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Knockout of a pectate lyase in tomato increases fruit firmness and reduces foliar susceptibility to pathogens

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ABSTRACT

As agriculture and food systems are facing growing challenges because of extreme weather conditions and unstable socioeconomic landscapes, improving crop quality and agronomic traits has become an increasingly important goal in plant breeding. Preventing post-harvest losses and finding new genetic sources for pathogen resistance are vital for the development of resilient and competitive varieties. In tomato (*Solanum lycopersicum* L.), fruit softening plays a key role in determining the post-harvest quality, with a significant impact on both food waste and food safety. To elucidate the role of pectate lyase (*SlPL*, *Solyc03g111690*) in the softening process and in susceptibility-to-pathogen mechanisms, knock-out mutants have been generated in a Micro-Tom genetic background by CRISPR/Cas9. Two CAS-free edited mutant lines (carrying a 1 bp insertion and a 2 bp deletion, respectively) showed a significant increase in fruit firmness at two different ripening stages (breaker+4 and red ripe) and lower weight loss during post-harvest storage. Furthermore, the observed overall reduction in the activity of six cell wall remodeling enzymes (pectate lyase, pectin methylesterase, polygalacturonase, α-D-galactosidase, β-D-galactosidase and β-D-glucosidase) and the reduced susceptibility to leaf infection caused by *Botrytis cinerea*, *Phytophthora capsici* and *Pseudomonas syringae* pv. *tomato* pathogens suggested that the alteration of the cell wall metabolism limits the pathogen's ability to establish infection. The obtained results open the possibility of developing new tomato varieties that meet both agronomic and quality needs while helping to clarify the complexity of the cell wall-disassembly process.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide. Excluding tubers, it is first in terms of both production, with 192 Mt produced in 2023, and cultivated area (5.4 Mha). Most of tomatoes are produced in China and India, followed by Turkey, USA, Egypt and Italy (FAOSTAT, 2024; last consultation on 01 December 2024). Tomatoes are nutritious foods that represent important sources of compounds beneficial for the human health, such as minerals and antioxidant molecules, i.e. lycopene, β-carotene, ascorbic acid, vitamin E and phenols (Frusciante et al., 2007).

Fruit ripening consists of a complex chain of events leading to deep changes in fruit metabolism, altering its physiology and biochemical composition (Kalaitzis et al., 2010). With ripening, pigments, like carotenoids and flavonoids, accumulate and the chlorophyll is degraded; softening occurs because of cell wall remodeling, and the contents of sugars and volatile compounds increase, resulting in flavor formation (Li et al., 2022).

Once the fruit has received appropriate stimuli to start the ripening cascade, catabolic and anabolic pathways are activated, and profound changes start to shape its new appearance. One of the fundamental events that occurs with ripening is softening. Softening takes place through a modification of texture, which is due to changes in the cell wall and cuticle composition (Giovannoni et al., 2017). The plant cell wall shows an intricate structure made up of three predominant polymers: i) cellulose, ii) hemicellulose, and iii) pectin. The interactions

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among different enzymes are responsible for the degradation of such polymers (Fig. S1), leading to cell wall remodeling during fruit ripening and softening. Cellulose is cleaved by β-glucosidases (β-Glu); hemicellulose, which in eudicots is prevalently composed of xyloglucan (Barnes and Anderson, 2018), is disassembled by different glycosidases, including β-galactosidase (β-Gal) and β-glucosidase. For pectin, different enzymes cooperate to depolymerize the homogalacturonan (HG) structure; pectin methylesterases (PMEs) remove the methyl-ester groups from the polygalacturonic acid chains, making them accessible to polygalacturonases (PGs) and pectate lyases (PLs), both of which require the de-esterification of pectate to hydrolyze the HG backbone. The contribution of β-Gal to pectin degradation through the cleavage of galactose residues in the rhamnogalacturonan-I (RG-I) has also been described; β-Gal increases pectin solubilization, thus facilitating the access of PMEs and PGs to their substrate (Brummell and Harpster, 2001). Finally, α-galactosidase (α-Gal) also plays a role in fruit ripening and softening; the enzyme is prevalently allocated in the cytosol and the vacuole, but it has been reported in the cell wall as well (Keller, Pharr, 2017; Marraccini et al., 2005). The capacity of α-Gal to hydrolyze cell wall materials and solubilize pectin has been described in orange (Burns, 1990), avocado (De Veau et al., 1993) and papaya (Soh et al., 2006).

aPreventing post-harvest losses, caused by fruit deterioration, premature softening, microbial decay or mechanical damage, is a major concern faced by all the stakeholders of the agri-food supply chain. Spanning from harvesting to consumption, losses up to 50 % have been estimated in the tomato market (Arah et al., 2016). To meet this challenge, tomato breeders have long focused on enhancing fruit firmness to improve harvesting and post-harvest procedures, to better preserve the quality of products and minimize food waste (Nie et al., 2024). Different strategies have been adopted over the years, and in tomato, the goal has been to reduce softening in order to increase fruit firmness and extend storage-life while preserving other desirable qualities. While spontaneous ripening mutants, such as *ripening inhibitor* (*rin*) and *non-ripening* (*nor*), have been largely used to develop commercial varieties with improved firmness and shelf-life (Wang et al., 2020; Wang and Seymour, 2022), targeting cell wall remodeling enzymes has appeared to be a more effective way to increase post-harvest quality without causing detrimental effects on fruit color and flavor (Uluisik et al., 2016). To achieve this goal, genes encoding for polygalacturonase (*SlPG2a*), β-galactosidase (*SlTBG4*) and xyloglucan endotransglucosylase/hydrolase (*SlXTH5*), have been the target of genetic engineering (Wang et al., 2023). Recently, Nie et al. (2022) reported delayed softening and higher firmness in CRISPR/Cas9 mutants for the *SlPG2a* gene, and different authors showed that the silencing of the pectate lyase-encoding gene *SlPL* (*Solyc03g111690*) in Ailsa Craig through CRISPR/Cas9 and in Micro-Tom through RNAi, can have a positive effect on delaying fruit softening (Uluisik et al., 2016; Yang et al., 2017; Wang et al., 2019). Notably, when combining mutations in *SlPG2a* and *SlPL*, Ortega-Salazar et al. (2024) increased firmness and reduced water-loss to a greater extent than in single mutants.

In tomato, 22 *PL* genes have been identified, however only *SlPL* is highly expressed in fruits, indicating a putative major role in softening (Yang et al., 2017). PLs were first described in the pathogens *Erwinia carotovora* and *Bacillus* spp. (Starr and Moran, 1962), where they act as virulence factors and play an essential role in infection processes (Uluisik and Seymour, 2020). Once the pathogen penetrates the cutin layer, pectins act as a barrier to prevent infections, stimulating the plant immune response (Wan et al., 2021). In *Arabidopsis*, the modification of the expression of genes encoding pectin-degrading enzymes, such as *PME3* and PME inhibitors enhanced the plant resistance to *Botrytis cinerea* and *Pectobacterium carotovorum* (McMillan et al., 1993; Lionetti et al., 2007). Moreover, the deletion of the PL-econding gene *PL1332* in *Alternaria brassicicola* reduced the pathogen virulence (Cho et al., 2015). As the threat of pathogens is likely to worsen because of the ongoing climate changes (Velásquez et al., 2018), finding new ways to face crops diseases has become of greater importance.

