can be positive or negative (Bai et al., 2018). Water limitation may affect the pathogen resistance of plants in different ways, depending on the crop, pathogen, and drought scenario (Choudhary and Senthil-Kumar, 2024). A study on the effects of drought on the interaction of tomato with the biotrophic fungus powdery mildew (*Oidium neolycopersici*) and the necrotrophic fungus (*Botrytis cinerea*) reported that drought led to significant suppression of infections by both pathogens, while increased soil salinity only affected the Oidium infection (Achuo et al., 2006).

We hypothesize that simultaneously achieving drought resistance and disease resistance in tomatoes through knocking out the SlDMR6-1 gene is synergistic due to the interplay between hormonal pathways including SA and ABA. An integration between SA and ABA signaling in guard cells of stomata has been proposed, but the effects of the mechanism of this integration remain to be elucidated (Prodhan et al., 2018). Studying the response of plants exposed to combinations of stress factors is thus essential to gain insight into stress response interactions and to improve crop yields under stressful field conditions. Further studies are need to shed light on mechanisms underlining the enhanced resistance to both biotic and abiotic stresses in *dmr6-1* mutants of tomato and other crops.

4. Conclusions

We demonstrated for the first time that *Sldmr6-1* gene knock-out may contribute to the development of new tomato varieties tolerant to drought stress due to an induced plant water saving strategy. The drought-avoidance mechanism observed in our *dmr6-1* mutants might be related to a successful coupling between the drought prevention strategy and an efficient ROS scavenging activity allowing stomatal control of photosynthesis and increasing the Water Use Efficiency. Moreover, our results add *P. infestans* to the list of pathogens to which *SlDMR6-1* gene knock-out can confer resistance (*P. syringae pv. tomato, X. gardneri, X. perforans, P. neolycopersici, P.capsici*) (Thomazella et al., 2021).

On the basis of our genomic analyses we can state that CRISPR/Cas9 represents a precise tool to introduce targeted mutations, since our edited line carries an insertion that makes it completely indistinguishable from spontaneous mutants.

In our experiment no phenotypic differences between *DMR6-1* mutants and wild type plants were observed, however we intend to pursue additional research to conduct a more thorough evaluation of the effects of *SlDMR6-1* inactivation on tomato plant production in field conditions and confirm its potential as a strategy for tomato breeding.

5. Materials and methods

5.1. Target identification and vector construction

Three gRNAs (Data S1) targeting the first three exons (Fig. S1) of *SlDMR6-1* (ID Solyc03g080190) were designed using the online tool CRISPR-P 2.0 (hzau.edu.cn). The transformation vector pDGB3_alpha1 was assembled through a Golden Braid (GB) cloning system (Sarrion-Perdigones et al., 2011; Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2017; Maioli et al., 2020) following GB software-directed procedures (https://gbcloning.upv.es/). Within the vector, the expression of h*Cas9* and *NptII* was driven by the CaMV 35S and *nos* promoters, respectively, while the gRNAs were placed in a polycistronic gRNA array under the control of the *At*U6-26 RNA PolIII promoter and sgRNA scaffold/terminator.

5.2. Plant material and genetic transformation

Seeds of the cultivar 'San Marzano' were provided by Agrion (www.agrion.it) and were maintained in the Germplasm Bank of DISAFA (University of Torino, Italy). Fifty tomato seeds were sterilized in 2.5% sodium hypochlorite soaking for 20′ and then rinsed in sterile water three times. Sterile seeds were placed on sterile germination medium (1/2 MS + 15 g/l sucrose + 8 g/l plant agar) in plastic boxes, that were kept at 25 °C in the dark for 72 h before being transferred to a day/night cycle of 16/8 h. After 10 days, plantlets presented fully grown cotyledons that were used for plant genetic transformation.

