



CRISPR/Cas-mediated polyphenol oxidase gene knockout in potato reveals divergent roles in resistance to bacterial wilt and late blight

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ARTICLE INFO

Keywords:

Biotic stress
Phytopathogen
Plant immune system
Phytophthora
PPO
Ralstonia

ABSTRACT

Polyphenol oxidases (PPOs) play a pivotal role in plant immune responses by catalysing the oxidation of phenolic compounds into cytotoxic quinones and melanin and contributing to the fortification of cell walls. Despite their biological significance, the high expression of PPOs in potatoes is not desirable due to their promotion of tuber browning. This study elucidates the relationship between PPO activity and defense mechanisms against the diverse pathogens *Ralstonia solanacearum* (Rs) and *Phytophthora infestans* (Pi) while mitigating enzymatic browning. CRISPR/Cas-mediated editing of the tuber- and root-specific PPO genes in the 'Desirée' and 'Balatoni Rózsa' potato cultivars considerably reduced enzymatic activity and browning. Among four PPO-edited mutant lines, three exhibited increased susceptibility to Rs while responses to Pi remained unchanged, underscoring the importance of PPOs in resistance to Rs. The PPO knockouts resulted in significant shifts in metabolite and hormone profiles characterized by elevated levels of dihydrokaempferol, coniferyl alcohol and taxifolin among other metabolites in the roots of Rs-susceptible mutants. Additionally, reduced PPO activity in these lines correlated with increased concentrations of salicylic acid, jasmonic acid and several antimicrobial compounds and alterations in flavonoid regulation. These findings highlight the complex role of PPOs in plant defense, establishing a positive correlation between PPO activity and resistance to Rs, while offering insights into the trade-offs associated with PPO gene editing in potatoes.

1. Introduction

Potato (*Solanum tuberosum*) is a globally important staple crop and the subject of intensive breeding programs aimed at improving yield and quality traits. Among these, enhancing resistance to pathogens is a major priority. One of the most serious threats to potato cultivation is the *Ralstonia solanacearum* (Rs) species complex, the causal agent of bacterial wilt. This pathogen affects more than 1.5 million hectares of potato fields worldwide, leading to significant economic losses, and has a wide host range encompassing over 250 plant species in 54 botanical families (Buddenhagen and Kelman, 1964). Rs is a water-borne, biotrophic bacterium classified as a quarantine organism all over Europe (Elphinstone, 2005). Entry into plants is usually via wounded root openings, followed by colonization of the xylem vessels in the vascular

system. Bacterial biofilm blocking the vessels is the primary cause of wilting and plant death (Álvarez et al., 2010; Buddenhagen and Kelman, 1964). Rs is widely regarded as one of the most destructive bacterial phytopathogens due to its high genetic diversity, adaptability, and broad host range (Mansfield et al., 2012). Another major pathogen affecting potato is the hemibiotrophic oomycete *Phytophthora infestans* (Pi), which causes late blight and is responsible for devastating crop failures (Agrios, 2005). Pi has a host range that includes other solanaceous species such as tomato, petunia, and *Nicotiana benthamiana* (Becktell et al., 2006). In potato, infections begin with lesions on lower leaves and rapidly progress through asexual sporulation. Sporangia are disseminated by wind and water, releasing zoospores that can infect foliage and tubers through direct contact or in the soil (Tsedaley, 2014). Under conducive environmental conditions, Pi can destroy an entire potato field's canopy

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within days, with continued tuber infection during post-harvest storage (Fry, 2008; Tseadaley, 2014).

Polyphenols are integral components of plant defense mechanisms against both biotrophic and hemibiotrophic pathogens. Their spatial and temporal accumulation is pivotal for effective resistance responses (Nicholson and Hammerschmidt, 1992). Polyphenol oxidases (PPOs), encoded by nuclear genes and localized primarily to plastids of plants and the cytosol of fungi (Burke and Cairney, 2002; Mayer, 2006), catalyse the hydroxylation of monophenols to *o*-diphenols (via tyrosinase or cresolase activity, EC 1.14.18.1) and their subsequent oxidation to *o*-quinones (via tyrosinase or catechol oxidase activity, EC 1.10.3.1) using molecular oxygen (Van Gelder et al., 1997). The resulting *o*-quinones are cytotoxic and highly reactive, capable of cross-linking macromolecules, thereby reducing the nutritional value of alkylated proteins and forming structural barriers (Felton et al., 1992). These quinones undergo spontaneous polymerization to produce melanin-like polymers, contributing to tissue browning and plant defense (Duffey and Felton, 1991; Golan-Goldhirsh et al., 1984; Pihet et al., 2009).

PPOs are involved in defense pathways against biotrophic and necrotrophic phytopathogens as well as herbivores. Their expression is modulated by signalling molecules such as salicylic acid (SA), jasmonic acid (JA), and systemin, reflecting their multifaceted role in plant immunity (Burke and Cairney, 2002; Duffey and Felton, 1991; Johnson et al., 2003; Mayer, 2006; Nicholson and Hammerschmidt, 1992; Taranto et al., 2017). One proposed mechanism of action involves melanin-based physical barriers that inhibit pathogen ingress (Johnson et al., 2003). Laccases, a subclass of PPOs (EC 1.10.3.2), catalyse the polymerization of *p*-diphenols and monolignols, facilitating lignin formation and thus cell wall reinforcement – a well-known protective strategy against many pathogens (Jia et al., 2016; Liu et al., 2020; Underwood, 2012). For example, tomato plants overexpressing a potato *PPO* gene exhibited increased resistance to *Pseudomonas syringae* (Li and Steffens, 2002), while antisense-mediated suppression of tomato *PPO* enhanced susceptibility (Thipyapong et al., 2004). Similarly, strawberries overexpressing one of the four *PPO* genes demonstrated improved resistance to grey mold (Jia et al., 2016).

In contrast, several studies have found no consistent correlation between PPO activity and resistance to various pathogens and pests (Melo et al., 2006; Richter et al., 2012), and some have even reported a negative relationship (Hakimi et al., 2002; Llorente et al., 2014). In *Rs*-resistant wild peanut, miR3508, which targets *PPO* transcripts, was significantly upregulated upon *Rs* infection, suggesting that PPO suppression may enhance resistance (Zhao et al., 2015). In potatoes, silencing PPO altered phenylpropanoid metabolism, particularly leading to the accumulation of chlorogenic acid, a diphenolic compound with antimicrobial properties (Llorente et al., 2014). Chlorogenic acid inhibited all known *Rs* races and *Escherichia coli* (Kabir et al., 2014; Yang et al., 2016), and suppressed *Monilinia* infection in peaches (Villarino et al., 2011). A broader spectrum of phenolic and polyphenolic compounds also demonstrated antimicrobial activity (Coppo and Marchese, 2014; Nakamura et al., 2015; Pagliarulo et al., 2016).

This raises the question of whether disease resistance is primarily mediated by PPO activity or by the phenolic substrates themselves. Further complicating this dynamic is the fact that several bacterial pathogens, including *Rs*, also possess their PPOs (Fairhead and Thöny-Meyer, 2012). The *Rs* strain GMI1000 carries at least three genes encoding PPOs, including an atypical tyrosinase preferentially oxidizing monophenols over diphenols (Hernández-Romero et al., 2006). These enzymes likely contribute to bacterial self-defense by detoxifying phenolic compounds through oxidation (Hernández-Romero et al., 2005), thereby neutralizing host-derived antimicrobials such as chlorogenic acid. This enzymatic degradation potentially undermines the host's chemical defenses, illustrating the complex interplay between plant and pathogen PPO systems.

Beyond their role in defense, PPOs are also responsible for enzymatic browning, a major cause of post-harvest losses in fruits and vegetables.

In potatoes, browning can compromise up to 20 % of the yield during storage and processing, with enzymatic browning responsible for as much as 50 % of quality-related losses in industrial processing (Holderbaum et al., 2010; Tomás-Barberán and Espín, 2001; Whitaker and Lee, 1995). This phenomenon negatively impacts sensory appeal and nutritional value, contributing to consumer rejection and financial loss. Degrading the amino acid tyrosine by PPOs and reducing the digestibility of food by forming protein-quinone adducts also decreases its dietary value (Friedman, 1996, 2003).

Potato *PPO* genes exhibit a spatially and temporally diverse expression profile. Among these, *StuPPO2* (originally *Pot32*, PGSC gene accession ID: PGSC0003DMG400018916) and *StuPPO3* (*Pot33*, PGSC0003DMG400018914) are primarily expressed in roots and tubers with moderate expression in flowers, while *StuPPO4* (*Pot72*, PGSC0003DMG400018917) shows root-specific expression (Thygesen et al., 1995; PGSC, 2011; Chi et al., 2014). Other members of the *PPO* gene family are expressed in leaves and flowers: *StuPPO1* (*POTP1/P2* or *Nor333*, PGSC0003DMG400029575) and *StuPPO9* (PGSC0003DMG400022430) have minor expression also in tubers, while *StuPPO5–8* and *StuPPO10* are low-expression and/or incomplete genes (Thygesen et al., 1995; PGSC, 2011; Chi et al., 2014; Chi et al., 2024). Identifying favourable allelic variants of *PPO* genes represents a promising strategy for breeding cultivars with reduced browning potential. However, traditional breeding in tetraploid potatoes is time-consuming and labor-intensive, often requiring over a decade to develop new cultivars (Mullins et al., 2006; Werij et al., 2007). Genome editing, particularly via CRISPR/Cas9-mediated knockout (KO) of *PPO* genes, offers a more expedient route to generate cultivars with enhanced resistance and reduced browning.

