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Analysing disease susceptibility genes to generate potato plants resistant to bacterial wilt and late blight

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1. BACKGROUND OF THE WORK AND ITS AIMS

The intricate interplay between plants and pathogens significantly impacts the cultivation of crucial crops like potato (*Solanum tuberosum* L.), the fourth most important global crop due to its high carbohydrate content. Bacterial wilt (BW), caused by *Ralstonia solanacearum* (*Rs*), and late blight, driven by *Phytophthora infestans* (*Pi*), are two diseases with historical and economic significance. Traditional breeding methods have struggled to control these pathogens effectively, highlighting the need for innovative solutions.

Pathogens, particularly biotrophs, rely on host cooperation to establish infection. Susceptibility (S) genes facilitate this process, and their mutation or loss can hinder pathogen success. CRISPR/Cas genome editing technology offers a new possibility for targeting S genes to control plant diseases. However, understanding plant defense mechanisms is crucial for identifying S gene candidates. Pathogens like *Rs* and *Pi*, known for their adaptability and resilience, require comprehensive study of their infection strategies, injected effectors, and the resulting changes in plant transcriptome, metabolome, and structural constitution. Based on this consideration the specific aim of our research was to understand the response of *Rs*-resistant potato varieties 'Calalo Gaspar' and 'Cruza 148' to *Rs* infection compared to that of the susceptible 'Désirée,' compiling susceptibility gene candidates for both *Rs* and *Pi*.

Polyphenol oxidases (PPOs) are enzymes involved in plant defense against pathogens and herbivores, though their role in *Rs* and *Pi* infections remains unclear. Our second research aim was to generate *PPO* mutants and analyse their responses to *Rs* and *Pi* infections.

MiR396, a non-coding RNA molecule, regulates plant growth, development, and responses to stress and diseases. Its involvement in disease resistance is noted in *Rs*-resistant peanut plants and its predicted targeting of

disease resistance genes in potatoes (Zhang *et al.*, 2013; Zhao *et al.*, 2015). Given its significance, we aimed to generate miR396 potato mutants and evaluate their resistance responses to both *Rs* and *Pi*.

Plant sulphate transporters (SULTR) are essential for sulphur absorption and distribution. Recent evidence suggests that plants may withhold sulphur from pathogens as a defense mechanism (Criollo-Arteaga *et al.*, 2021). Our research focused on analysing *Arabidopsis* SULTR mutants to understand their responses to *Rs*, supported by literature suggesting sulphate transporters as susceptibility genes (Cernadas *et al.*, 2014).

In a general term, this integrated approach aims to contribute to a deeper understanding of plant-pathogen interactions, resistance mechanisms, and the potential for innovative strategies to combat these devastating diseases, ultimately benefiting both producers and consumers contributing to the ultimate aim of this study.

The following were the set objectives for the study:

1. Analysing resistance mechanisms of *Rs*-resistant potato lines
2. Studying the effect of PPO knockout in tetraploid potato on resistance to *Rs* and *Pi*
3. Studying the effect of miR396 knockout in tetraploid potato on resistance to *Rs* and *Pi*
4. Studying the effect of sulphate transporters in resistance response to *Rs* in *Arabidopsis thaliana*

2. MATERIALS AND METHODS

2.1. Plant material

Table 1. Plant materials used in the study

No.	Name of variety	Origin	Resistance type
1	*Solanum tuberosum* L. 'Désirée' (DES)	WUR, Wageningen, The Netherlands	Partially resistant to *Pi*, susceptible to *Rs*
2	*Solanum tuberosum* L. 'Balatoni Rózsa' (BR)	Potato Research Centre, Keszthely	Moderately susceptible to *Pi*. No data for *Rs*
3	*Solanum tuberosum* L. 'Botond' (Bt)	Potato Research Centre, Keszthely	Moderately resistant to *Pi*. No data for *Rs*
4	*Arabidopsis thaliana* Col-0 ecotype	Nottingham Arabidopsis Stock Centre	Susceptible to *Rs*
6	*At*SULTR3.1 (SULTR3.1 T-DNA insertion line in *Arabidopsis thaliana* Col-0 ecotype)	Nottingham Arabidopsis Stock Centre: 4839483; SALK_023190C	No data
7	*At*SULTR1.2 (SULTR1.2 T-DNA insertion line in *Arabidopsis thaliana* Col-0 ecotype)	Nottingham Arabidopsis Stock Centre: 4839483; SALK_122974	No data

Table 2. Potato accessions tested for *Rs* resistance as listed by Jose *et al.* (2023)