To increase our understanding of the effects of *SlPL* knockout on fruit firmness, shelf-life, cell wall remodeling and pathogen susceptibility, a gene editing approach was set up in a Micro-Tom genetic background. *SlPL* was hereby chosen to generate CRISPR/Cas9 mutants and two lesions in the second exon of the gene, which resulted in a higher fruit firmness and lower weight-loss in comparison to wild type, have been described. In the mutants, overall reduction in the activity of cell wall remodeling enzymes occurred and reduced susceptibility at foliar level to three different tomato pathogens (*B. cinerea*, *Phytophthora capsici* and *Pseudomonas syringae* pv. *tomato*) was reported for the first time.

2. Materials and methods

2.1. Plant material

Solanum lycopersicum cv. Micro-Tom (hereafter referred to as wild type, WT) seeds were *in vitro* germinated at 25°C to obtain cotyledons for transformation. Edited plants were grown in growth chamber under standard conditions (16 h light/8 h dark at 25°C) and fruits at the ripening stage of breaker (BR)+ 4 and BR+ 7, equivalent to red ripe (RR), were collected for the phenotypic characterization and biochemical analysis.

2.2. Construct assembly and plant transformation

2.2.1. SgRNA design and CRISPR/Cas9 vector construction

The construct for the CRISPR/Cas9 transformation was assembled using the GoldenBraid (GB) cloning system (Sarrion-Perdigones et al., 2011) as described by Gianoglio et al. (2022). A single guide RNA (sgRNA) was designed to target the second exon of *SlPL* using the CRISPR-P v2.0 tool (Liu et al., 2017) and selected on the basis of the best on-target efficiency score and the lowest number of predicted off-target sites. The final pDGB3-ω1 construct, containing the transcriptional units Tnos:NptII:Pnos-35S:Cas9:Tnos-PAtU626:sgRNA:scaffold, was transferred by electroporation into *Agrobacterium tumefaciens* EHA105 electrocompetent cells and used for the transformation of cotyledonary explants.

2.2.2. Agrobacterium-mediated transformation

Transformation was carried out according to Olimpieri et al. (2011). Sterile Micro-Tom seeds were germinated in Petri dishes and transferred in a growing medium containing 1 X Murashige and Skoog (MS) salts, 3 % sucrose and 7 g/L plant agar (pH 5.8). Once emerged, cotyledons were dissected into four pieces and incubated in the dark on preconditioning medium supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4D), 0.35 mg/L indole-acetic-acid (IAA), 0.7 mg/L trans-zeatin (T-zea) and 200 μM acetosyringone. After 48 h, explants were inoculated for 5 min with the *Agrobacterium* culture and incubated on the same medium in the dark for 24 h. Then, the cotyledons were maintained on induction medium supplemented with 0.35 mg/L IAA, 0.7 mg/L T-zea, 200 mg/L cefotaxime and 50 mg/L vancomycin for one week and finally transferred onto regeneration medium, supplemented with 0.35 mg/L IAA, 0.7 mg/L T-zea, 500 mg/L carbenicillin and 100 mg/L kanamycin. Regenerated shoots of approximately 1 cm in length were transferred to rooting medium supplemented with 0.35 mg/L IAA, 0.1 mg/L indole-butyric-acid (IBA), 200 mg/L cefotaxime and 50 mg/L kanamycin.

2.3. Molecular screening and sequencing

DNA was extracted from rooted shoots according to Doyle and Doyle (1987) and screened for the transgenic cassette by amplification of the Cas9 and NptII regions, using primer pairs shown in Table S1. The PCR reaction was set up in a final volume of 10 μL containing 1 μL of gDNA (50 ng/μL), 1 μL of each primer (10 pmol/μL), 5 μL of 2 × Green GoTaq buffer (Promega Corporation, Madison, WI, USA), and 2 μL of ddH_2O.

The reaction included an initial denaturation step at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, an annealing at the specific temperature for each primer pair (Table S1) for 30 s, and an elongation at 72°C for 30 s, and a final elongation step at 72°C for 7 min. Transformed T_0 plants were transferred *in vivo* into pots, and their sgRNA target sites (amplified using primers listed in Table S1) were Sanger-sequenced (Eurofins Genomics, Luxemburg City, Luxemburg) and analyzed by the Tracking of Indel by DEcomposition (TIDE) tool (https://tide.nki.nl/). Seeds from edited plants were then collected. T_1 plants were self-pollinated until the mutations were fixed in a homozygous state. A 10X whole genome resequencing of T_3 Cas-free plants (one for each obtained mutant genotype, plus WT as control) was performed (BGI Genomics, Shenzhen, China) and screened for on-target/off-target mutations. Possible Cas9 off-target sites were identified using the Cas-OFFinder software (http://www.rgenome.net/cas-offinder/), using the *S. lycopersicum* SL2.50 genome assembly as reference and setting the maximum number of mismatches (MMs) to five. For the T7EI endonuclease assay, primers were designed to amplify an 816 bp region containing the CRISPR target site, with the expected Cas9 cut site located at 266 bp downstream of the 5′ end of the WT fragment (Table S1). The assay procedure was carried out according to the protocol supplied by New England Biolabs Inc.

2.4. RNA extraction, cDNA synthesis and qPCR analysis

For RNA extraction, pericarp tissue of fruits at the RR stage and leaves from five-weeks old plants were collected in triplicates. Samples were frozen and extracted using TRIzol™ (Invitrogen™, Thermo Fisher Scientific, USA) following the manufacturer's protocol. RNA quantification and quality control were assured by Qubit™ RNA Broad Range and Integrity and Quality Assay Kits (Invitrogen™, Thermo Fisher Scientific, USA). Two hundred ng of RNA were used for cDNA synthesis by yourSial® cDNA synthesis kit (S.I.A.L. Srl, Rome, Italy). The relative expression of *SlPL* (*Solyc03g111690*) was assessed by RT-qPCR using yourSIAL® Green Mix Plus ROX run on a QuantGene 9600 real-time PCR system (Bioer Technology, Hangzhou, China). Samples were amplified in triplicate and three independent runs were performed for each tissue, proceeding with an initial incubation at 95°C for 20 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. *Solyc11g008430* (*Q-actin*) was used as the reference gene for the CT comparison (Shang et al., 2021) and previously described primers for *SlPL* (Yang et al., 2017) were utilized (Table S1). The expression of additional genes (*Solyc03g123630*, *Solyc05g005170*, *Solyc04g008730*, *Solyc11g069270*, *Solyc01g074030*), selected to represent the PME, PG, α-Gal, β-Gal and β-Glu families in leaves according to available databases (TomExpress database http://tomexpress.toulouse.inra.fr; Fumelli L. and Mazzucato A., unpublished data), was analyzed in leaf tissues using the same protocol. The relative primers are reported in Table S1.