Despite these technological advances, a key question persists: is disease resistance in potato primarily mediated by PPO enzymes, or by the phenolic compounds that serve as their substrates? This study seeks to elucidate the role of PPO activity in resistance to *R. solanacearum* and *P. infestans*, while also considering post-harvest browning. To this end, CRISPR/Cas9 was employed to edit *PPO* genes in two commercial potato cultivars, and disease response was assessed in combination with metabolomic profiling. The differential susceptibility of four edited lines to the two pathogens highlights the pathogen-specific and possibly gene-specific contributions of PPOs to potato defense.

2. Materials and methods

2.1. Plant material and in vitro culture

Two tetraploid potatoes (*Solanum tuberosum*) were chosen for this study. 'Désirée', a widely grown cultivar of Dutch origin (<https://potatoes.agricrops.org/varieties/view/Desiree>), has become an experimental model due to its excellent tissue culture amenability and competence for genetic transformation. 'Balatoni Rózsa' is a commercially successful Hungarian-bred cultivar (<https://unipatatas.com/variety/balatoni-rozsa/>). Both 'Désirée' and 'Balatoni Rózsa' are highly susceptible to *Rs* based on (Jose et al., 2023) as well as on breeder's communication and moderately susceptible to *Pi* (Ali et al., 2012; Polgár et al., 2010).

The plants were maintained and propagated *in vitro* from nodal segments or apical stem cuttings in 500 mL jars containing MS medium with vitamins (Duchefa Biochemie, Haarlem, the Netherlands, M0222), supplemented with 2 % (w/v) sucrose and 7 g L⁻¹ agar. Growth conditions were a constant 23 °C at a 16-h photoperiod (100 μmol m⁻² s⁻¹ PPFD). For *Agrobacterium*-mediated transformation, leaves of four-week-old plants were utilized as explants.

2.2. Generation of vector constructs

Total DNA extraction from plants of the two potato cultivars was conducted using the GeneJET Plant Genomic DNA Purification Mini Kit

(Thermo Scientific, Waltham, MA, USA). Subsequently, fragments of tuber- and root-specific *PPO* genes were amplified by PCR using the Phire Plant Direct PCR Master Mix (Thermo Scientific) with conserved primers (PPOallF: 5'-AAATGTTCTTCTGGCTTAGG-3', PPOallR: 5'-ACTCGGAGTTCAACCAATC-3') designed to target a 1-kb region in the first part of the genes (ca. 300–1200 bp of the coding sequence). The PCR products were then cloned using the CloneJET PCR Cloning Kit (Thermo Scientific) and Sanger sequenced with the PPOallR primer. Sequence data were blasted in the EnsemblPlants database (http://plant.ensembl.org/Solanum_tuberosum/Info/Index) to identify the corresponding *PPO* genes. Based on the obtained sequence information, a universal CRISPR target (5'-CAATATGGCAAAGCGAAAGTTGG-3') was designed. The two strands (upper: 5'-ATTGCAA-TATGGCAAAGCGAAAGT-3' and 5'-AAACACTTTCGCTTTGCCATATTG-3' for the lower strand) of the target, without protospacer-adjacent motif (PAM), were synthesized with overhangs to create sticky ends (Xing et al., 2014). The CRISPR target was integrated into the pKSE401 binary vector (comprising a 35S promoter-driven and codon-optimized *zCas9* gene (Grützner et al., 2021)) and fused to the AtU6–26 promoter, in front of the single-guideRNA scaffold (Xing et al., 2014). The T-DNA construct was assembled in *E. coli* before transfer into the *Agrobacterium tumefaciens* strain LBA4404 (Weigel and Glazebrook, 2006). The presence of the target was confirmed at each step by PCR using the U6–26p-F (5'-TGTCCCAGGATTAGAAATGATTAGGC-3') and CRISPR-target lower-strand primers (Xing et al., 2014). The final constructs were Sanger-sequenced with U6–26p-F.

2.3. Plant transformation and molecular analysis

Leaf explants from four-week-old 'Désirée' and 'Balatoni Rózsa' plants were co-cultivated with LBA4404 containing the *PPO* editing construct. Shoots were regenerated on kanamycin-containing medium ($75 \mu\text{g mL}^{-1}$) to screen for transformed lines, then transferred to MS medium with vitamins for rooting (Dietze, 1995). Verification of the sgRNA-coding transgene presence employed PCR with U6–26p-F and CRISPR-target lower strand, while *zCas9* presence was confirmed by PCR (forward: 5'-GACACGGCAGATCACTAAGC-3', reverse: 5'-GCGATCATCTTCTCCTCACATC-3', $T_m = 66^\circ\text{C}$, product size: 300 bp). PCR amplification of the *Agrobacterium* chromosomal *picA* gene (Yusibov et al., 1994) was used to detect/exclude *Agrobacterium* contamination.

Total RNA extracted from leaves with the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), it was then reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The expression of the *zCas9* transgene was tested by 30-cycle semi-quantitative RT-PCR using primers: ZCas9QF1 (5'-GACACGGCAGATCACTAAGC-3') and ZCAS9RTR1 (5'-GCGATCATCTTCTCCTCACATC-3'), having T_m at 58°C , and the amplicon size was 300 bp. Sequencing at the target region in the *PPO* genes (using the primers PPOallF and PPOallR) was performed with plants actively expressing the *zCas9* transgene.

2.4. PPO enzymatic activity

Triplicate tuber samples, 0.1 g each, were collected randomly from pooled tubers of three plants per line. Simultaneously, triplicate root and leaf samples, also each 0.1 g, were pooled from 10 *in vitro* plants per line. The measurement of enzyme activity followed the protocol outlined (González et al., 2020), incorporating 2 % (v/v) Triton X-100 for tubers and leaves and 0.02 % (w/v) SDS for roots in the extraction buffer and using 15 mM 4-methylcatechol as substrate. For statistical analysis, ANOVA and Student's *t*-test were employed to identify the level of significance for comparisons between control and edited plant samples.

2.5. Browning assay

Three 5-mm-thick tuber slices were hand-cut from the harvest of five

randomly selected plants per line. Complete individual roots of five *in vitro* plants per line were also included. To initiate the assay, 100 μL of 0.01 M disodium tyrosine substrate solution was pipetted onto the test samples within 5 min, and the samples were incubated for 1–2 h at 30°C in darkness (Busch, 1999).

2.6. Pathogen inoculation tests

The GFP-expressing transgenic *Ss* strain UW551 (race 3, biovar 2; highly virulent on potato) was inoculated on 20 plantlets per line across four rectangular Petri dishes, following our methodology (Jose et al., 2023). Briefly, the inoculation was performed by pipetting the bacterial suspension into the cut sites at the root tip. The progression of bacterial invasion and symptom development in the edited 'Désirée' lines 14 (Des14) and 17 (Des17), 'Balatoni Rózsa' lines 12 (BR12) and 25 (BR25), together with unedited control plants, was monitored daily until complete wilting of the control plants. Disease intensity was quantified by calculating the relative frequency of wilted plants at each time point and recording the percentage of wilted plants at the time point when the differences in disease symptoms were distinguishable and evident. This experiment was repeated to confirm the results. Photos were taken under visible and UV light, showing the spread of GFP-expressing *Ss* bacteria. Bacteria were quantified by measuring GFP fluorescence using ImageJ (Schneider et al., 2012; Shiha et al., 2021). The UV images underwent inversion and background removal under default conditions. The ROI, representing individual plants in the Petri plate, was defined, and the average grey area was measured and subsequently normalized by subtracting the average grey value from an identical background ROI of the plate.

For the *Pi* inoculation assay, potato plants were cultivated in pots containing peat-enriched soil within a glasshouse maintained at 20°C , under ambient spring conditions supplemented with 12 h of illumination daily. At eight weeks of age, five plants per line were challenged with the P1217 *Pi* isolate (collected from tomatoes in Hungary in 2022). The pathogen was cultivated and rejuvenated on ca. 7-mm-thick potato ('Hópehely') tuber slices. These slices were inoculated on the abaxial side with 0.5 cm diameter mycelial agar plugs obtained from colonies thriving at $18\text{--}20^\circ\text{C}$ on V-8 juice agar (Miller, 1955). The inoculated tuber slices were incubated at $18\text{--}20^\circ\text{C}$ in 100 % relative humidity for a week to facilitate fungal colonization on the upper side. Subsequently, sporangia were extracted by washing with distilled water, filtering out debris, and adjusting the inoculum concentration to 1.5×10^4 sporangia mL^{-1} . The release of zoospores commenced shortly before spraying 115 mL of the inoculum suspension on the plants. Post-inoculation, the plants were kept at 17°C , in nearly 100 % relative humidity and complete darkness for the first 24 h. They were then transitioned to a 12-h light regime in a climate chamber at 17°C with a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. The degree of infection was measured by the spread of lesions across the leaves and by estimating the percentage of infected plants, where 9 = no blight and 1 = 100 % blighted.