No.	Name of Variety	Taxonomy	Accession ID	Reference, source
1	'Calalo Gaspar' (CG)	*S. stenotomum* ssp. *stenotomum*	CIP 700670	Martin, (1979)
2	'Cruza 148' (CR)	*Solanum* hybrid (*S. demissum*)	CIP 720118, PI 619136	Martin, (1979); Jackson (2012

2.2. Plant growth conditions

Potato plants were grown in 500-mL containers with MS (Murashige and Skoog, 1962) medium, 2% sucrose, and agar under controlled conditions (23°C, 16-h light, 100 μE light intensity). Twenty plants per genotype were multiplied from nodal segments for *Rs* infection. For whole plant infection assays, potato plants were grown in pots with peat-containing soil in a

glasshouse under ambient light conditions (12 h light, 20°C). At 8 weeks old, five plants per genotype were subjected to *Pi*.

For *A. thaliana* ecotype Columbia (Col-0) and *AtSULTR3;1*, *AtSULTR1;2* mutants, seeds were surface sterilised and stratified at 4°C for 48 hours. They were then planted on Petri dishes with ½ MS medium and 1% sucrose. Germinated seedlings were transferred to Jiffy pots at 21°C under short-day conditions (8-h light, 16-h dark), with high humidity, and irrigated with ¼ strength MS solution. *AtSULTR3;1*, *AtSULTR1;2* and Col-0, were cultivated on solid ½ MS plates without the inclusion of sucrose at 22°C, under 100 µE light intensity for suberisation assessment. The experiments were carried out employing methodologies shown in **Figure 1**.

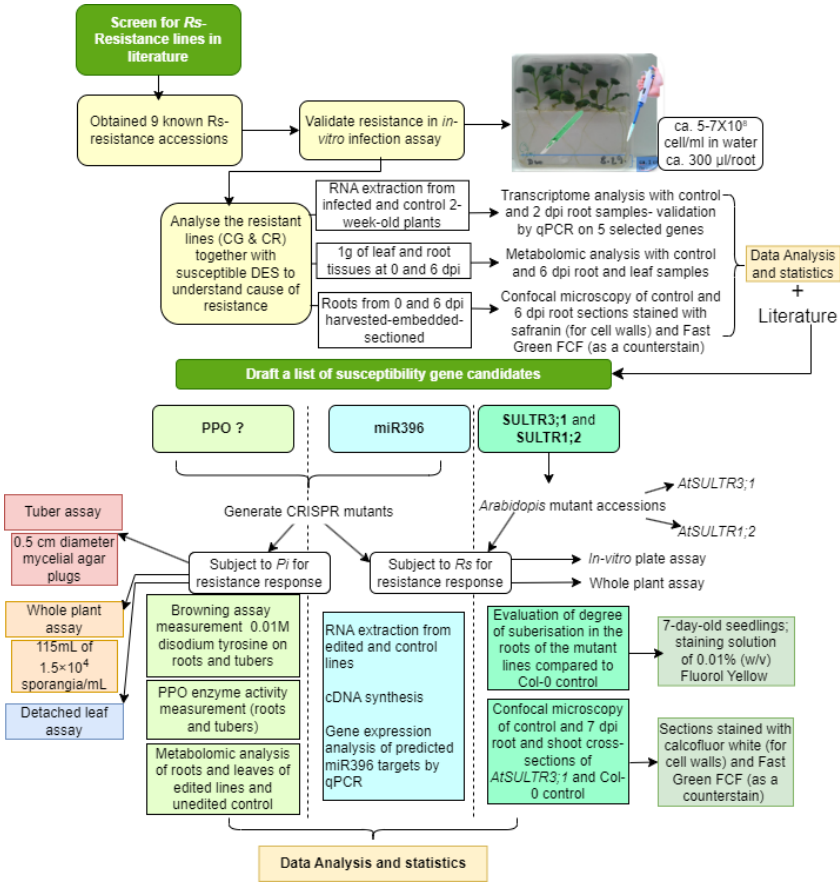

Figure 1. Systematic flow of methodologies employed

2.3. CRISPR constructs

CRISPR constructs for all target genes were created by following a standardised protocol, as originally described by Xing *et al.* (2014), followed by transformation on four-week-old leaves of commercially grown potato cultivars as proposed by Dietze (1995). They were regenerated on callus induction and shoot induction selection medium.

3. RESULTS AND DISCUSSION

3.1. Molecular analysis of *Rs*-resistant potato cultivars

Among the 9 accessions known to possess *Rs* resistance in the field, CG is a traditional diploid landrace originating from Peru, belonging to the species *S. stenotomum* (Jackson *et al.*, 1978). CR is categorised as a 'tuberosum type' and originates from Toluca, Mexico. It was the first cultivated potato to exhibit tolerance to bacterial wilt (Jackson *et al.*, 1979). Our *in vitro* inoculation tests with CG, CR, and *Rs*-susceptible commercial cultivar DES corroborated these prior findings with a 100% survival rate for CG and CR (**Figure 2A)** at 21-days post-inoculation (dpi) compared to only 32% survival of the susceptible control DES and the more intense spread of *Rs* bacteria spread throughout the DES plants (**Figure 2B**).