The final vector pDGB3_alpha1_Tnos:NptII:Pnos_U6-26:tRNA:gRNA1-2-3_P35S:hCas9:Tnos was introduced by heat shock into the *Agrobacterium tumefaciens* LBA4404 strain. Bacteria inoculum was prepared as follows. On the first day, *A. tumefaciens* was cultured in MGL (Data S1) supplemented with streptomycin 50 mg/l and kanamycin 50 mg/l and incubated at 28 °C over night (ON). On the second day, an aliquot of the culture was inoculated (1:50) in TY (Data S1) supplemented with 200 μM acetosyringone and incubated at 28 °C ON. The OD_{600} was evaluated and the bacterial solution was diluted to a final OD_{600} of 0.10–0.15 in TY medium supplemented with 200 μM acetosyringone. Cotyledons of the seedling were cut in pieces of about 0.5 cm, which were dipped in bacterial culture for 10′, blotted dry on sterile paper and placed for 48 h on a co-culture medium in the dark. Callogenesis, shoot induction, elongation and rooting were obtained as previously described (Qiu et al., 2007). After regeneration, fully developed plantlets (T$_0$ plants, Data S1) were transplanted to soil and acclimated to *ex vitro* environment. By

selfing T_0_2 plant (selected on the basis of editing outcome, Data S1), 14 plantlets were obtained (T_1 generation) (Data S1). T_2 plants were obtained from selfing of T_1_6 (homozygous, Cas positive) and T_1_7 plant (homozygous, Cas free).

5.3. Molecular screening

Genomic DNA was extracted from T_0/T_1 plants' leaves using E.Z.N.A. ® Plant DNA Kit (Omega Bio-Tek, Norcross, USA). The screening for h*Cas9* presence was performed using primers reported in Data S1 by PCR using KAPA HIFI Taq (Kapa Biosystems, Boston, USA) with the following program: 95 °C/3′, 30 cycles of 98 °C/15′′, 60 °C/20′′, 72 °C/1′ and 72 °C/5′.

Editing efficiencies were evaluated by PCR amplification of gRNA-targeted regions according to Maioli et al. (Maioli et al., 2020) (Data S1). PCR products were sequenced by Sanger method and chromatograms were analysed using the TIDE online tool (Brinkman et al., 2018).

5.4. Whole genome sequencing and analysis

A T1 plant (T_1_7), carrying a homozygous mutation in two target regions and hCas9 segregation, together with a wild type (WT) plant were whole genome sequenced with an Illumina sequencer (Illumina Inc., San Diego, USA). One μg of DNA was used to prepare short insert (length 350 bp) genomic libraries (Novogene, Hong Kong), which were sequenced with paired-end chemistry (2 × 150 bp). Cleaning of the raw reads was conducted using Scythe (v0.991, https://github.com/vsbuffalo/scythe) and Sickle (v1.33, https://github.com/najoshi/sickle). SRA files (Project: PRJNA846963), containing raw data was submitted to NCBI.

A de novo genome assembly was carried out using the MegaHit assembler (v1.2.9, available at https://github.com/voutcn/megahit). This assembly process involved specific parameters, including k-min = 27, k-max = 141, k-step = 10, disconnect-ratio = 0, and cleaning-rounds = 1. Subsequently, a Blast analysis was conducted on the assembled scaffolds (T_1 and WT) to identify potential insertions, utilizing the T-DNA sequence as the query.

5.5. Target, off-target analysis, and SNP statistics

To analyze the identified target genomic variants and allele frequencies, we employed CRISPResso2 (accessible at http://crispresso2.pinellolab.org (Clement et al., 2019)). Fastq reads were extracted within a 100 bp window around each gRNA. For the identification of potential off-target regions in the tomato genome (SL4.0), we utilized the CasOT script (available at https://github.com/audy/mirror-casot.pl). All designed gRNAs were considered as baits in a single-gRNA mode, adhering to the default "A" PAM type (allowed PAM type:A: -NGG only; B: -NGG and -NAG; C: -NGG, -NAG and -NNGG; N: no limit) and specific permissible mismatches in both the non-seed (2) and seed (2) regions. Each identified off-target locus is defined by a Mismatch Type code (e.g.: A12). For example, "A12" means the PAM is A (-NGG), the counts of mismatches in the seed and non-seed regions are 1 and 2, respectively; the gene ID and symbol are listed as output if the site is located in an exon. Coordinates of all potential target and off-target genomic regions were intersected with the vcf file using the bedtools intersect command (accessible at https://bedtools.readthedocs.io) to eliminate monomorphic regions among edited and WT plants. The results were then inspected through custom bash scripts.