The Eucablight protocol was followed for tuber inoculation assays (Leontine Colon, 2004). Tubers of approximately $2 \times 3 \text{ cm}$ were harvested from 16-week-old potted plants grown in the glasshouse. The tubers were washed in tap water, disinfected in 70 % (v/v) ethanol for 45 s, and rinsed with sterile distilled water. Seven-mm-thick slices were then excised from the disinfected tubers, rinsed in sterile distilled water, and dried in a decontaminated laminar flow cabinet. Subsequent inoculation on the abaxial side utilized 0.5 cm diameter mycelial plugs from a colony grown at 20°C on V-8 agar medium. The inoculated tubers were placed in plastic containers with moistened paper towels at the bottom to maintain 100 % relative humidity; the entire setup was kept at 20°C in the dark for 5 days before evaluating *Pi* colonization. The spread of the mycelial mat corresponded to the ease of *Pi* colonization, giving a perception of susceptibility to *Pi*. The mycelial mat was quantified using ImageJ by calculating the intensity of the 8-bit image of individual tubers with a constant area of ROI across all examined tubers.

2.7. Sample preparation and metabolite analyses

For the measurement of abundant phenolics (in total 140 compounds), 0.1 g of root and leaf samples were taken in four replicates from six-six plants of ‘Désirée’ and ‘Balatoni Rózsa’ control and edited lines grown simultaneously *in vitro*. The studied potato genotypes were measured in two separate experimental batches (first batch: DesC, Des17, BRC, BR25, second batch: DesC, Des14, BRC, BR12), and the results of the mutant genotypes were compared to their respective controls. The metabolite analysis was done using ultra-performance liquid chromatography-ESI tandem mass spectrometry (UPLC-MS/MS) as published (Pál et al., 2019), based on (Vrhovsek et al., 2012). The respective multiple-reaction monitoring (MRM) transitions used for the quantification of compounds, and the details of UPLC and mass spectrometric conditions are listed in Table S1. Student's *t*-test (two-tailed) was carried out on the metabolite amount of the edited lines and their controls, and a value of $p < 0.05$ was considered significant.

For the measurement of gibberellic acid forms, sample preparation and UPLC-MS/MS analyses were done according to (Cseh et al., 2024) with minor modifications. Triplicate samples (0.1 g each) of shoot tips from six plants of ‘Désirée’ and ‘Balatoni Rózsa’ were collected from control and edited lines grown *in vitro* and homogenized in 2-mL safety Eppendorf tubes with two pieces of inert borosilicate glass beads (ϕ 3 mm) with TissueLyser II (Qiagen, Hilden, Germany) in a liquid nitrogen-cooled rack. The samples were stored at -80°C until extraction, when a 30- μL internal standard containing 5 ng of $[^2\text{H}_4]\text{GA}_1$ and $[^2\text{H}_2]\text{GA}_4$ in methanol (OlChemIm s.r.o., Olomouc, Czech Republic) was added for quality control purposes. Extraction was performed with 1 mL of 75 % (v/v) ice-cold methanol (UPLC gradient grade; VWR, Radnor, PA, USA). The tubes were vortexed, inserted into a pre-cooled (-18°C) tube holder, and shaken in a 1600 MiniG® - Automated Tissue Homogeniser and Cell Lyser (SPEX, Rickmansworth, UK) at 1250 rpm for 3 min. The samples were centrifuged at 16,500 g for 15 min at 4°C , and the supernatants were recovered and placed in separate Eppendorf tubes. The residues were re-extracted as described above. The two supernatants for each sample were pooled and partitioned against 1 mL of petrol ether:diethyl ether 4:1 (v/v) by vortex mixing, followed by centrifugation. The lower methanol:water phase of 2 mL was subjected to a solid-phase extraction (SPE) type flow-through clean-up as follows: an ENVI-18 SPE tube (1 mL; Supelclean, Merck-Sigma Group, Schnell-dorf, Germany) was first conditioned with 2 mL of methanol and equilibrated with 2 mL of 75 % (v/v) methanol. The pooled extracts were loaded and flown through into a 15-mL centrifuge tube. The SPE cartridges were washed with 1 mL of 75 % (v/v) methanol; this washing solution was introduced into the same centrifuge tube. The total (approximately 2.6 mL) extracts were evaporated under vacuum at 35°C , reconstituted in 400 μL (final sample concentration of 0.25 g/mL) of 30 % (v/v) methanol containing 0.1 % (v/v) formic acid (MS grade; VWR), filtered through a 0.22 μm disposable PTFE syringe filter, and immediately submitted for analysis into the UPLC-MS/MS set-up by injecting 3 μL . The analytical method details, retention times, and MRM channels used for quantitative determination are summarized in Table S2.

3. Results

3.1. Design of CRISPR target for potato PPO genes

To ensure the precise, cultivar-specific design of the CRISPR-target, a 1-kb PPO gene fragment was amplified with primers conserved for the three major root- and tuber-specific genes (*StuPPO2-StuPPO4*, Table S3), both in ‘Désirée’ and ‘Balatoni Rózsa’. A total of 49 fragments, i.e., 23 + 26 for the two cultivars (Fig. S1 and Fig. S4), respectively, were Sanger-sequenced, and the corresponding individual PPO genes were identified. The CRISPR-target was perfectly homologous in both cultivars with the *StuPPO2* sequence, the most highly expressed PPO gene in

potato roots and tubers (Thygesen et al., 1995). The CRISPR target exhibited zero or one mismatch with *StuPPO3* and *StuPPO4* sequences (Fig. S1 and S4). Gene IDs of other members of the potato PPO gene family are shown in Table S3. The CRISPR-target has 2 mismatches with *StuPPO1* and 3 mismatches with *StuPPO9* in the seed region adjacent to the PAM, therefore it is unlikely that it would mutate these sequences, while *StuPPO5–8* and *StuPPO10* were characterised being are low expression and/or incomplete genes (Thygesen et al., 1995; PGSC, 2011; Chi et al., 2014; Chi et al., 2024). Sequence alignment of the CRISPR-target to *StuPPO1–4* and *StuPPO9* is provided in Table S3. The seed region has been variously defined as 8–14 nt proximal to the PAM where even a single mismatch could abolish cleavage (Wu et al. 2014; Rabinowitz and Offen, 2021), here we apply the term for the region 12 nt adjacent to the PAM, as it is in the CRISPOR target-designer program (<https://crispor.gi.ucsc.edu/>). Other potential non-PPO CRISPR off-targets were also predicted using the CRISPOR program and with BLAST searches in SpudDB (<https://spuddb.uga.edu/blast.shtml>). The search yielded no additional potential off-targets with an identical sequence to our target or with 1–2 mismatches in the seed region.

3.2. Generation of PPO-edited potato lines

Twenty-three plants from the ‘Désirée’ variety and twenty-eight plants from ‘Balatoni Rózsa’ cultivar were regenerated after *Agrobacterium*-mediated transfer of the CRISPR/Cas9 vector into the ‘Désirée’ and ‘Balatoni Rózsa’ cultivars. These plants were selected by kanamycin for a preliminary screening of the transgenes. PCR analyses on the regenerants confirmed the presence of sgRNA and *zCas9* sequences, and *zCas9* expression was also detected (data not shown). Thirteen ‘Désirée’ and nine ‘Balatoni Rózsa’ plants were positive in the above tests, and also free of *Agrobacterium* contamination, as confirmed by *pica* PCR. Among these plants, we selected for further experiments those with a clear phenotype, based on preliminary browning assays and PPO enzyme activity measurements. Out of the transgenic plants, two were selected and micropropagated from each cultivar for more detailed characterization. Accordingly, ‘Désirée’ lines Des14 and Des17 and ‘Balatoni Rózsa’ BR12 and BR25, alongside their unedited control lines, formed the core of our investigations.

These lines harboured various CRISPR-induced mutations near the Cas9 PAM in the *StuPPO2* gene, as revealed by nucleotide sequencing (Data S1, Fig. S1–S6). For all the genotypes, at least 20 amplicons per line were sequenced, and at least eight of them were identified by BLAST searches as *StuPPO2*, the strongest expressing PPO gene in roots and tubers. Alignment of these sequences with the CRISPR target site revealed various mutations near the Cas9 PAM region. All four edited lines exhibited mutations in the *StuPPO2* gene, and one of them, BR25, also contained mutations in *StuPPO3* and *StuPPO4*.

Phenotypically, all edited lines maintained normal characteristics but displayed a slightly smaller stature than the control lines. An exception to this trend was BR25, which exhibited rapid growth and a tall plant structure.

3.3. PPO enzymatic activity

All four lines, particularly BR25, exhibited significantly declined PPO enzyme activity in their roots compared to the respective controls, with *p*-values of 0.0014 and 0.007 for Des14 and Des17 and $p \leq 0.0285$ and 0.0005 for BR12 and BR25, respectively (Fig. 1). Significantly reduced PPO activity was also measured in the tubers of Des14 and Des17 relative to their controls, with a *p*-value of 0.0114 and 0.0081, respectively. The tubers of BR25 also showed a highly significant ($p \leq 0.0005$) reduction in PPO activity. PPO activity was the lowest in the leaves among the three studied organs, and it decreased in only one mutant, Des17, by less than 10 % (Fig. 1C). Lower PPO enzyme activity was also reflected in less browning of tubers (Fig. 2) and roots (Fig. 3), especially in the case of Des17 and BR25.