2.5. Protein prediction

The protein sequences of mutants were predicted with the Expasy Translate tool (https://web.expasy.org/translate/) and aligned against the WT sequence on Clustal Omega Multiple Sequence Alignment (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). The effect of mutations on the protein structure and ligand-binding sites of native Micro-Tom and mutated *PL* proteins were inferred by I-TASSER (https://zhanggroup.org/I-TASSER/).

2.6. Fruit phenotyping for quality traits

For the quality trait phenotyping all fruits were rinsed with distilled water and cleaned with ethanol prior to analysis. The weight of 20 red ripe T_3 fruits per genotype was recorded. For fruit shape index (FSI) and total soluble solids (TSS) determination, ten representative fruits from each line were analyzed. FSI was calculated as the ratio of the polar to the equatorial diameter of the fruit, and the TSS were obtained by two measurements of the juice per each fruit, taken by a digital pocket refractometer (ATAGO™ PAL-1, ATAGAO CO., LTD, Tokyo, Japan). pH was measured in three biological and three technical replicates, by a Fisherbrand™ Set pH meter accumet™ AE150 (Thermo Fisher Scientific, USA), on 250 mg of frozen pericarp resuspended in 15 mL of pH 7.0 distilled water.

The external color was determined on 20 fruits per genotype at each ripening stage (BR+4, RR) with a Minolta colorimeter (Minolta C2500; Konica Minolta, Ramsey, NY, USA) and two values, taken from the equatorial region on two opposite sides of the fruit were recorded for each CIEL*a*b* parameter.

Fruit firmness was measured with an Instron® Universal Testing Machine (model 4301, Instron, Canton, MA, USA). Thirty fruits per genotype were harvested at BR+ 4 and RR stages. Fruits at BR+ 4 were stored at room temperature and firmness was measured over a period of 10 d, for a total of four measurements. RR fruits were measured and immediately frozen for further analysis. The tomato fruit was compressed with a flat compression anvil (55 mm diameter) at a rate of 10 mm min^{-1}. The deformation of the whole fruit to a load of 3 N was recorded and reported as "deformation to 3 N".

Weight loss assessment was performed only on RR fruits; 15 fruits per genotype were collected and weighed every 3 d up to 18 d post-harvest (dph). The weight loss percentage was calculated as follows:

$$\frac{W_0 - W_1,\ W_2,\ \ldots,\ W_n}{W_0} \times 100$$

where W_0 is the weight recorded at harvest, W_1 is the weight recorded at 3 dph, W_2 is the weight at 6 dph, and so on until $n = 6$.

2.7. Enzymatic activity

To evaluate the activity of the main cell wall remodeling enzymes, fruits from the WT and mutant lines were harvested at the RR stage, and pericarp tissue was grinded by an IKA® A11 basic analytical mill (IKA, Staufen, Germany) and stored at −80°C. Assays were carried out on three biological and three technical replicates. Protein quantification was performed with a Qubit™ Protein Broad Range (BR) Assay Kits (Invitrogen™, Thermo Fisher Scientific, USA). PL activity was assessed with the Pectate Lyase Microplate Assay Kit (#CAK1093, Cohesion Biosciences) following the manufacturer's instructions. To determine the level of the 4,5-unsaturated reaction products, the absorbance of the supernatant was measured at 235 nm. For the PME activity, the Hagerman and Austin (1986) protocol was followed. The linear decrease in absorbance at 620 nm that occurred during the first minute of the reaction was recorded, and a standard curve of D-galacturonic acid was used to calculate the concentration of the reaction product. For PG activity, the protocol described by Shakir et al. (2022), with slight modifications, was followed and the product concentration was assessed via a standard curve of D-galacturonic acid. Glycosidase extraction was performed according to Fils-Lycaon and Buret (1991) with slight modifications, and the activity was calculated utilizing a molar extinction coefficient of 1.77×10^4 (Konozy et al., 2012). The activity of all enzymes was expressed as U (1 U = µmol of reaction product liberated per min) per mg of protein under the assay conditions.

2.8. Pathogenicity assays

Seeds from WT and from each of the T_3 homozygous mutated lines were surface-sterilized with 70 % ethanol, followed by 50 % bleach treatment. After thorough rinsing with distilled water, seeds were incubated on wet filter paper for 48 h in darkness at room temperature. Germinated seeds were transferred to autoclaved soil and grown in a controlled growth chamber with a 16/8 h light/dark photoperiod at 25°C. Leaves from five-week-old plants were collected in triplicate and

infected with *B. cinerea*, *P. capsici*, and *P. syringae* pathogens. For the *B. cinerea* assay, the protocol of Lian et al. (2018) was followed with slight modifications. The fungal strain SF-1 was maintained on malt extra agar medium at 20°C under a 12 h photoperiod prior to spore collection. The spore concentration was measured with a counting chamber under a microscope and adjusted to 5×10^5 spores/mL before inoculation. Leaflets from the third true leaf were detached from the plants and placed onto 0.8 % agar medium. For *P. capsici* infection, the pathogen was cultured on V8 agar plates in the darkness at 25°C for 7 d. Portions of fungal cultures were transferred to fresh plates containing sterile water and incubated under continuous light for 3 d to induce sporangia formation. Sporangia were observed under a microscope and harvested by centrifugation at 5000 ×*g* for 10 min. The pellet was dissolved in 2 mL of sterile water to prepare the spore suspension. For both *B. cinerea* and *P. capsici*, 5 µL of spore suspension was used to infect leaves, and, after 48 and 72 h of incubation, respectively, disease symptoms were evaluated by assessing lesion development and lesion area on the infected leaflets via ImageJ software (https://imagej.net/ij/index.html). Sterile distilled water was used as a control treatment.

P. syringae pv. *tomato* (Pst) DC3000 was grown on LB liquid medium, supplemented with 50 µg/mL rifampicin, at 28°C for 36–48 h. After growth, bacterial cells were suspended in sterile distilled water to a final optical density (600 nm) of 1.0. Leaves of each genotype were syringe-infiltrated with the Pst suspension or sterile water as a control. Two leaf discs with a total area of 0.4 cm^2 were collected 3 d post inoculation, surface sterilized with 70 % ethanol, rinsed with sterile distilled water, and subsequently homogenized in 1 mL of sterile water. Serial dilutions of the homogenate were prepared, and 5 µL of each dilution were plated onto LB agar plates containing 50 µg/mL rifampicin. After 48 h of incubation at 28°C, bacterial colonies were counted.

2.9. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA), and significant differences were determined by Dunnett's Multiple Comparison *post hoc* test, implemented on the DescTools R package (Signorell, 2024) and performed in RStudio v4.3.3 (Posit team, 2022). The same statistical analysis was performed on phenotypic data, gene expression analysis, enzymatic activity and pathogenicity assays.