For the edited plant samples, clean reads were aligned to the tomato reference genome (SL4.0, available at https://solgenomics.net) using the Burrows-Wheeler Aligner (BWA, v0.7.17, accessible at https://sourceforge.net/projects/bio-bwa/files). The 'mem' command with default parameters was employed for this purpose. Subsequently, BAM files were processed and used for SNP calling through Samtools (v1.9–166-g74718c2) mpileup, utilizing default settings with the

exception of the minimum mapping quality ($Q = 20$) and filtering out multimapping events (-$q > 1$). This process resulted in the generation of a vcf (variant call format) file.

5.6. Evaluation of agronomic traits

Nine WT and 9 T_2_7 plants were grown in a greenhouse (mean temperature 25 °C), in 10 l pots containing a substrate mixture of horticultural substrate and perlite (3:1 v/v) from March to September 2022. Standard horticultural practices were applied. Experimental plots were arranged in a randomized complete block design with nine replications. Two different classes of variables were analyzed: growth variables (plant height) and yield component variables (fruit weight; number of fruits per plant). Fruits were collected at the commercial ripening stage. Each value represented the mean of nine biological replicates compared through a one-way analysis of variance (ANOVA) test ($p \leq 0.05$).

5.7. Drought stress analysis

Six WT, 6 T_2_6 and 6 T_2_7 plants were grown in a climate chamber (temperature 25 °C, RH 60%, 16 h light: 8 h dark photoperiod cycle, light intensity of 300 μmol m^{-2} s^{-1} PPFD) in pots containing perlite and soil-substrate (van Egmond universele potgrond) 1:5 v/v (Fig. S3). An aliquote of this soil was used to determine the maximum water holding capacity of the pots (Patono et al., 2022). Plants were grown in a well-watered state by watering to field capacity (above 75% of soil relative water content SRWC, daily at 8am) for 6 weeks prior to the experimental imposed drought. Starting the drought, plants were allowed to slowly experience water stress by withholding irrigation. The measurement of petiole water potential identified two levels of water stress: a moderate water stress, when petiole water potential reached −0.3 MPa (day 1 in WT plants, day 4 in mutants), and a severe stress when petiole water potential had reached −0.5 MPa (day 3 in WR plants, day 6 in mutants). Plants at day zero (well-watered conditions) showed approximately – 0.1 MPa in both WT and mutants according to Secchi et al. (2013). At the end of the drought stress, leaves were detached from plants and scanned. Pictures obtained were analysed with Image J software for leaf area evaluation. Leaves, stems and roots of single plants were collected, dried separately and weighted according to Huang et al. (2019).

Steady state measurements of plant-to-atmosphere gas exchange were conducted on replicate plants from 10:0am to 02:00pm with a portable Infra Red Gas Analyzer - IRGA (GFS-3000, Walz, Germany) on single leaves, under 300 ± 5 μmol m^{-2} s^{-1} light, adjusted by the additional IRGA light source (Patono et al., 2023). Assimilation rate (A), transpiration rate (E), stomatal conductance (Gs) and CO_2 concentration in the sub-stomatal chamber (Ci) were calculated following von Caemmerer and Farquhar's equations (Von Caemmerer et al., 1981); water use efficiency (WUE) was calculated as A/E.

Measurements were taken in watered condition (Day 0) and daily following drought stress application (Day 1–7). One leaf per plant was sampled at each measurement, frozen in liquid nitrogen and stored at −80 °C. The total weight (soil, pot, plant) was also measured daily and soil relative water content (soil SRWC) calculated as percentage of moisture in the soil compared to the maximum water holding capacity.