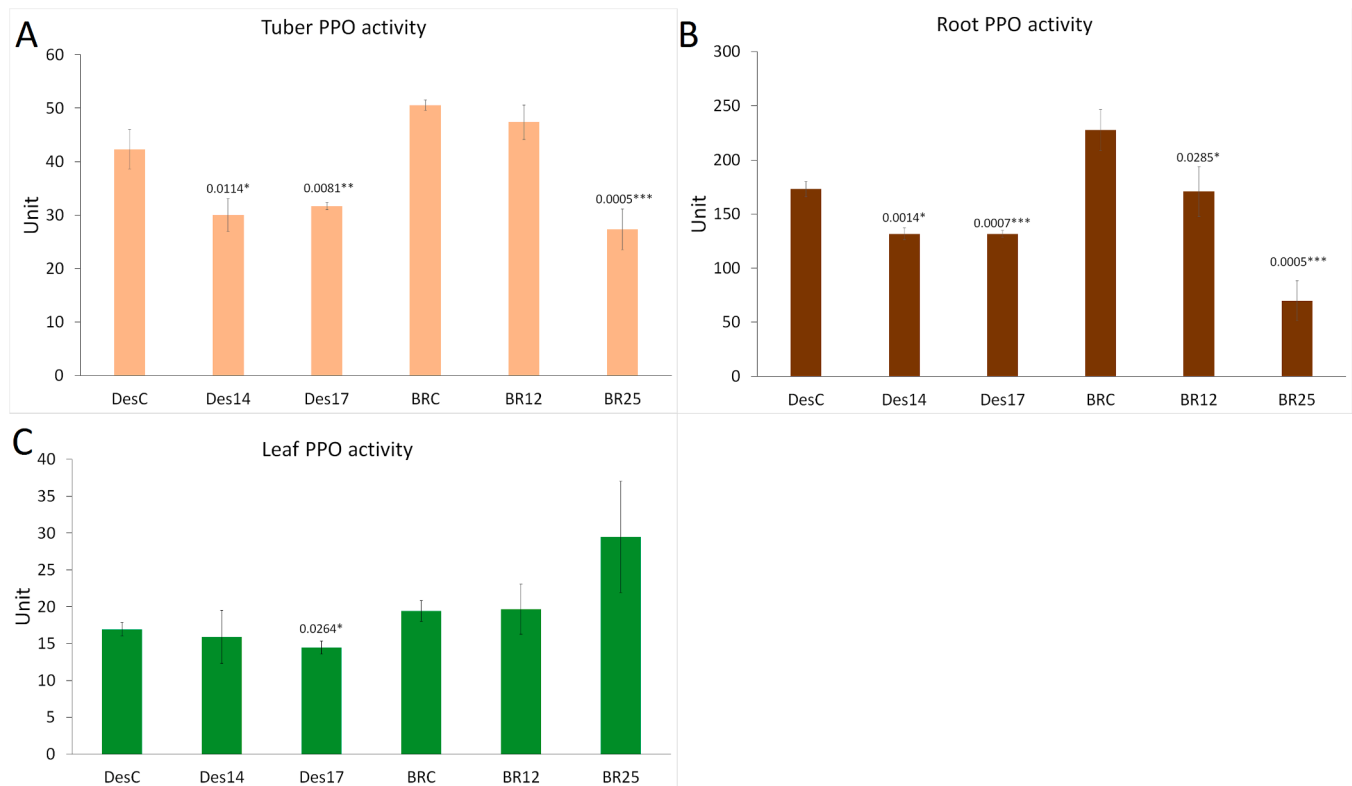


Fig. 1. PPO enzymatic activities in tubers (A), roots (B), and leaves (C) of control and *PPO*-edited ‘Désirée’ and ‘Balatoni Rózsa’ potato plants. Results are from three technical replicates per line, with error bars representing the standard deviation (Mean $\pm$ SD). Significance values of statistical differences from respective controls are indicated.

3.4. Differential disease responses to inoculation by *Rs* and *Pi*

The progression of bacterial wilt disease across the *PPO*-edited lines and their respective controls was quantified by assessing the infection through the percentage of wilting (Table 1). According to the complete wilting and susceptibility data, distinct time points were recorded between the two cultivars, ranging from 2 to 21 days post-inoculation (dpi) overall. A discernible difference in disease response among the ‘Désirée’ lines manifested earlier, at 11 dpi, compared to ‘Balatoni Rózsa’ which exhibited apparent differences at 13 dpi. At this time point, both Des14 and Des17 displayed increased susceptibility, with 87 % and 60 % wilting, respectively, compared to 40 % in the control. This susceptibility intensified in a repeated experiment, as the wilting percentage in the two edited lines reached 100 %, surpassing the 75 % level in the control. Similarly, BR25 exhibited elevated susceptibility, with 73 % and 100 % wilting percentages relative to the control’s 13 % and 80 % in experiments 1 and 2, respectively. BR12 deviated from this pattern, displaying no evident increase in susceptibility. The fewer mutations seen among the sequenced *StuPPO2* amplicons compared with the other edited lines indicate less mutations, and these may not have been enough to cause a detectable decrease in the pathogen resistance. Furthermore, the other mutant from the same cultivar, BR25 harboured both mutant *StuPPO2* and *StuPPO3* genes, while in BR12, only the *StuPPO2* was mutant (Fig S1-S6).

To confirm these data visually, the progression of the GFP-tagged *Rs* strain UW551 was monitored. The internal bacterium load was quantified by the GFP fluorescence on UV images, revealing higher levels in all *PPO*-edited lines compared to their controls (Fig. 4). The Des14 and Des17 lines were more susceptible to *Rs*, with an earlier spread of bacteria to the shoots relative to the ‘Désirée’ control (Fig. 4. and Fig. 5). Similarly, BR25 exhibited increased susceptibility, with plants wilting faster and succumbing to rapid *Rs* spreading, indicative of *Rs* in the shoots much earlier than the control.

Whole plant assays and assessments of potato tubers exposed to *Pi* yielded diverse responses. The ‘Balatoni Rózsa’ plants exhibited delayed symptom development compared to ‘Désirée’, prompting symptom recording at 4 dpi and 2 dpi, respectively. The overall progression of infection across the lines (Fig. 6) revealed distinct symptoms, including leaf lesions, darkened leaf edges, and complete necrosis, in ‘Désirée’ by 2 dpi. However, by 4 dpi, both cultivars were fully infected, leading to the near-total death of plants across all lines by 7 dpi. However, none of the *PPO*-edited lines exhibited considerable differences from the inoculated controls at any time point (Fig. 6).

As for the tuber infection tests, *Pi* vigorously grew through the slices; its mycelia first appeared on the upper side at 2 dpi, continued to grow, and covered the entire surface by 5 dpi on all tuber slices from all lines. The mycelium mat on ‘Balatoni Rózsa’ appeared to be slightly denser than on ‘Désirée’, but the *PPO* mutants again did not show a difference from their respective controls for *Pi* colonization, except for Des14 having higher *Phytophthora* growth (Fig. 7 and Table S4). The pathogen produced sporangia abundantly on all tuber slices.

3.5. Secondary metabolite analysis

The UPLC-US-MS/MS analysis revealed overall differences in the concentrations of various phenolic compounds between control and *PPO*-edited potato plants (Table S5 and Table S6). The level of more metabolites (phenolic acids, phenolic alcohols, and flavonoids) changed significantly in the roots as a result of mutating *PPO*-genes (Fig. 8) compared to the leaves (Fig. 9) in agreement with the editing of root- and tuber-specific *PPO* genes. Roots of ‘Désirée’ mutants contained higher amounts of chlorogenic acid, caffeic acid and dihydrokaempferol, which are among the major metabolites (>1000 ng g fresh weight, FW⁻¹). The levels of the following medium-range metabolites (100–1000 ng g FW⁻¹) were also higher in Des14 and Des17 compared to their controls: acetovanillone, coniferyl alcohol, taxifolin,