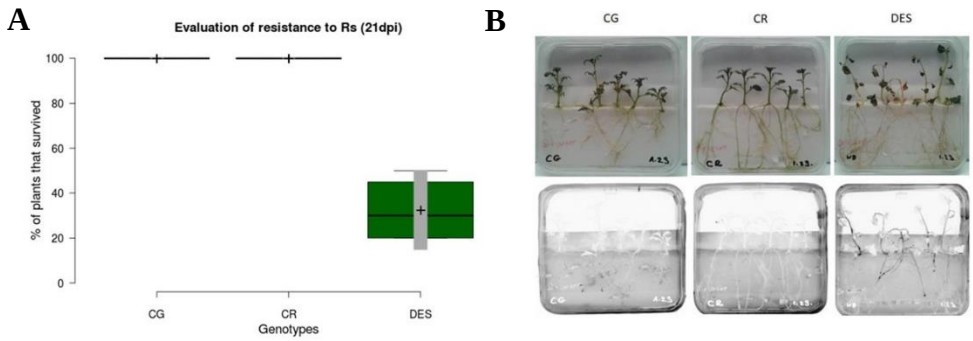

Figure 2. Survival of two selected potato accessions in an *in vitro Rs* inoculation assay at 21 dpi (A). Visual evaluation of resistance to *Rs* infection (21 dpi) in the *in vitro* inoculation bioassay under visible light for the observation of wilting (upper row) and UV light for the detection of GFP-expressing *Rs* bacteria (lower row). CG, 'Calalo Gaspar'; CR, 'Cruza 148'; DES, 'Désirée'(C) (Jose *et al.*, 2023).

To uncover the molecular basis of *Rs* resistance in CG and CR a transcriptome analysis was performed. The *Rs* infection impacted each cultivar's transcriptome differently revealing only 580 common differentially expressed genes upon infection (DEGs) and 2,142, 1,242, and 616 unique DEGs in CG, CR, and DES, respectively. RT-qPCR experiments on five randomly selected genes across the three potato cultivars confirmed the RNA-

seq data, with a high correlation coefficient (R^2 = 0.9102), affirming the reliability of our transcriptome analysis.

Gene ontology (GO) enrichment analysis showed notable expression patterns: oxidative stress response genes were downregulated in CG, while cell wall and chitin metabolic processes were induced in CR and DES (**Figure 3A**). KEGG pathway analysis (**Figure 3B**) supported these findings, highlighting downregulation of phenylpropanoid and plant-pathogen interaction pathways in CG, upregulation of the phenylpropanoid pathway and glutathione metabolism in CR. This indicates varying responses to *Rs* infection among the potato cultivars, with distinct gene expression and pathway activations reflecting their resistance levels.

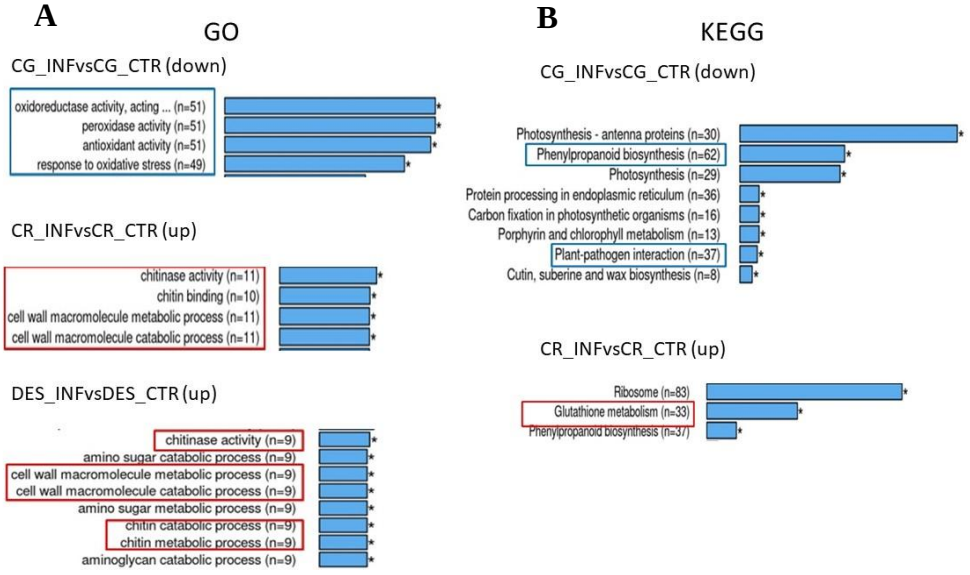

Figure 3. Upregulated and downregulated GOs upon *Rs* infection (2 dpi) in the roots of 'Calalo Gaspar' (CG), 'Cruza 148' (CR), and 'Désirée' (DES). Asterisks indicate that all enrichments are significant. Categories with enrichments specific to each cultivar are boxed (Jose *et al.*, 2023).