3. Results

3.1. Two non-sense editing events in SlPL were generated by CRISPR/Cas9 in Micro-Tom

A 20 nt sgRNA targeting the second exon of *SlPL* was designed, based on the best on-target and lowest off-target scores (Table S2), and the final pDGB3-ω1 construct was used to transform Micro-Tom cotyledons. Five independent shoots (PL1, PL7, PL8, PL9, and PL17) were positive to the Cas9/NptII PCR screening. Sanger sequencing of the target locus of T_0-transformed plants revealed no editing events in PL7 or PL8. Furthermore, two chimeric events occurred, one in PL1, which retained the WT allele and exhibited a 1 bp insertion and a 3 bp deletion, and one in PL17, which retained the WT allele and exhibited a 1 bp insertion and a 2 bp deletion. Finally, PL9 was heterozygous for the WT and a 1 bp insertion allele (Table S3). Seeds were collected from each T_0 line, and T_1 progeny plants were grown and further analyzed as described above. Alleles predicted in T_0 were not yet fixed, with all lines showing the same allelic distribution as T_0, except for PL1, which lost the 1 bp insertion allele, and PL7, in which a new allele appeared (Table S3). As PL1 and PL7 did not produce seeds and PL8 did not carry any mutations, only PL9 and PL17 were grown to the T_2 generation. T_2 DNA was screened for mutations by the T7EI endonuclease assay (Fig. S2), and none of the PL9-derived plants displayed the mutation digestion pattern. Consequently, only PL17 Cas-free plants, confirmed to be edited by the T7EI assay, were sequenced in the target region to assess the type of mutation. By TIDE decomposition, all the screened plants exhibited a heteroallelic configuration (Fig. S3), and ultimately, only T_3 homozygous lines could be recovered and phenotypically evaluated (Table S3; Fig. S4). In Fig. 1, the two fixed mutations are shown; the 1 bp insertion mutant will be hereafter referred to as PL^{INS} and the 2 bp deletion mutant as PL^{DEL}.

SlPL encodes a 403 amino acid (aa) protein. The two mutations caused a predicted frameshift that introduces a premature stop codon, with the 1 bp insertion resulting in a 176 aa truncated protein and the 2 bp deletion resulting in a 175 aa truncated protein. As shown by the alignment of the native and mutated PL proteins, PL^{INS} and PL^{DEL} are identical, except for an isoleucine (I) residue that is retained in PL^{INS}, as in the WT sequence (Fig. S6). Additionally, the Interpro domain IPR002022 Pectate lyase/Amb_all (from aa 128–325) resulted to be truncated in both the mutated proteins, lacking the substrate binding-activity (D224, K248, L251, R285) and the active site (D202, M280) residues, as predicted by I-TASSER (Fig. S6).

Cas-OFFinder identified a total of 20 potential off-target sites, one with 4 MMs (falling within the first intron of *Solyc02g088550*) and 19 with 5 MMs (all falling in intergenic regions). No mutations were detected at either the 4 MMs (Fig. S5) nor in the 5 MMs (not shown) predicted off-target sites in the two CRISPR-edited lines.

3.2. SlPL knock-out mutants showed no changes in fruit phenotype except for post-harvest traits

Fruit weight (FW), FSI, TSS and pH were measured at the RR stage in WT, PL^{INS} and PL^{DEL} fruits. No significant difference was detected in any of the analyzed traits between the WT and the mutated lines (Fig. 2). With respect to color, PL^{INS} showed higher values for the L* and b* parameters in comparison to WT at BR+ 4 and RR stages, while PL^{DEL} showed lower values of a* and b* at RR stage (Table S4). In terms of fruit firmness, both the PL^{INS} and PL^{DEL} fruits were highly significantly different ($p < 0.001$) in comparison to WT. Fruit deformation was 19 % and 17 % lower than that of the WT fruits for PL^{INS} and PL^{DEL} at BR+ 4 and about 20 % lower than that of the WT fruits at RR (Fig. S7). For firmness decay, PL^{DEL} fruits always exhibited a significantly reduced softening compared to WT, while PL^{INS}, even showing a general increase in firmness, resulted to be significantly firmer only at the first, third and fourth measurement (Fig. 3A).

Similar to fruit firmness, the PL^{DEL} fruits always exhibited a significantly lower percentage of weight loss than the WT fruits, while PL^{INS} showed a significant difference compared to WT starting from 9 dph (Fig. 3B). Fruits of all the genotypes appeared wrinkled after 18 dph, but for PL^{INS} and PL^{DEL} wrinkling was visibly reduced (Fig. 3C). Furthermore, the relative expression of *SlPL* in fruit pericarp was significantly reduced by more than 55 % in both the mutated lines in comparison to the WT (Fig. 3D).

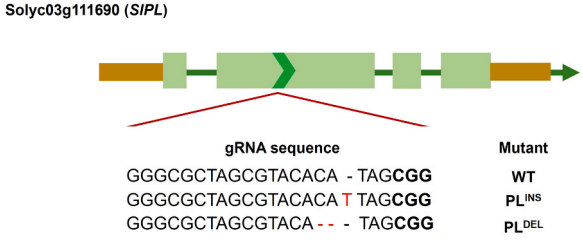

Fig. 1. Target sequence of the *Solyc03g111690* (*SlPL*) gene and genetic lesions obtained. The PAM sequence is reported in bold. Mutations obtained by insertion (PL^{INS}) and deletion (PL^{DEL}) are reported in red.