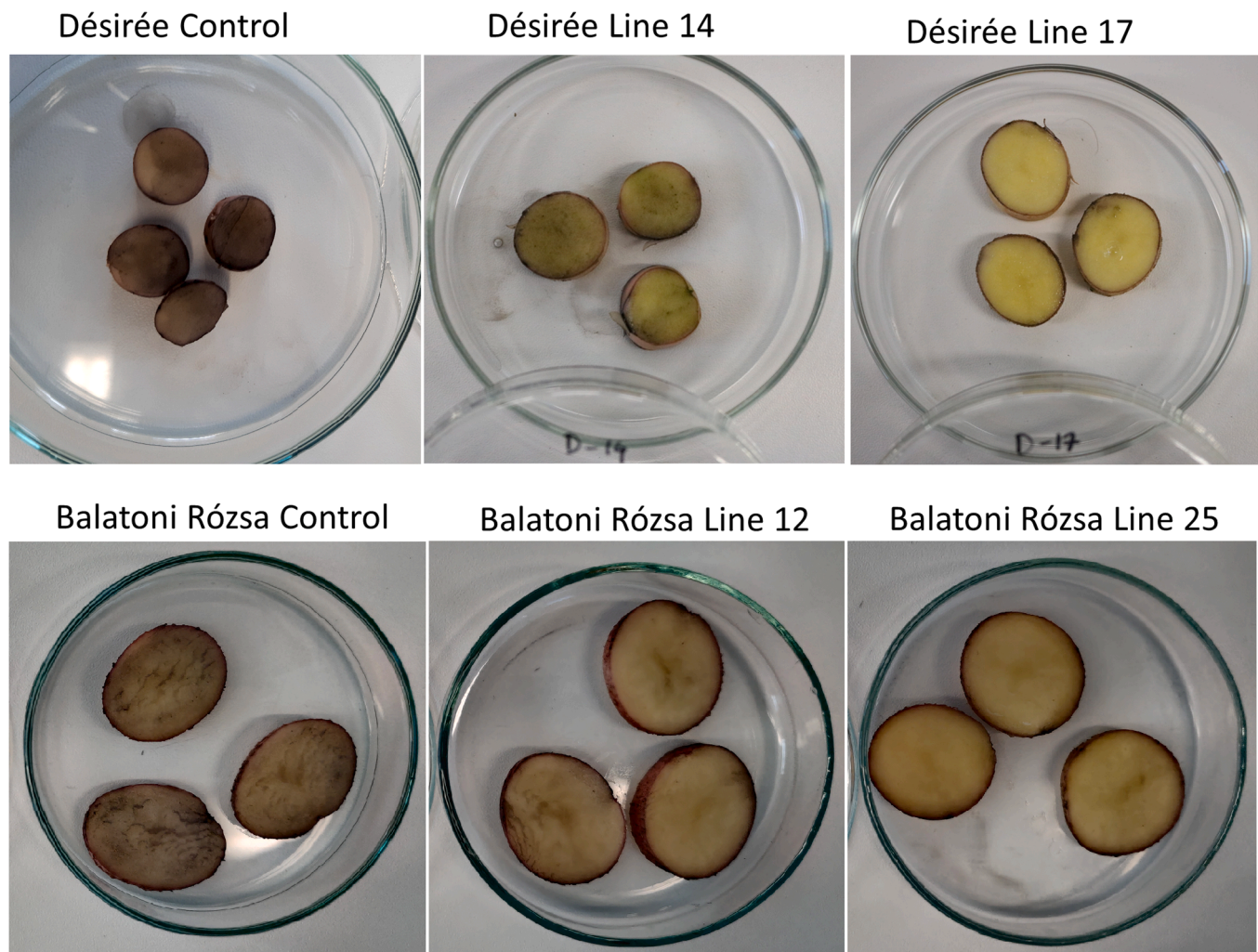


Fig. 2. Tuber browning assay of control and *PPO*-edited ‘Désirée’ (upper row) and ‘Balatoni Rózsa’ (bottom row) potato plants. Tuber slices were treated with L-tyrosine to induce browning.

and isorhamnetin, but the level of 4-hydroxybenzoic acid was lower. Des14 and Des17 also produced higher amounts of the minor metabolites ($<10 \text{ ng g FW}^{-1}$) naringenin and phloridzin, and the level of phaeic acid was also higher in BR25. Among the hormones, higher level of JA was detected in the roots of the ‘Désirée’ mutants, but also in BR12, compared to their respective control. The leaves contained higher levels of JA and SA hormones than the roots, and the mutants Des17 and BR12 even produced more in the leaves. In the leaves, the medium range ($100\text{--}1000 \text{ ng g FW}^{-1}$) of vanillic acid increased in the ‘Désirée’ edited lines, but decreased in BR25, and 4-hydroxybenzoic acid increased in Des14 and BR12.

In shoot tips, we also found significantly higher levels of the gibberellic acid GA_8 , both in ‘Balatoni Rózsa’ edited lines and GA_{20} in BR25 only (Fig. 9).

4. Discussion

Our study had the dual aim of CRISPR/Cas-mediated knockout of *PPO*-genes from potato in order to reduce browning of tubers and to improve their quality, and also to understand the role of *PPO* in the defense against pathogens *Rs* and *Pi*. Our CRISPR-target was specifically designed for the root and tuber-specific *StuPPO2*, *StuPPO3*, and *StuPPO4* genes, primarily *StuPPO2*, the highest expressing one. Importantly, the design intentionally avoided targeting the main leaf-specific *StuPPO1* and *StuPPO9* genes, allowing us to interpret our findings in the context

of root- and tuber-specific *PPO* functions, and also because the role of *StuPPO1* and *StuPPO9* have already been characterised for pathogen resistance (Li and Steffens, 2002; Chi et al. 2024). The transformation and genome editing were successful: *PPO*-mutant potato lines were established in both of the studied cultivars, ‘Désirée’ and ‘Balatoni Rózsa’, with decreased *PPO* enzyme activity and reduced browning of roots and tubers. Notably, among the *PPO* mutants, despite detecting some intact copies of the *StuPPO2* gene, our partial mutants already exhibited considerable reductions in enzyme activity and browning. Interestingly, tubers of BR12 showed decreased browning (Fig. 2), but their *PPO* enzyme activity did not decrease significantly (Fig. 1A). The *StuPPO2* gene had been edited in this line; however the fewer mutant amplicons among the ones generated from this line indicates less mutations, compared to the other edited lines. We believe the change in *PPO* activity in this genotype was around the detection limit, which is why the change was significant in some tests but was not significant in others. While the lowering of browning in potatoes via *PPO* gene manipulation is not entirely new (Bachem et al., 1994; Chi et al., 2014; González et al., 2020; Llorente et al., 2014, 2011; Shepherd et al., 2015), our dual-cultivar approach provides a more comprehensive understanding of the role of *PPO* in potatoes and enhances the applicability of our results. Browning in eggplant was observed to correlate with the total phenolic content and *PPO* activity (Mishra et al., 2013; Prohens et al., 2007a). Additionally, there is a suggestion that the pursuit of reduced browning in newer commercial eggplant cultivars may



Fig. 3. Root browning assay of control and *PPO*-edited ‘Désirée’ (upper row) and ‘Balatoni Rózsa’ (bottom row) potato plants. Roots were treated with L-tyrosine to induce browning.

Table 1
Number of wilted plants among *Ralstonia-solanacearum*-infected ‘Désirée’ (at 11 dpi) and ‘Balatoni Rózsa’ (at 13 dpi) potato plants.

Genotype	Experiment 1					
	DesC	Des14	Des17	BRC	BR12	BR25
Total plants	15	15	15	15	15	15
Wilted plants	6	13	9	2	3	11
Wilted plants (%)	40	87	60	13	20	73
Genotype	Experiment 2					
	DesC	Des14	Des17	BRC	BR12	BR25
Total plants	20	10	20	20	20	20
Wilted plants	15	10	20	16	13	20
Wilted plants (%)	75	100	100	80	65	100

inadvertently lead to the indirect selection of materials with lower concentrations of phenolics. This occurs despite the recognized health benefits associated with many of these phenolic compounds (Prohens et al., 2007b). Our findings suggest that integrating older potato cultivars into breeding programs, coupled with CRISPR/Cas-mediated gene editing, could address the intricate balance between browning, phenolic content, and *PPO* activity. This nuanced understanding contributes to ongoing discussions on precision breeding and biotechnological interventions in agriculture, emphasizing the potential for genotype-specific responses to gene editing interventions in diverse

potato cultivars as demonstrated by the editing in ‘Désirée’ and ‘Balatoni Rózsa’ cultivars.

After establishing *PPO* mutant potato lines, we then studied their metabolic profile as altered metabolome after lowering *PPO* enzyme activity have also been published by other scholars and some of the produced metabolites could act with antimicrobial and antifungal properties (Araji et al., 2014; Coppo and Marchese, 2014; Kabir et al., 2014; Llorente et al., 2014; Nakamura et al., 2015; Pagliarulo et al., 2016; Villarino et al., 2011; Yang et al., 2016). Shepherd and co-workers (Shepherd et al., 2015) reported that the level of a few metabolites, including chlorogenic acid and some free amino acids, changed as a result of down-regulation of *PPO* gene expression, and overall, mechanical damage to tubers had a considerably greater impact on the metabolite profile. Similar effects may have caused the considerable differences between the metabolite content of our control root and leaf samples from the two measurements; nevertheless, we were able to identify many metabolites with identical changes in more than one of the *PPO* mutants, compared to their respective controls. The level of known *PPO* substrates, chlorogenic acid, caffeic acid, and taxifolin (Altunkaya and Gökmen, 2008; Duangmal and Apenten, 1999; Lourenco et al., 1992), did increase in the roots of Des14 and Des17, as expected. The elevated accumulation of coniferyl alcohol in the roots of the ‘Désirée’ mutants is particularly noteworthy. It not only stimulates virulence in certain bacterial species (Song et al., 1991; Gafni and Levy, 2005), but also serves as a substrate for polyphenol oxidases (PPOs) and