Previous studies align with our findings, noting upregulation of glutathione metabolism in resistant cultivars of eggplant and tobacco upon *Rs* infection (Gao *et al.*, 2019; Li *et al.*, 2021; Peng *et al.*, 2021). Glutathione, a

crucial antioxidant, likely contributes to CR's *Rs* resistance (Dorion *et al.*, 2021).

Plants produce a wide range of secondary metabolites, such as phenolics and flavonoids via the phenylpropanoid pathway, which contribute to resistance against biotic and abiotic stresses (Mierziak *et al.*, 2014; Zaynab *et al.*, 2018; Yadav *et al.*, 2020). Plant hormones, particularly ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA), also play critical roles in stress responses (Verma *et al.*, 2016).

Metabolomic analysis on CG, CR, and DES with 30 specific metabolites from the phenylpropanoid pathway and the plant hormones ABA, SA, JA, and IAA revealed that *Rs* infection caused more pronounced metabolite changes in roots than leaves, particularly in CG and DES compared to CR.

We found higher concentrations of chlorogenic acid, known for its antimicrobial and antioxidant properties (Kabir *et al.*, 2014), in *Rs*-resistant cultivars and its level increased in roots of all cultivars upon infection. Dihydrophaseic acid (PHA) decreased in CG and CR but increased in the susceptible DES, suggesting an inverse relationship of PHA to resistance. Increased phaseic acid (PA) concentration in DES leaves and higher ABA levels in DES roots may contribute to basal defense and drought-stress protective pathways as in the sensitive peanut genotype during *Rs* infection (Zhao *et al.*, 2015). Naringenin, a flavonoid, increased in amount in the leaves of all cultivars upon infection, but especially in CR, while quercetin and its derivatives increased in CG, highlighting their roles in the resistance response of CG. Flavonoids have both antioxidant and antipathogenic properties and are stress generated metabolites (Mierziak *et al.*, 2014) which can significantly contribute to resistance (Li *et al.*, 2021). *Rs* infection led to increased SA levels in roots of all cultivars, indicating its role in basal defense, while JA levels,

initially higher in DES leaves, decreased upon infection in all cultivars (**Figure 4 and 5**).

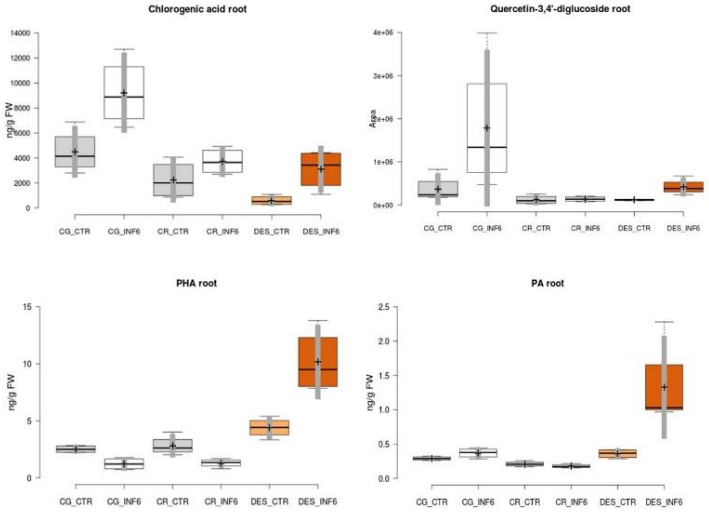

Figure 4. Concentration differences of four selected secondary metabolites between non-infected and *Rs*-infected 'Calalo Gaspar', 'Cruza 148', and 'Désirée' roots (brown) and leaves (green) at 6 dpi. CG_CTR, 'Calalo Gaspar' control (grey); CG_INF6, 'Calalo Gaspar' infected (white); CR_CTR, 'Cruza 148' control (grey); CG_INF6, 'Cruza 148' infected (white); DES_CTR, 'Désirée' control; DES_INF6, 'Désirée' infected.