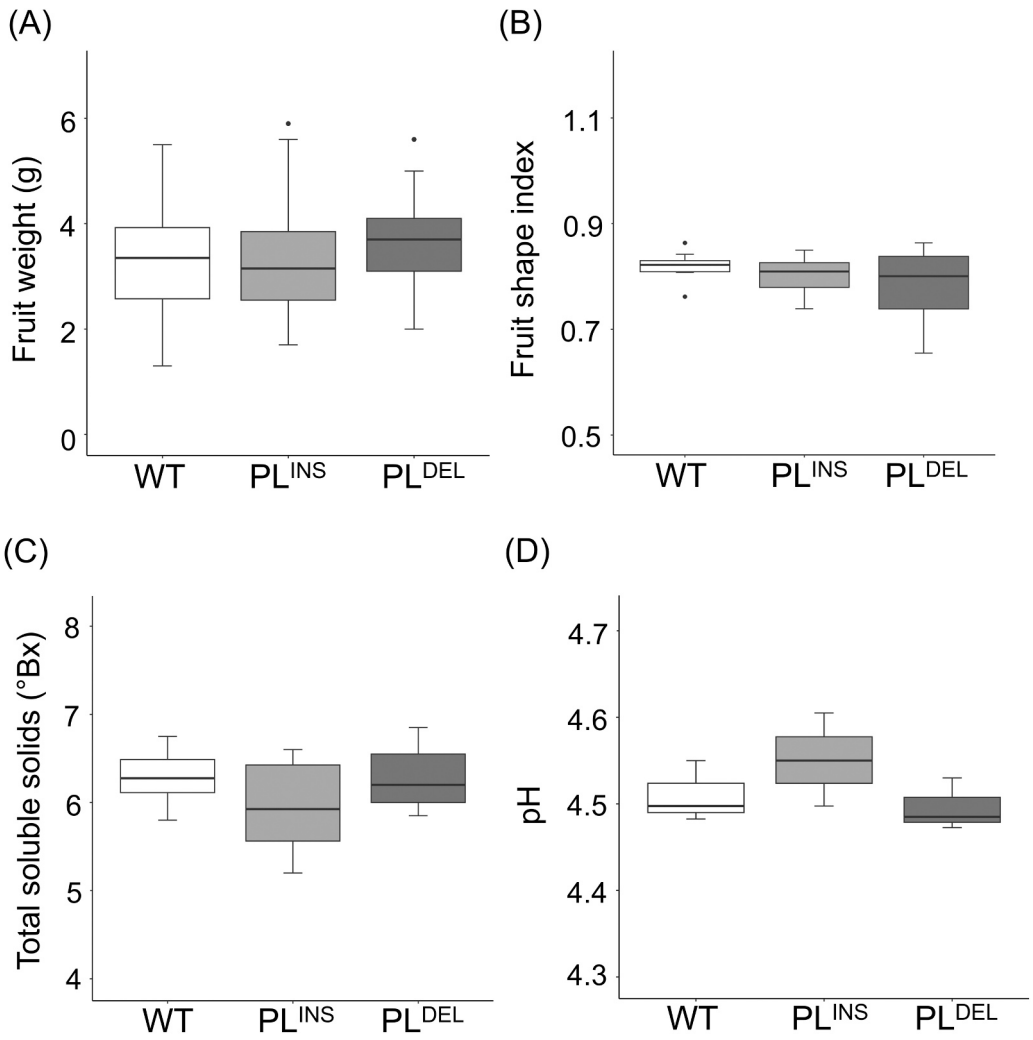

Fig. 2. Fruit quality parameters in the wild type (WT) and mutated lines by insertion (PL^{INS}) and deletion (PL^{DEL}). (A) Fruit weight; (B) fruit shape index; (C) total soluble solids; (D) pH.

3.3. Cell wall remodeling enzymes showed reduced activity in SlPL mutant fruits

Both PL^{INS} and PL^{DEL} exhibited a significant reduction in the activity of all the tested enzymes at the RR harvesting time. The PL activity was reduced by 37.4 % and 16.6 % in the PL^{INS} and PL^{DEL} lines, respectively (Fig. 4A). For PME, the activity decreased by about 40 % in comparison to WT (Fig. 4B), while for PG the activity decreased approximately by 50 % (Fig. 4C). Finally, all the analyzed glycosidases showed lower activity in the mutated lines in comparison to the WT: α-Gal activity was reduced by 46 % in both mutants; β-Gal activity decreased by more than 55 % and the decrease in β-Glu activity was about 60 % (Fig. 4D-F). PME was the enzyme that presented the highest activity in all the genotypes, followed by α-Gal, PG, PL, β-Gal and lastly β-Glu.

3.4. SlPL mutants showed reduced susceptibility to pathogens at the vegetative level

Compared to WT plants, both the PL^{INS} and PL^{DEL} lines presented reduced susceptibility to *B. cinerea* and *P. capsici* infection (Fig. 5A-B; Fig. S8). For *B. cinerea*, mutants showed a reduction in lesion size of about 20 %. For *P. capsici*, the disease severity was reduced by 20 % in PL^{INS} and by 30 % in PL^{DEL}. In the bacterial infection assay involving Pst, both *SlPL* mutants exhibited higher levels of resistance, outdoing the WT genotype (Fig. 5C), with PL^{INS} and PL^{DEL} showing 22 % and 27.4 % fewer bacterial cells, respectively.

These effects were coupled with a reduction in the relative expression of *SlPL* in leaves of about 43 % and 52 % for PL^{INS} and PL^{DEL}, respectively (Fig. 6A). In contrast, no significant differences were detected in the expression of PME, PG, α-Gal, β-Gal and β-Glu encoding genes that were selected to represent those enzyme families inhibited in mutant fruits (Fig. 6B-F).

4. Discussion

The chromatogram decomposition analysis carried out on TIDE revealed that in T_0 three plantlets were edited at the target *SlPL* site, but no homozygous events occurred. For PL7, a new allele (1 bp insertion) appeared in the T_1 generation. This was probably due to residual Cas9 activity, since the obtained PL7 T_1 plant retained the Cas9 sequence. Mutations not detectable in T_0 but appearing in the T_1 generation were previously reported in wheat by Zhang et al. (2021).

In plants, the most common CRISPR/Cas9-induced mutations are single nucleotide insertions, mainly A or T, as reported for different crop species (Ma et al., 2015; Gianoglio, 2018; Dono, 2020), and up to 10 nucleotides deletions (Jansing et al., 2016), with breakpoints mainly located 3 bp upstream of the PAM sequence. PL^{INS} and PL^{DEL} have a 1 bp insertion (+T) and a 2 bp deletion (-AC), respectively, occurring 3 bp upstream of the PAM. Insertion mutations have been previously described in tomato (Uluisik et al., 2016; Wang et al., 2019) but no