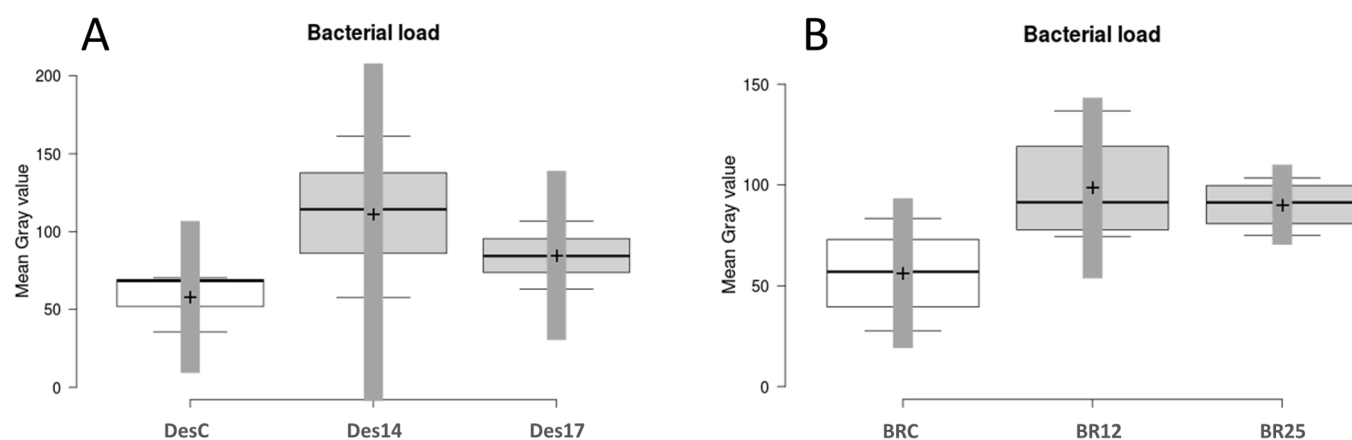


Fig. 4. Bacterial spreading in 'Désirée' (A) and 'Balatoni Rózsa' (B) control and *PPO*-edited plants. GFP-tagged *Rs* UW551 was quantified by GFP fluorescence on inverted colour images using ImageJ software. The 'Désirée' plants were compared at 11 dpi, while the 'Balatoni Rózsa' plants were at 13 dpi. Each line was represented by 20 individual plants (biological replicates) and evaluated with boxplots.

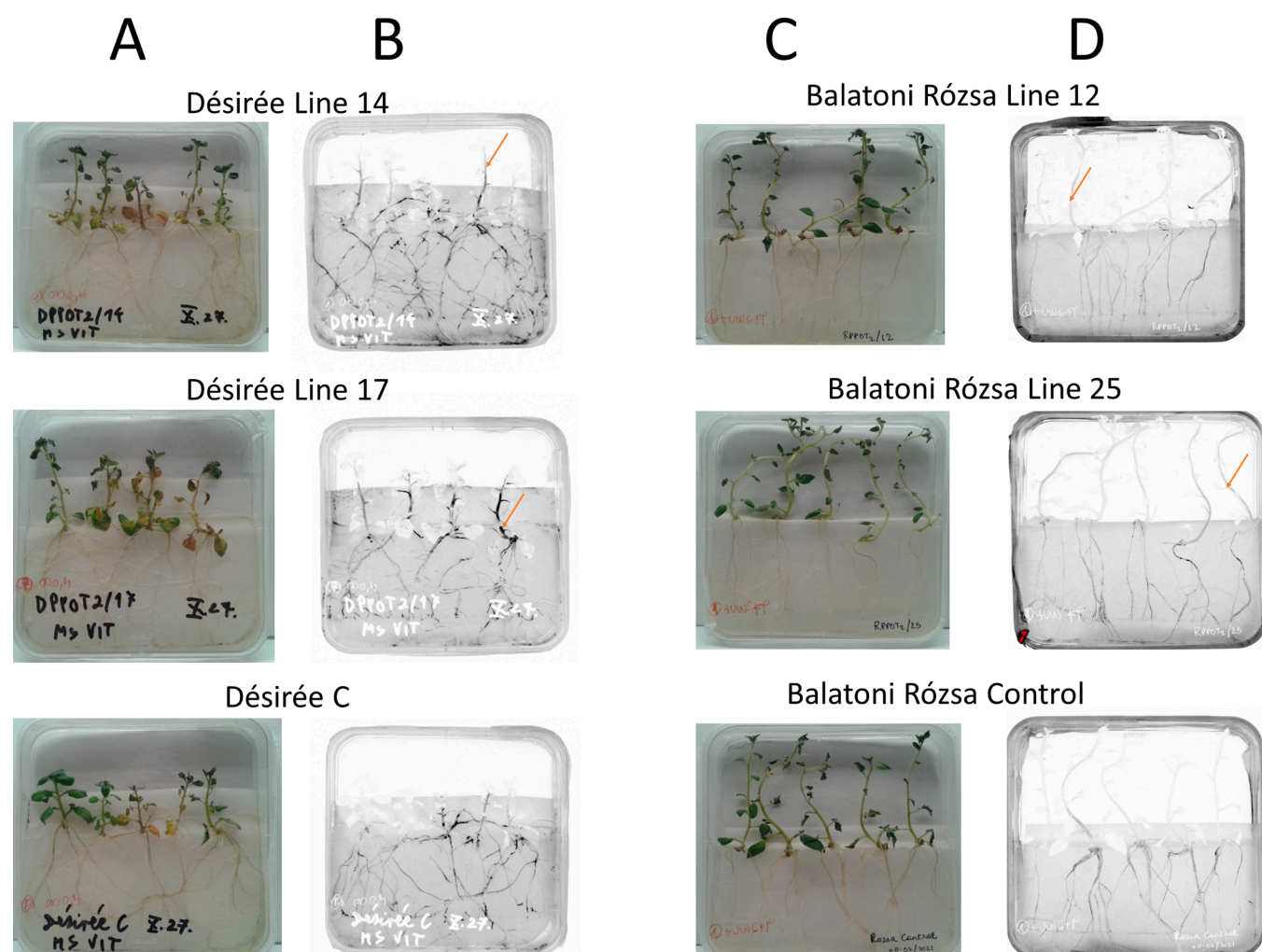


Fig. 5. Representative images of *R. solanacearum*-infected 'Désirée' control and *PPO*-edited (Des14 and Des17) plants at 11 dpi, as well as Balatoni Rózsa' control and *PPO*-edited (BR12 and BR25) plants at 13 dpi. Photos taken in visible light (A, C) show the progression of disease symptoms (wilting). The black and white images taken in UV light (B, D) indicate the distribution of the GFP-expressing *Rs* bacteria (black colour).

laccases, and its oxidation is essential for lignin biosynthesis (Bao et al., 1993). The higher residual level of coniferyl alcohol, therefore, suggests a reduced rate of lignin biosynthesis, potentially rendering the roots more susceptible to *Rs* invasion. As reviewed by Miedes et al. (2014),

lignin is widely recognized as a key structural barrier that impedes initial pathogen colonization, and tomato varieties resistant to *Rs* infection typically exhibit higher lignin content in their roots than susceptible ones. Consistent with this, numerous studies, including our

Day2



Day4



Day7

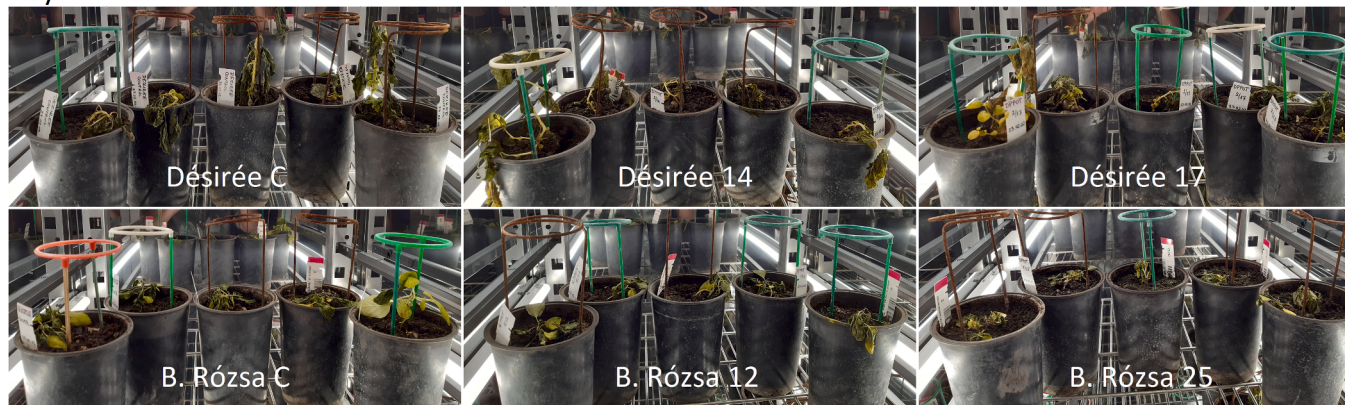


Fig. 6. Disease symptoms (2, 4, and 7 dpi) of *P. infestans* on control and *PPO*-edited 'Désirée' and 'Balatoni Rózsa' potato plants.

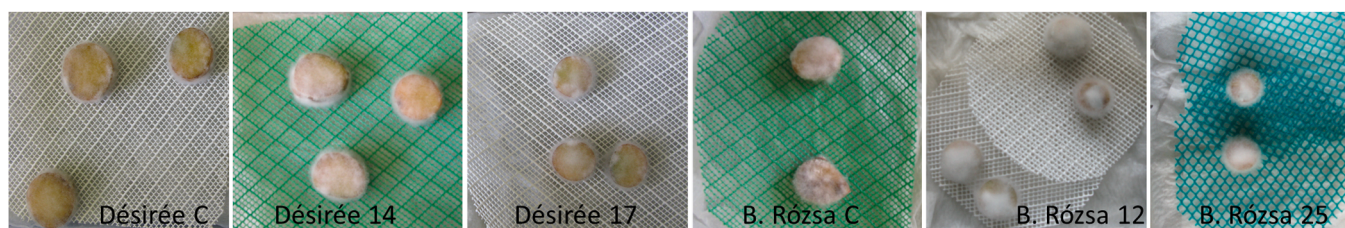


Fig. 7. Mycelial growth (5 dpi) of *P. infestans* on tuber slices of control and *PPO*-edited 'Désirée' and 'Balatoni Rózsa' potato plants.