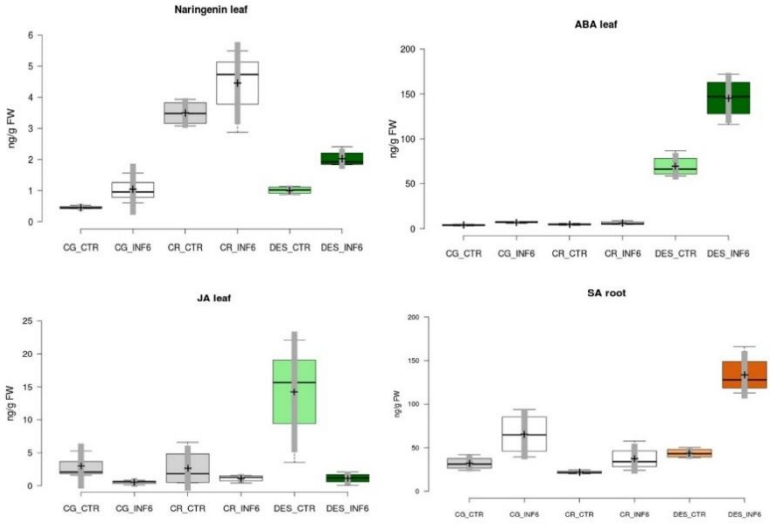

Figure 5. Concentration differences of naringenin and 3 hormones ABA, JA, and SA between non-infected and *Rs*-infected 'Calalo Gaspar', 'Cruza 148', and 'Désirée' roots (brown) and leaves (green) at 6 dpi. CG_CTR, 'Calalo Gaspar' control (grey); CG_INF6, 'Calalo Gaspar' infected (white); CR_CTR, 'Cruza 148' control (grey); CG_INF6, 'Cruza 148' infected (white); DES_CTR, 'Désirée' control; DES_INF6, 'Désirée' infected.

Our findings align with previous studies indicating that metabolites and hormones like glutathione, flavonoids, and SA contribute to *Rs* resistance, while JA may be less critical (**Figure 5**) (Lowe-Power *et al.,* 2016; Pan *et al.*, 2021). The role of plant hormones in defense against *Rs* remains complex and not fully understood (Hirsch *et al.,* 2002; Hernández-Blanco *et al.,* 2007).

Based on GO data, and metabolomic analysis showing involvement of cell wall reinforcement fuelling *Rs* resistance, we studied it using confocal microscopy. Root cross-sections stained with safranin revealed lignification patterns (**Figure 6**). It was found that in CG, CR, and DES, the central root parts, especially the xylem, showed lignification even without *Rs* infection, with the lowest lignin levels in DES (**Figures 6A-C**). *Rs* infection did not alter lignification in CG (**Figure 6D**), but significantly increased it in the stele of CR (**Figure 6E**) and dramatically in the xylem of DES (**Figure 6F**), where a large quantity of *Rs* bacteria was detected. This suggests that the basal lignification level in DES may have been insufficient to prevent *Rs* invasion.

Lignin, a crucial secondary metabolite synthesized via the phenylalanine/tyrosine metabolic pathway, plays a pivotal role in plant response to various stresses (Cesarino, 2019). The cell wall serves as the initial physical defense layer against pathogens. Studies on *Arabidopsis* mutants with impaired cell wall-cellulose synthesis have shown that alterations in cell wall formation can confer resistance against vascular pathogens, including *Rs* (Hernández-Blanco *et al.,* 2007; Denancé *et al.,* 2013).

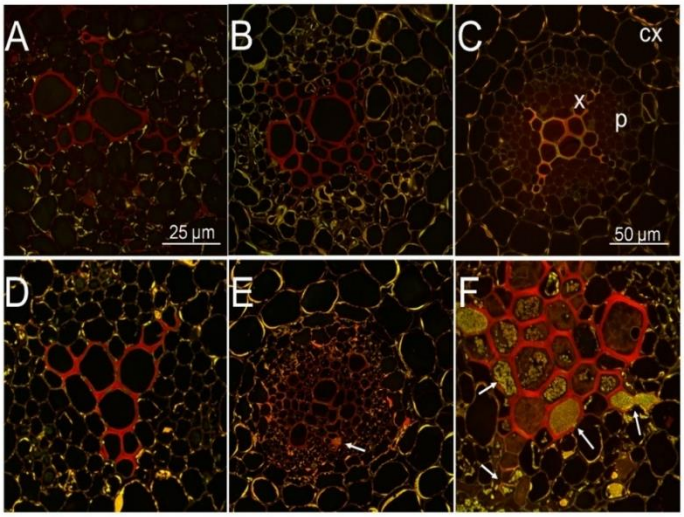

Figure 6. Confocal microscopy images of potato root cross-sections from non-infected control (A-C) and *Rs*-infected (D-F) plants at 6 dpi. Samples from 3-week-old, *in vitro* plants were stained with safranin for lignin and Fast Green for contrast. (A) 'Calalo Gaspar' control, (B) 'Cruza 148' control, (C) 'Désirée' control, (D) 'Calalo Gaspar' infected, (E) 'Cruza 148' infected, (F) 'Désirée' infected. cx, cortex; p; parenchyma; x, xylem; arrows, *Rs* bacteria (Jose *et al.*, 2023).