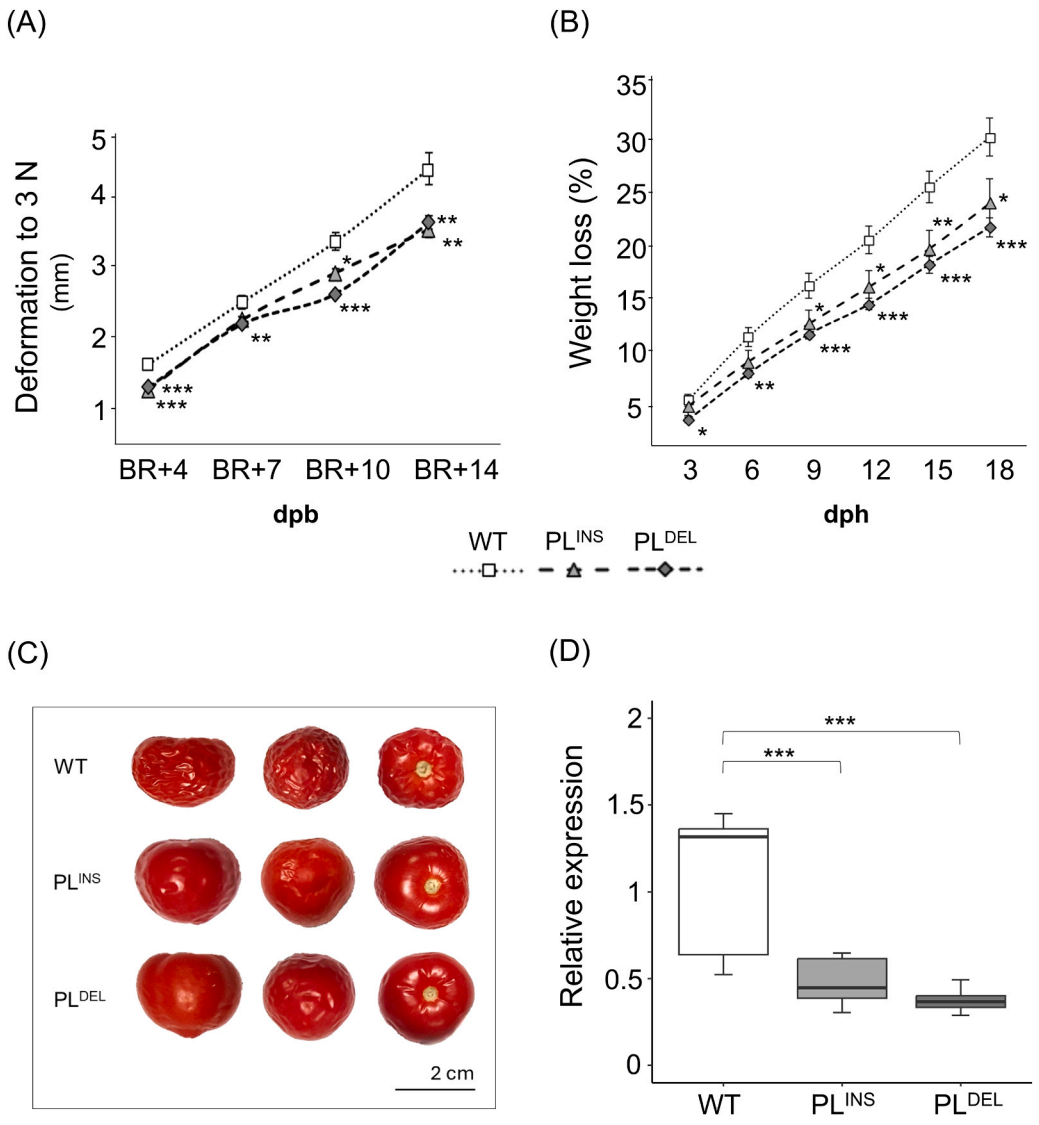

Fig. 3. Dynamics of post-harvest traits and relative expression of *SlPL* (*Solyc03g111690*) in wild type (WT) and mutated lines (PL^{INS}, PL^{DEL}). (A) Fruit firmness at different d post-breaker (dpb), (B) weight loss in fruits stored at room temperature for 18 d post-harvest (dph), (C) lateral, stylar, and stem side views of representative fruits at 18 dph and (D) relative expression of *SlPL* in fruit pericarp at the RR stage. Asterisks indicate significant differences, determined by Dunnett's test between each mutated line and the WT (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

deletions have yet been reported at the target region of the *SlPL* locus.

4.1. *SlPL knock-out improved fruit firmness maintaining morphological and quality traits unchanged*

As the world is facing growing challenges, like climate changes, overpopulation and food scarcity (United Nations, 2024), improving quality, agronomic, and post-harvest traits of crop species has become a very important goal in plant breeding (Cardi et al., 2023). In tomato, preserving fruit firmness during handling and transporting, and improving storage-life for large-retailer distribution are among the key factors that affect the marketability of the product. Since spontaneous ripening mutants, largely exploited in the tomato breeding, can strongly affect the ripening process and have negative pleiotropic effects on the organoleptic quality (Juan-Cabot et al., 2022), new approaches to better face the softening issue have been developed and targeting the enzymes responsible for cell wall remodeling has appeared a more effective way to avoid fruit color and flavor alteration (Uluisik et al., 2016).

In our experiment, no differences were detected between fruits of the two mutants and those of the WT in the TSS content, an important index for taste and overall tomato fruit quality (de Brito et al., 2021), indicating no pleiotropic effects of the *SlPL* knockout, nor unwanted events from *in vitro* transformation on this indicator. Moreover, *SlPL* editing did not affect FW, FSI and pH, proving that CRISPR/Cas9-induced specific mutations can be obtained without altering non-target traits. Previous authors demonstrated that silencing *SlPL* in Ailsa Craig through CRISPR/Cas9, and in Micro-Tom by RNAi, can have a positive effect on delaying fruit softening (Uluisik et al., 2016; Yang et al., 2017; Wang et al., 2019). Here, the same effect on fruit phenotype, associated with a significant reduction of the gene expression in the pericarp in comparison to WT, was achieved in two *SlPL* mutants obtained in a Micro-Tom background by CRISPR/Cas9 system. Indel occurrence may introduce premature stop codons and truncated transcripts induce nonsense-mediated mRNA decay mechanisms, leading to mRNA degradation before a significant amount of protein is produced (Popp and Maquat, 2016). This mechanism can likely affect PL activity, with visible effects on fruit texture at different ripening stages, including higher firmness in mutated genotypes even after 10 d of storage and a reduced level of weight loss in comparison to the WT over a period of 18 d.

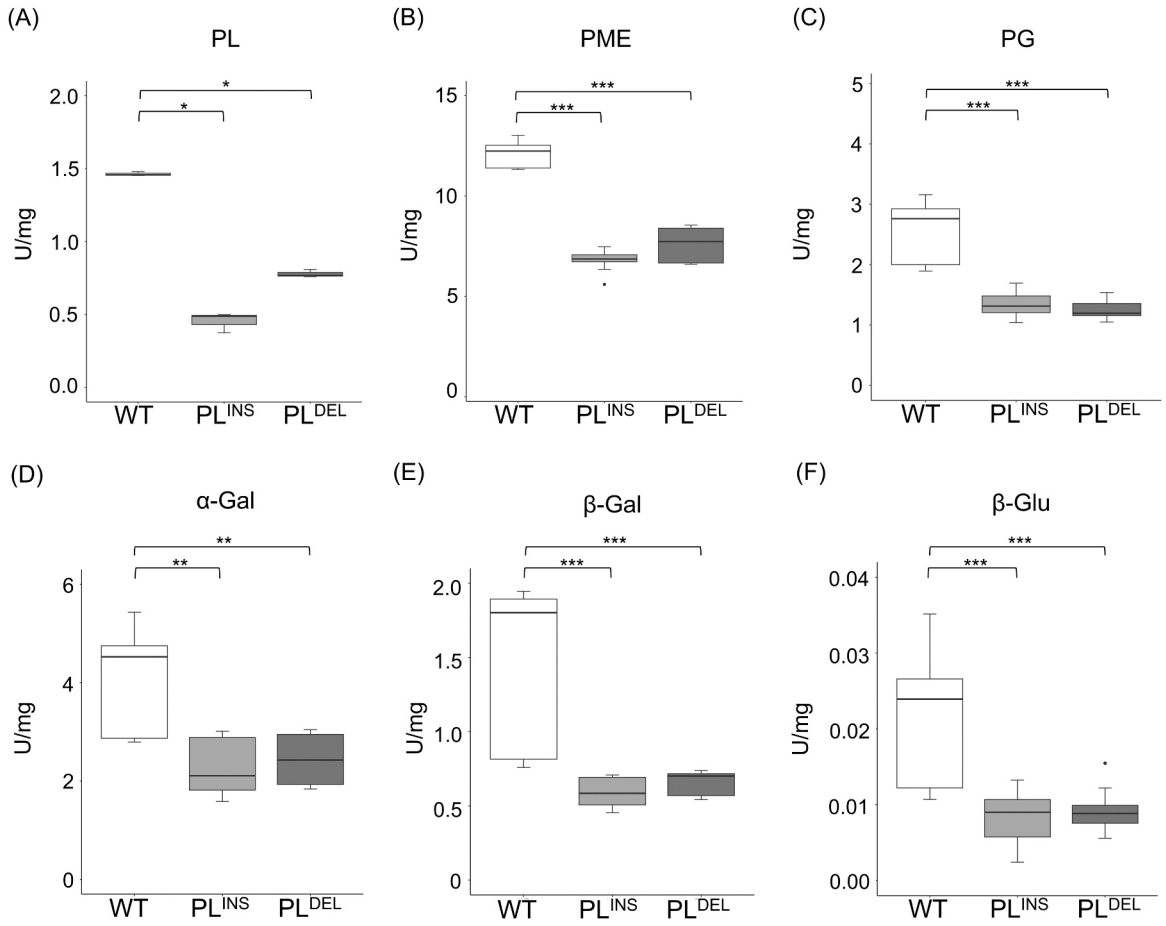

Fig. 4. Activity of cell wall-remodeling enzymes. (A) Pectate lyase (PL), (B) pectin methylesterase (PME), (C) polygalacturonase (PG), (D) α-galactosidase (α-Gal), (E) β-galactosidase (β-Gal) and (F) β-glucosidase (β-Glu). Asterisks indicate significant differences, determined by Dunnett's test, between each mutated line (PL^{IN}, PL^{DEL}) and wild type (WT) (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