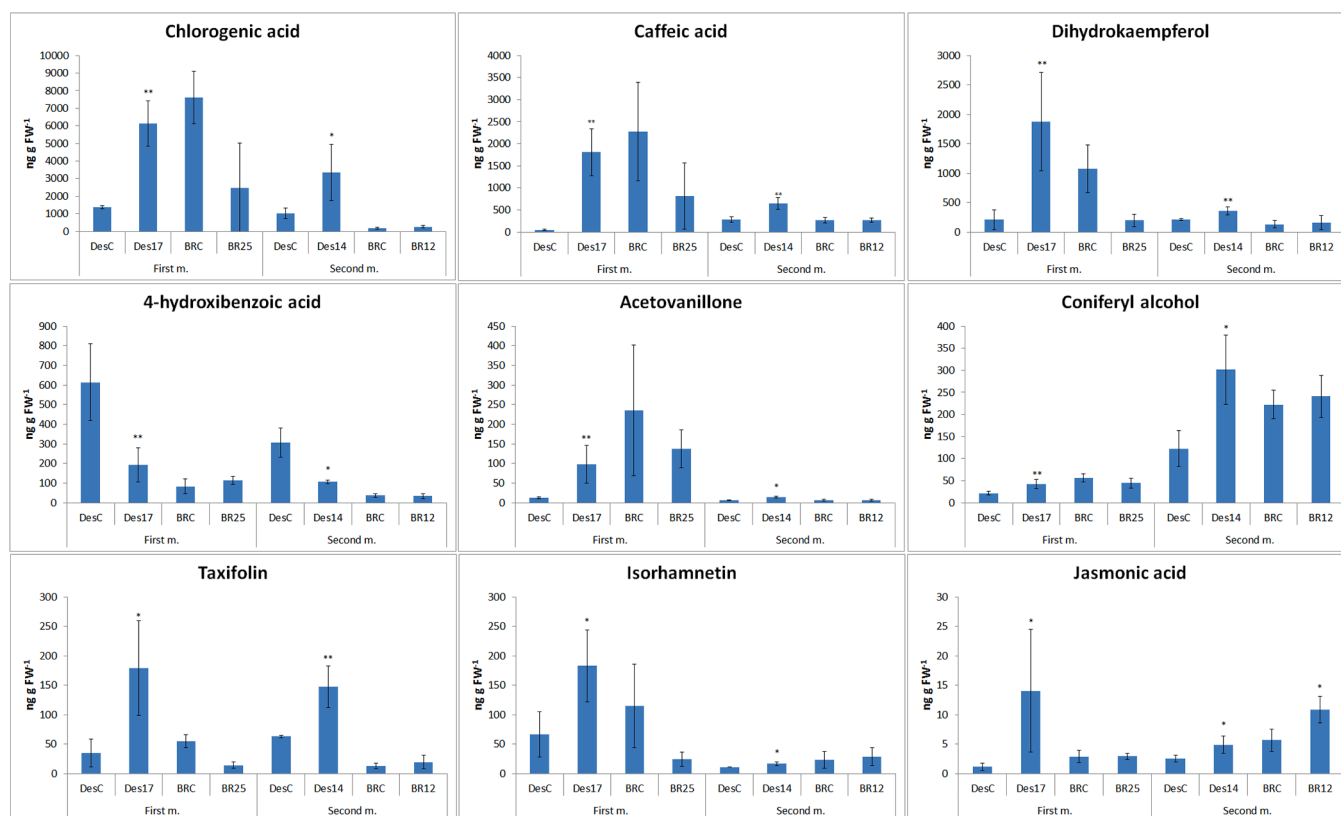


Fig. 8. Quantities of significantly changed phenolic metabolites in roots of control and PPO-edited 'Désirée' and 'Balatoni Rózsa' potato plants from two measurements. Results are from four technical replicates per line, with error bars representing the standard deviation (Mean $\pm$ SD). Significant differences are shown compared to the controls.

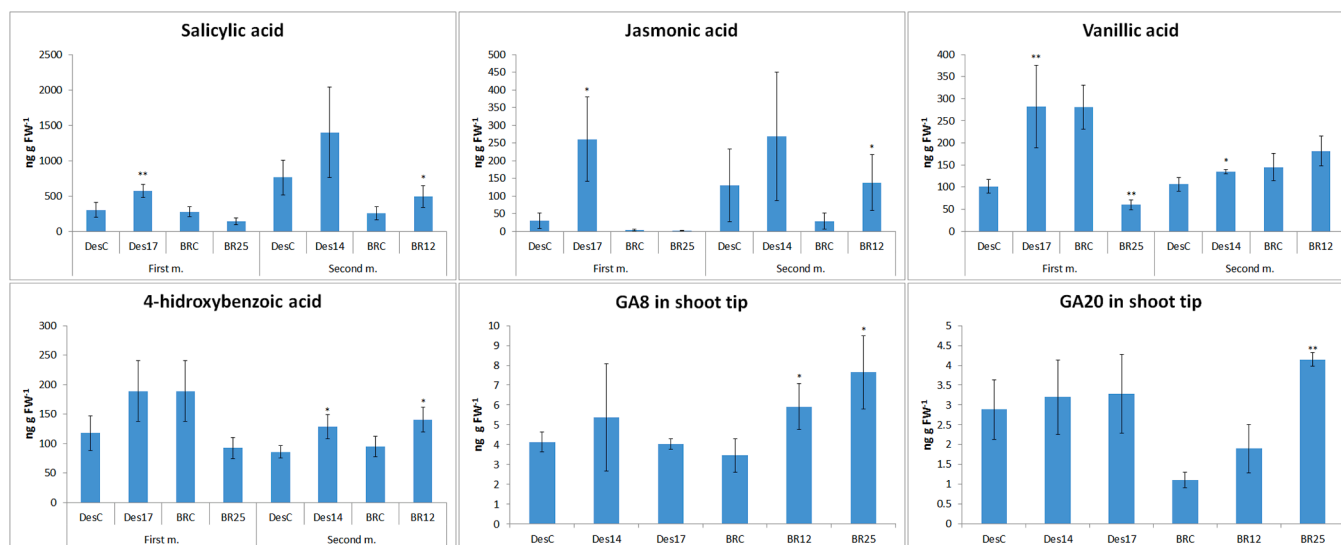


Fig. 9. Quantities of significantly changed phenolic metabolites in leaves and gibberellic acid forms GA₈ and GA₂₀ in shoot tips of control and PPO-edited 'Désirée' and 'Balatoni Rózsa' potato plants from two measurements. Results are from four technical replicates per line, with error bars representing the standard deviation (Mean $\pm$ SD). Significant differences are shown compared to the controls.

own, have demonstrated the involvement of lignin and lignin biosynthetic pathways in resistance to *Rs* and other bacterial pathogens. Moreover, infection often induces the expression of lignin biosynthesis genes and promotes enhanced lignification (Ishihara et al., 2012; Kashyap et al., 2022; Jose et al., 2023; Li et al., 2025).

However, even the level of hormones (JA in roots of Des14 and Des17, in leaves of Des17 and BR12, SA in leaves of Des17 and BR12)

increased, which are not PPO substrates. Notably, PPO editing increased the level of 4-hydroxybenzoic acid in the leaves but decreased it in the roots. Therefore, metabolic changes could not be explained by simple biochemistry. The production of PPOs is known to be influenced by SA, JA, and systemin, as reviewed (Taranto et al., 2017), and in certain species, by gibberellins (Stewart et al., 2001; Taneja and Sachar, 1974). We hypothesize that plants could sense the PPO KO effect in the absence

of PPOs, possibly through redox regulation, triggering a negative feedback regulation. This feedback mechanism may activate alternative defense mechanisms against pathogens. In a comparable work (Araji et al., 2014), the observed necrotic lesions on PPO-silenced walnut leaves suggested a role for SA in programmed cell death induction. 'Balatoni Rózsa' lines displayed a higher level of gibberellic acid (GA), suggesting a unique response in comparison. GA might have inhibited metabolite production in 'Balatoni Rózsa' and influenced the fast-growing phenotype of BR25. GAs were reported to inhibit flavonoid biosynthesis (Hinderer et al., 1984). Conversely, JA enhances secondary metabolism (De Geyter et al., 2012). These effects may have caused us to observe most of the metabolic changes only in the 'Désirée' mutants. In other experiments where PPO activity was reportedly reduced, 'Désirée' was also used in one work (González et al., 2020), and various other varieties were used in other studies (Bachem et al., 1994; Chi et al., 2014; Llorente et al., 2011). Our results indicate that PPO-editing of different cultivars may lead to remarkably different metabolic profiles.