Our study highlights differences in cell wall fortification as a resistance response to *Rs* infection, varying between resistant cultivars. CR demonstrated a self-protective mechanism through cell wall reinforcement, while CG exhibited primarily a metabolic response, both viable strategies against the pathogen.

Based on the transcriptomic data and simultaneous analysis of relevant literature, we screened for candidate susceptibility genes from the DEGs that were downregulated among the resistant lines contrary to susceptible DES and could be potentially used for CRISPR/Cas9 editing to confer resistance against the pathogens *Rs* and *Pi*. The three candidates from this combined analysis, namely *POLYPHENOL OXIDASE* (*Pot32*, literature), *stu-miR396* (literature) and *SULPHATE TRANSPORTER 3;1* (transcriptome data) were selected for further studies.

3.2. Studying the effect of *PPO* knockout in tetraploid potato on resistance to *Rs* and *Pi*

The transformation was carried out on ‘Désirée’ (DES) and ‘Balatoni Rózsa’ (BR) for the *Pot32* PPO gene as mentioned in 2.3. Two DES lines (D14 and D17) and two BR lines (BR12 and BR25) were selected for further examination to assess the effects of *PPO* editing. We observed that, all four selected lines had mutations at the target site within the *Pot32* PPO gene. D14 and D17 had mutations in two of four alleles, BR12 had a monoallelic mutation, and BR25 had mutations in three alleles. The examined lines, especially D17 and BR25 displayed significantly reduced browning in tubers compared to controls (**Figure 7A**) in correlation with a significant decrease in PPO activity. D14 and D17 had reductions of 10.9% and 33.8%, while BR12 and BR25 had reductions of 10.9% and 13.2%, respectively (**Figure 7A**). Three mutant lines (D14, D17, and BR25) also showed reduced browning in their roots (7.6%, 11.5%, and 8.6% reductions), but BR12 did not (**Figure 7B**).

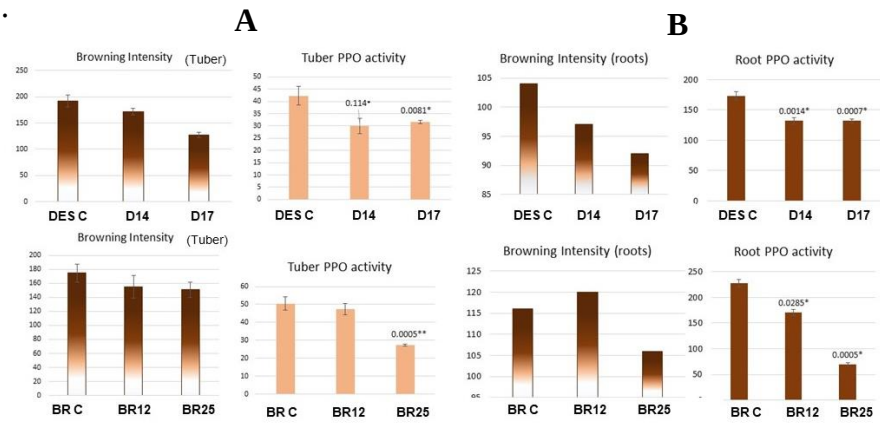

Figure 7. Quantification of browning assay done and PPO enzyme activity in tubers (A) and roots (B). Browning as observed with substrate solution containing 0.01M disodium tyrosine and PPO enzyme activity against 15 mM 4-methylcatechol of ´Désirée’ control (DES C) and *PPO*-mutants ´Désirée' Line14 and 17 (D14, D17) (Row 1), Balatoni Rózsa’ control (BR C) and PPO-mutants, ´Balatoni Rózsa’ Line12 and 25 (BR 12 and BR25) (Row 2). * indicates the level of significance of p-value<0.05

While previous studies have used RNA interference or CRISPR-related techniques to decrease PPO activity in potatoes (Bachem *et al*., 1994;

Llorente *et al*., 2014), our dual-genotype approach offers a more comprehensive understanding of PPO's role in potatoes and extends this knowledge to the Hungarian cultivar, BR.