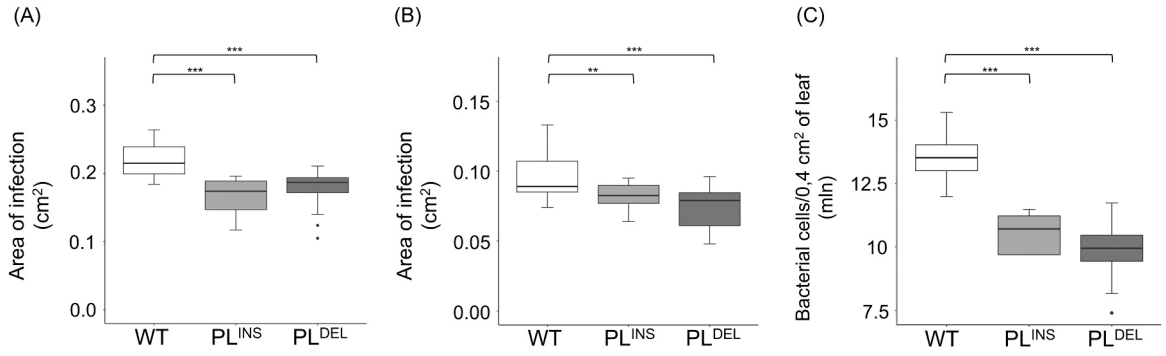

Fig. 5. Response of wild type (WT) and mutated lines (PL^{INS}, PL^{DEL}) leaves to pathogen infections. (A) *B. cinerea*, (B) *P. capsici* and (C) *P. syringae* pv. *tomato*. Asterisks indicate significant differences, determined by Dunnett's test, between each mutated line and WT (** $p \leq 0.01$, *** $p \leq 0.001$).

Similar to pectin, changes in cuticle composition also affect the fruit softening process, reducing water loss and delaying softening (Lara et al., 2019). The cuticle interacts with the cell wall of the outer epidermal layer (Domínguez et al., 2011), and changes in the cell wall structure might affect its permeability, decreasing transpiration and consequently weight loss. With respect to the external fruit color, the decrease in L* and b* values together with the a* value increase, obtained from BR+ 4 to RR in all genotypes, reflected the darkening of the fruits due to carotenoid (mainly lycopene) biosynthesis and chlorophyll degradation (Ayuso-Yuste et al., 2022). The difference between the mutated lines and the WT may be caused by possible changes in the lycopene β-cyclase activity, that can affect β-carotene and lycopene accumulation (Wang et al., 2019).

4.2. SlPL knock out reduced the overall activity of cell wall remodeling enzymes in fruits

The processes of plant cell wall remodeling and reorganization involve many players, ranging from phytohormones (especially ABA and ethylene) to enzymes and ripening-related expansins (EXPs) (Forlani et al., 2019). During plant growth, cell walls continuously change, and the disassembly mechanism peaks during fruit softening. In fleshy fruits,

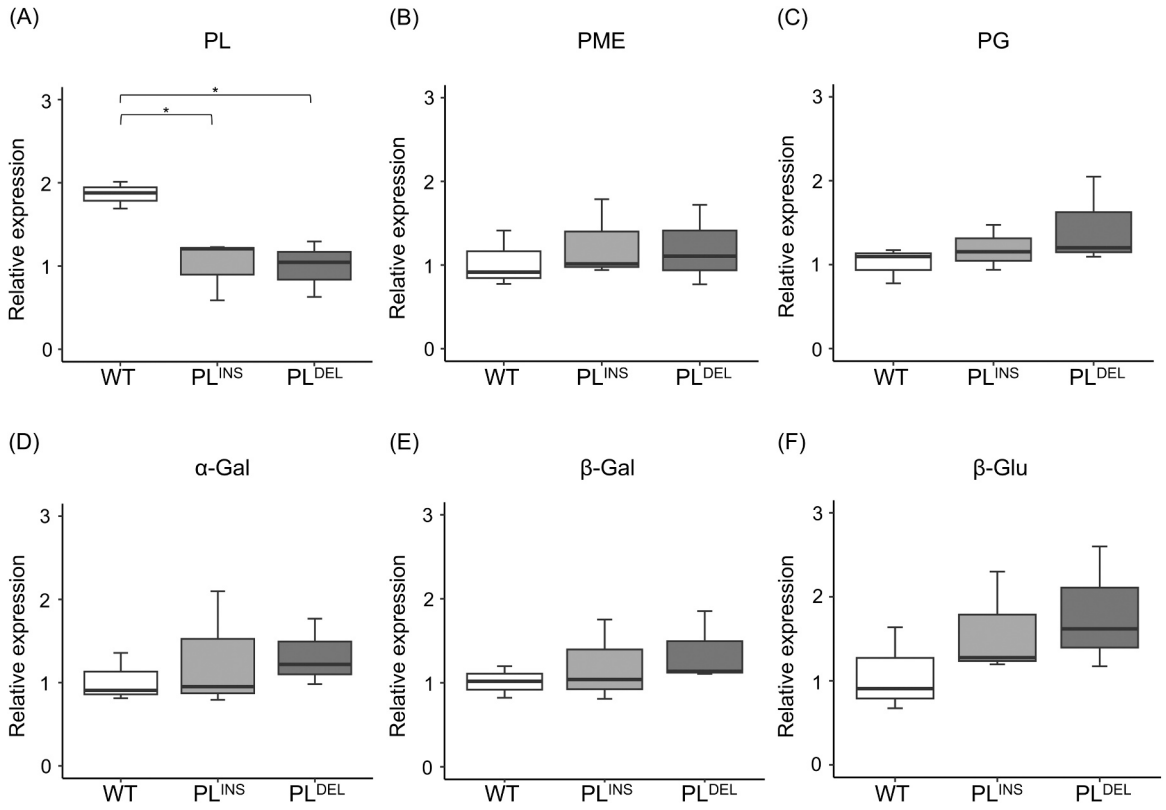

Fig. 6. Relative expression of (A) *Solyc03g111690* (*SlPL*), (B) *Solyc03g123630* (PME), (C) *Solyc05g005170* (PG), (D) *Solyc04g008730* (α-Gal), (E) *Solyc11g069270* (β-Gal) and (F) *Solyc01g074030* (β-Glu) in five-weeks-old leaves of Micro-Tom wild type (WT) and mutated lines (PL^{INS}, PL^{DEL}). Asterisks indicate significant differences, determined by Dunnett's test, between each mutated line and the WT (* $p \leq 0.05$).

pectin depolymerization occurs, and the integrity of the middle lamella is affected, leading to a decrease in cell-to-cell adhesion and thus a reduction in fruit texture (Brummell and Harpster, 2001). Pectin undergoes the most relevant changes, being remodeled, disassembled and solubilized by the action of a variety of enzymes, such as PGs, PLs, PMEs, β-Gal and EXP (Wang et al., 2018). In our *SlPL* CRISPR mutants, PL alteration caused a decrease in the overall activity of several cell wall remodeling enzymes, being consistent with the higher cellulose and hemicellulose content found by Yang et al. (2017) in *SlPL*-RNAi plants and resulting in firmer fruits and lower weight loss during storage.