RNA was extracted from leaf samples (three biological replicates for each genotype) and using Spectrum Plant Total RNA Kit (Sigma Aldrich, Saint Luis, USA) following instruction. qPCR analysis was performed according to Maioli et al. (2020). Chosen targets belong to two groups: anti-oxidant related genes (*SlGST, SlPOD, SlSOD, SlAPX, SlCAT*) and ABA-related genes (*SlNCED1, SlNCED2, SlNCED3, SlCYP707.A1, SlCYP707.A2, SlCYP707.A3*). Tomato *Actin* and *β-Tubulin* were used as housekeeping genes. Information about primer sequences and target can be found in Data S1. Transcript levels were quantified through the 2$^{-\Delta\Delta Ct}$ method. Each value represented the mean of three biological replicates compared using Student's *t*-test ($p \leq 0.05$).

5.8. Pathogen assay with Phytophthora infestans

The isolate of *Phytophthora infestans* (Westerdijk Fungal Biodiversity Institute strain CBS 120920) was maintained in Cornmeal medium (Data S1) at 18 °C in dark. *P. infestans* was inoculated on Rye Agar (Data S1) one week before pathogenicity assay and kept at 18 °C in the dark. The plate was then flooded with tap sterile water (refrigerated at 4 °C) and kept for 2–3 h at 4 °C to induce zoospore release. Subsequently, the plate's liquid was passed through two layers of cheesecloth, and the quantity of zoospores was determined using a hemocytometer. The concentration was diluted to 2.5×10^4 spores/ml (Karki et al., 2021).

A detached leaf assay was set up using 5 leaves from the six selected T_1 plants (Fig. 1) and WT plants according to the procedure described by Foolad et al. (2015). The leaves were washed with sterile water, gently dried using sterile paper, and then positioned in plastic trays containing water agar (20 g/l). About 250 zoospores (10 μl) were placed on each leaf. Plastic trays were then covered with lids and incubated at 20 °C in the dark in a growth chamber. The trays were examined every day. Picture and samples were collected three days post inoculation.

To quantify the pathogen infection rate, the ratio between fungal and plant DNA was evaluated according to Pavese et al. (2021). Disk samples around infection site were taken and DNA extraction was performed using an E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, Norcross, USA). For the quantification of DNAs standard curves were prepared using primers designed as follows: *SlActin* for tomato DNA, *PiO8* (Llorente et al., 2010) for *P. infestans* DNA. Extracted DNAs were analyzed through real-time qPCR both with pathogen gene (*PiO8*) and tomato's one (*SlActin*). qPCR reaction was carried out as described in the previous paragraph and information about primer sequences can be found in Data S1. Fungal and plant DNA was quantified using standard curves and the ratio fungus DNA/plant DNA calculated. One-way analysis of variance test (ANOVA)was performed through IBM SPSS statistical software. Each value represented the mean of 5 biological replicates compared using Tukey's HSD Test ($p \leq 0.05$).

Funding

CRediT authorship contribution statement

Alex Maioli: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Federica De Marchi:** Writing – review & editing, Investigation, Data curation. **Danila Valentino:** Writing – review & editing, Investigation. **Silvia Gianoglio:** Writing – review & editing, Validation, Investigation, Data curation. **Davide Lucien Patono:** Writing – review & editing, Investigation. **Fabio Miloro:** Writing – review & editing. **Yuling Bai:** Writing – review & editing. **Cinzia Comino:** Writing – review & editing. **Sergio Lanteri:** Writing – review & editing. **Claudio Lovisolo:** Writing – review & editing, Data curation. **Alberto Acquadro:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Andrea Moglia:** Writing – review & editing, Writing – original draft, Supervision, 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.

Data availability

Sequencing data used in this study are openly available in the NCBI database (PRJNA846963).

Acknowledgment

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100541.

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