Previous research demonstrated that downregulating *PPO* genes using RNAi leads to diverse metabolic alterations in potato tissues, redirecting metabolic pathways and elevating specific metabolites such as chlorogenic acid, fraxin, naringenin, phaseic acid, and taxifolin (Araji *et al.*, 2014; Llorente *et al.*, 2014). Our UPLC-MS/MS measurements showed variations in the concentrations of 26 phenolic compounds between control and PPO-edited potato plants, with PCA analysis revealing significant divergence in the root metabolome of most PPO mutants (D14, D17, and BR25).

Key findings include (**Figure 8**):

- Certain metabolites, like flavonols (isorhamnetin-3-O-rutinoside and quercetin-3,4'-diglucoside), increased in three edited lines (D14, D17, and BR12). However, BR25 showed significantly reduced flavonoid levels (naringenin, dihydrokaempferol, and taxifolin).
- Phaseic acid levels were elevated in the roots of D14, D17, and BR25.
- Genotype-specific differences were noted: naringenin, dihydrokaempferol, and taxifolin increased in the roots of D14 and D17 but decreased in BR12 and BR25.
- The root metabolomes of the two potato cultivars exhibited marked distinctions, emphasizing the impact of *PPO* editing, with BR12 showing no significant differences from its control.

The impact of *PPO* silencing on hormonal levels had not been previously documented. *PPO* genes respond to stress-related hormone signalling. Our study did not find significantly elevated SA levels but noted regulatory changes due to *PPO* editing. JA levels were heightened in DES

mutants, while various forms of GAs were increased in PPO-edited BR plants. Elevated metabolite production was mainly observed in the PPO-edited DES lines, suggesting a genotypic differential response.

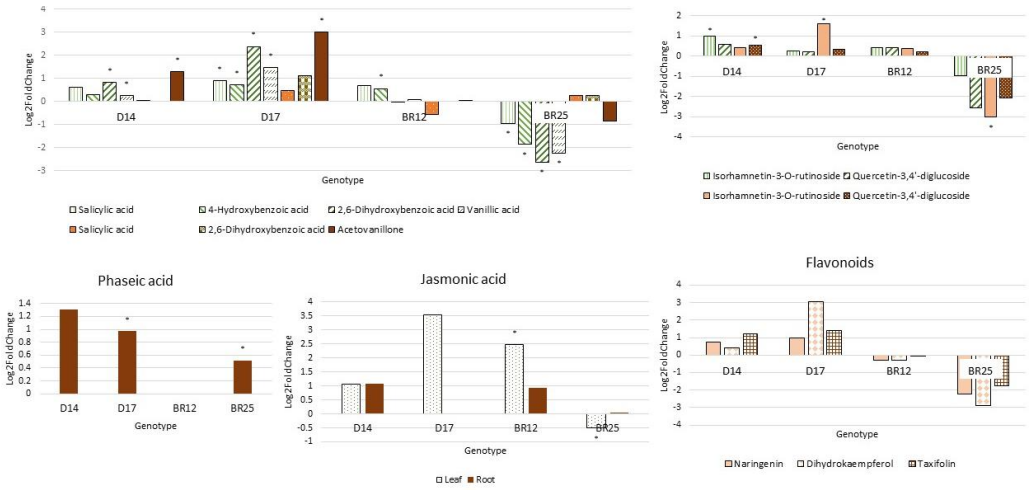

Figure 8. Differential regulation of selected significant metabolites calculated by the Log2Fold change with respect to the control in the leaves (stripes and lines) and roots (solid fill,dots,checks) of the mutant lines Désirée Line 14 (D14), Désirée Line 17 (D17), Balatoni Rózsa Line 12 (BR12) and Balatoni Rózsa Line 25 (BR25). * indicates significant changes at $p \leq 0.05$

Comparing the higher levels of phaseic acid (catabolic product of ABA) in D14 and D17 *PPO* mutants with the findings of previous studies on *Rs*-resistant lines (CG and CR) and susceptible DES, we noted that dihydrophaseic acid, also a catabolic product of ABA, exhibited an inverse relationship with resistance in resistant lines. This highlights the interplay of ABA-related metabolites in defense mechanisms against *Rs*.

We found that the *PPO*-edited potato lines D14, D17, and BR25 displayed higher susceptibility to *Rs* infection compared to their unedited controls. This increased susceptibility was evidenced by a higher number of wilted plants and faster bacterial spread to the shoots. Specifically, D14 and D17 showed wilting percentages of 87% and 60%, respectively, compared to the control's 40%. BR25 had a wilting percentage of 73%, versus 13% in the

control. In contrast, BR12 showed no significant differences from its control (**Figure 9**).