The second aim of the study was to decipher the role of PPOs in the resistance against pathogens. Previous research has implicated PPOs in plant response to late blight disease, with increased enzyme activity observed during the hypersensitive reaction in potatoes (Érsek et al., 1973). However, doubts persist regarding whether this reaction is a cause or consequence of resistance to *Pi* infection (Király et al., 1972). PPO activity was also associated with early blight disease protection of tomato plants (Narendra Babu et al., 2015). The current study, however, aligns with the view of no correlation of PPO to *Pi* infection, though the growth of the mycelium was higher on tuber slices of a single mutant, Des14. It is also possible that mutation of another PPO gene, *StuPPO9*, could have had a higher effect on *Pi* resistance. However, our CRISPR-target has 3 mismatches with that gene, located in the seed region adjacent to the PAM; therefore, it is highly unlikely that the *StuPPO9* gene was also mutated. Chi et al. (Chi et al., 2024) reported that the *StuPPO2* gene, primarily mutated in our study, was induced in potato leaves as a result of *Pi* infection; however, expression of *StuPPO9* peaked at a higher level. The *StuPPO2* gene, highly expressed in roots and tubers, may thus function more in the resistance against *Rs*, while the leaf-based *StuPPO9* is more important for the resistance against *Pi*. An alternative explanation for the lack of *Pi* resistance changes in our mutants comes from the fact that the level of JA, SA, and vanillic acid increased in the leaves as a result of the PPO-knockout, and so the effects of decreased PPO activity and increased production of other defense compounds may have counterbalanced each other. These hormones are known to have a role in the resistance against *Pi* (Cohen et al., 1993; Halim et al., 2007; Maksimov et al., 2011; Shibata et al., 2010), while vanillic acid inhibited the growth of *Phytophthora* species (Pusztahelyi et al., 2015).

Our investigation into the progression of bacterial wilt disease in PPO-edited potato lines, Des14, Des17, BR12, and BR25, along with their respective controls, unveils a noteworthy surge in susceptibility among the mutants, particularly in Des14, Des17, and BR25. Within the 'Balatoni Rózsa' lines, the resistance level to *Rs* seemed directly proportional to the mutations in the PPO genes. The unedited wild-type control, having no induced mutations, demonstrated the highest resistance. Following this trend, BR12, a mutant with mutations only in *StuPPO2* gene, displayed a higher resistance level than the BR25, the only plant harbouring both mutant *StuPPO2* and *StuPPO3* genes. This correlation underscores the pivotal role of PPO mutations in influencing metabolite regulation and, consequently, shaping the resistance profile against *Rs*. In the initial experimental round, the edited Des14 and Des17 exhibited a substantial rise in susceptibility to bacterial wilt, with wilting percentages reaching 87 % and 60 %, respectively, surpassing the control's 40 %. Similarly, BR25 displayed increased susceptibility, with wilting percentages of 73 % and 100 % in experiments 1 and 2, respectively, compared to the controls' 13 % and 80 %. These findings provide compelling evidence of the increased susceptibility of PPO mutants in both 'Désirée' and 'Balatoni Rózsa' cultivars to *Rs* infection.

Our results suggest that the loss of PPO activity may have outweighed any potential benefits arising from the increased production of antimicrobial compounds like taxifolin and dihydrokaempferol (Tajuddeen et al., 2014; Yang et al., 2023). While resistance to *Rs* in host species has been linked to elevated levels of salicylic acid, chlorogenic acid, activation of the phenylpropanoid pathway, increased antioxidants, and modulation of glutathione metabolism (Cao et al., 2020; Chen et al., 2022; Jose et al., 2023), our findings underscore the inadequacy of these factors alone for achieving resistance.

The study could also broaden our understanding of how to safeguard plants against PPO-expressing pathogens such as *Rs*. As mentioned earlier, the race 1 reference *Rs* strain GMI1000, uncommonly for bacteria, contains at least three functional genes encoding PPO (laccase, tyrosinase, and catechol oxidase) activity (Hernández-Romero et al., 2006). The atypical tyrosinase (UniProt Q8Y2J8, 496 aa) and catechol oxidase (Q8XZA3, 412 aa) are hypothesized to form a two-component system for a more efficient degradation of host antimicrobial phenolics and the tailored synthesis of bacterial melanin (Hernández-Romero et al., 2006). A search in the genome sequence and the reference proteome (UniProt UP000216264) of the UW551 race 3 *Rs* strain (Hayes et al., 2017) used in this study identified two PPO proteins (A0AB33VFM8, 543aa, and A0AB33VCY9, 412 aa) with 92 % and 87 % identity to the two GMI1000 PPO enzymes, respectively (Fig. S7). This observation not only confirms the widespread existence of such a two-component system in the *Rs* species complex but also provides implications for the observed *Rs* susceptibility of our PPO-edited lines. In all four edited lines, the level of decreased PPO enzymatic activity aligned well with their *Rs* susceptibility. Contrary to our initial hypothesis, which suggested that *Rs*, having its own PPO enzyme (Hernández-Romero et al., 2005), might tolerate the quinones produced by PPOs but would be inhibited by higher levels of other antimicrobial compounds present in plants with suppressed PPO activity, our findings revealed a different reality. As mentioned above, the genome of the applied *Rs* strain UW551 encodes two PPO enzymes, a tyrosinase, and a catechol oxidase. Hernández-Romero et al. (2005) reported that these enzyme activities, especially the tyrosinase, protected the wild-type *Rs* strain from the effects of L-tyrosine supplied to the medium, while the growth of the PPO-mutants was inhibited. The wild-type strain grew well while producing large amounts of melanin, obviously tolerating both it and the intermediate products, the quinones. The question may then arise, how could the PPO activity of a plant be protective against such a pathogen? Apart from a study that already established PPOs as molecular markers for resistance against *Rs* (El-Argawy and Adss, 2016), we assume that, given the obvious phylogenetic difference, the studied potato PPOs may be different enzymes from the bacterial ones and also synthesise some other products against which the bacterium remains susceptible. Melanin production can also be cited as an explanation. Melanin-based physical barriers may inhibit pathogen ingress and involved in the alkylation of proteins driven by PPOs, which could reduce the bioavailability of plant proteins (Johnson et al., 2003; Janusz et al., 2020). Subsequent PPO-activity of the pathogen may not enable it to overcome these limitations, also including the already discussed supposed PPO-derived lignin production in non-edited 'Désirée'.

The principal question of the study is whether PPOs or simple phenols/polyphenols represent the more important and effective defense mechanism against pathogens. Our study clearly shows the former to be true and underlines the importance of PPOs as a resistance mechanism against *Rs*. As stated earlier, the accumulation of these polyphenolics might certainly be part of the defense response as precursors for essential compounds, but spatial and temporal regulation and the corresponding additive effect of other enzymes like PPO might be required for achieving resistance. PPOs may exert this function by producing cytotoxic quinones, which potentially bind to proteins and amino acids and thereby provide the advantage of depriving pathogens of their supply (Felton et al., 1992). PPOs also participate in lignification and cell wall building (Bai et al., 2023; Bao et al., 1993), and our previous work has

already established the importance of lignification in the resistance against *Rs* (Jose et al., 2023). The significance of PPOs in plant defense mechanisms is undeniable, and the intricate nature of resistance mediated by polyphenols should not be underestimated.

5. Conclusion

Decreasing food browning via the downregulation or knockout of PPOs is a proven strategy; however, our study underlines the cost of this approach on *Rs* resistance. Resistance to pathogens can be conferred by understanding the underlying mechanism, and PPOs have a positive effect on *Rs* resistance together with plausible metabolite regulation, including flavonoids and phenolic acids. However, *Pi* infection was not affected in the *PPO* KO mutants.

CRedit authorship contribution statement

Jeny Jose: Conceptualization, Investigation, Writing – original draft. **Kamirán Áron Hamow:** Investigation, Validation, Writing – review & editing. **Csaba Éva:** Conceptualization, Supervision, Investigation, Writing – review & editing. **Blanka Moncsek:** Investigation. **Tetiana Kyrpa:** Investigation. **Liesel Gamarra Reinoso:** Investigation. **Zoltán Bozsó:** Conceptualization, Investigation, Writing – review & editing. **József Bakonyi:** Investigation, Writing – review & editing. **Ervin Balázs:** Conceptualization, Project administration, Funding acquisition. **László Sági:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Funding

The project was supported by grants NKFI K-132829, ATK-MATE 0205K0035P, and the Hungarian National Laboratories Program (grant number RRF-2.3.1–21–2022–00007).

Declaration of Competing Interest

The authors declare that they have no competing financial and non-financial interests.

Acknowledgements

The authors thank Ms. Szilvia Sipos and Mrs. Julianna Kurucz for their excellent technical assistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.plantsci.2025.112944](https://doi.org/10.1016/j.plantsci.2025.112944).

Data availability

Data will be made available on request.

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Glossary

- ABA: abscisic acid
 dpi: days post infection
 GA: gibberellic acid
 JA: jasmonic acid
 KO: knockout
 MRM: multiple-reaction monitoring
 PAM: protospacer-adjacent motif
 Pi: *Phytophthora infestans*
 PPO: polyphenol oxidase
 ROI: Region of Interest
 Rs: *Ralstonia solanacearum*
 SA: salicylic acid
 UPLC-US/MS/MS: Ultra Performance Liquid Chromatography-UniSpray tandem mass spectrometry