PMEs act upstream of PLs and PGs and are necessary for their activity to take place (Barnes and Anderson, 2018). PMEs remove the methyl-ester groups from the HG polygalacturonic acid chains, making them accessible to PL and PG. The reduction in PG and β-Gal activity observed in the mutated lines may be explained by the indirect interaction of these two enzymes with PL, which acts on de-methylated pectin chains and fosters a more extensive degradation of HG and RG-I (Ortega-Salazar et al., 2024). Based on the immunolocalization in the cell wall compartments and the signal intensity of the different pectic elements, Wang et al. (2019) concluded that PL is necessary to facilitate the normal degradation of β-1,4-galactans and to solubilize pectin, allowing the disaggregation of polymers necessary for subsequent depolymerization by PG.

Moreover, it has been observed that pectin physically and functionally interacts with cellulose fibrils, and hemicellulose (Broxterman and Schols, 2018). Taken together, these findings, along with an increase in cellulose and hemicellulose content found in Micro-Tom *SlPL*-silenced lines (Yang et al., 2017), indicate the intricate interaction among the different cell wall polymers and that the lack of PL activity may impair the capacity of other enzymes, such as α-Gal and β-Glu, to access their substrates.

The decrease in PME activity in PL-silenced plants remains of difficult explanation, as PME operates upstream of the pectin degradation process. Plausible hypotheses for this feedback regulation could reside in a lower availability of esterified HGs in a reduced PL-activity background or in a transcriptional tuning that affects the expression of genes which are responsible for cell wall remodeling. In this view, *PL* expression is necessary to maintain the transcriptional activity of enzyme-encoding genes and to repress the expression of inhibitor proteins (Yang et al., 2017).

4.3. Altering pectin metabolism reduced tomato susceptibility to different pathogens

The tomato is vulnerable to numerous pathogens that can severely impact crop production. Among these, *B. cinerea* and *P. syringae* are particularly harmful, leading to widespread infections at different stages of plant growth (El-Fatah et al., 2023, Rhouma et al., 2023). Effective management of these diseases requires a deep understanding of the pathogen life cycle and of their interactions with host plants. *P. capsici* is an oomycete responsible for a range of destructive diseases in vegetable crops such as peppers, cucumbers, squash and tomatoes (Lamour et al., 2012). Although it is not a true fungus, it presents many fungal-like traits in terms of infection patterns and life cycle. The response of tomato plants to pathogen attack is largely determined by their genetic background, which influences both susceptibility and resistance (Campos et al., 2021).

Pathogenicity assays revealed that the *SlPL* knockout mutants exhibited reduced susceptibility to three taxonomically different pathogens at vegetative level. The enhanced resistance to all tested pathogens suggests that the loss of PL activity impairs the pathogen's ability to infect, potentially by increasing cell wall integrity, which reduces the ability of the pathogen to degrade the plant cell wall through pectin degradation, a key process in *B. cinerea* pathogenicity (Silva et al.,

2023). Additionally, the reduced susceptibility of the mutant genotypes to *P. capsici* indicates that the knockout of *SlPL* can also confer resistance to oomycete pathogens. The mechanism underlying this phenotype could involve altered pectin structure, which may limit the pathogen's ability to establish infection or to efficiently penetrate plant tissues. Finally, the higher level of resistance to *P. syringae* pv. *tomato*, displayed by both PL^{INS} and PL^{DEL} mutants, indicates that the disruption of *SlPL* activity possibly leads to a stronger defense response or reduced susceptibility to bacterial colonization.

Reduced susceptibility to grey mold was previously reported in *SlPL*-silenced fruits (Yang et al., 2017; Ortega-Salazar et al., 2024), but effects after foliar infection have not been described to date. *Solyc03g111690* is mainly expressed in fruit pericarp during ripening (Uluisik et al., 2016; Yang et al., 2017), but our analysis carried out in Micro-Tom disclosed the presence of *SlPL* transcripts also in leaf tissues, with a significantly reduced transcription level in the mutated CRISPR lines. These result supports that PL plays a significant role in the susceptibility of Micro-Tom plants to fungal, bacterial, and oomycete pathogens in vegetative organs. PL is known to break down pectin in plant cell walls, facilitating the invasion and spread of pathogens (Uluisik and Seymour, 2020). The silencing of *SlPL* likely hampers this process, resulting in a cell wall that is less prone to pathogen degradation.

In contrast to fruits, where a decrease in activity of other cell wall remodeling enzymes was reported, no alterations in the expression of the respective genes were detected in mutant leaves, suggesting a specific role of PL in the reduced susceptibility of these organs. However, further investigation on the function of the modules regulating cell wall metabolism in leaves are needed, to provide a more extensive understanding of the mechanisms underlying the plant—pathogen interaction at the vegetative level.

5. Conclusions

Tomato is a widespread crop that is consumed worldwide. Among the main challenges of the Century, reducing food waste, increasing food security and facing agricultural threats such as pathogen attacks are of utmost importance, with relevance for fleshy fruits like tomato. Knocking out the pectin-degrading-enzyme-encoding gene *SlPL* in the Micro-Tom genetic background produced a multiple effect on fruit texture, cell wall remodeling enzyme activity and pathogen susceptibility. The insights gained from this study will help elucidate the complexity of the cell wall remodeling pathway and guide future breeding efforts to develop tomato varieties that are not only improved in fruit quality, but also more resilient towards biotic stresses, contributing to more sustainable agricultural systems. These results can also serve as a basis for future efforts to improve the resilience of other commercially important tomato varieties, including landraces, using similar CRISPR-based approaches.

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CRediT authorship contribution statement

Fabrizio Olivieri: Writing – review & editing, Methodology, Data curation. **Barbara Farinon:** Writing – review & editing, Methodology, Data curation. **Muhammad Ilyas:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Antonio Granell:** Writing – review & editing, Methodology. **Giulia De Lorenzo:** Writing – review & editing, Methodology, Data curation. **Roberto Forniti:** Writing – review & editing, Methodology, Formal analysis. **Rinaldo Botondi:** Writing – review & editing, Methodology, Data curation. **Ludovica Fumelli:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Andrea Mazzucato:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2025.112868.

Data availability

Data will be made available on request.

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