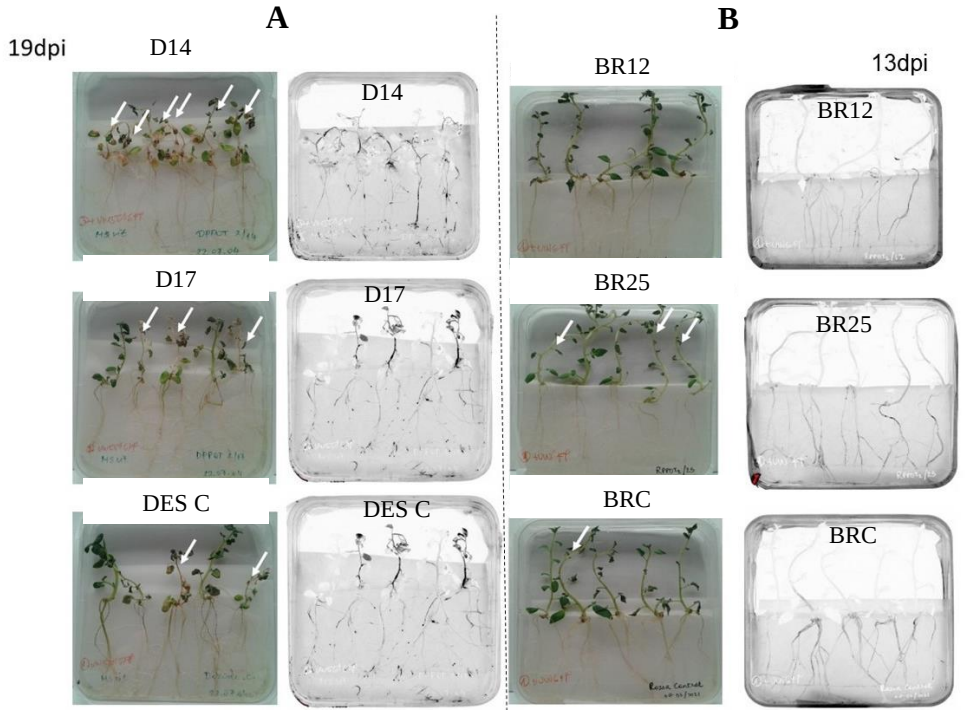

Figure 9. Visual evaluation of 'Désirée' (A) and 'Balatoni Rózsa' (B) along with its *PPO* mutants in response to *Rs* infection and bacterial spreading (19 dpi and 13dpi respectively) in the *in vitro* inoculation bioassay under visible light for the observation of wilting (1st column) and UV light for the detection of *GFP*-expressing *Rs* bacteria (2nd column). Arrows point to the wilted plants. (Z. Bozsó, NÖVI).

Initially, we hypothesized that *Rs* might be susceptible to antimicrobial compounds produced in the absence of PPOs. However, our findings indicated that PPO activity in potatoes is a crucial protective mechanism against *Rs*, similar to its protective effect against *Pseudomonas* bacteria (Li and Steffens, 2002). The extent of *PPO* gene mutation appeared to correlate with metabolite regulation: BR12, with a monoallelic mutation, showed the least changes, while D14 and D17, with two intact alleles, exhibited increased metabolite production as a compensatory response. BR25, with three mutated alleles, faced challenges in metabolite regulation.

Our research underscores the vital role of PPOs in potato resistance against *Rs*. The effectiveness of polyphenolic compounds in defense appears to depend on their spatial and temporal regulation and their interaction with other enzymes like PPO. The loss of PPO activity seems to outweigh any potential benefits from increased antimicrobial phenolic compounds and JA production in the edited lines. Overall, PPOs play a complex, yet critical role in plant defense mechanisms against pathogens.

The response of potato plants and tubers to *Pi* infection varied between cultivars. In whole plant assays, the BR cultivar showed delayed symptom development compared to the DES cultivar. By 4 dpi, both cultivars displayed damage, and nearly all plants succumbed to the infection by 7 dpi. In tuber infection tests, by 5 dpi, *Pi* mycelium uniformly covered all tuber slices in both cultivars, with a slightly denser mat on BR compared to DES. Notably, *PPO*-edited plants did not show significant differences from their respective controls at any observed time points. Contrary to literature suggesting increased *Pi* resistance attributed to *PPO* knockouts (Llorente *et al.*, 2014), our findings indicate no discernible effect of the knockout on the resistance response.

3.3. The effect of *miR396* knockout in tetraploid potato on resistance to *Rs* and *Pi*

A two-target genome editing vector was generated using the protocol of Xing *et al*. (2014), resulting in 10 CRISPR/Cas9-edited mutant plant lines in two commercial potato cultivars, 'Désirée' (DES) and 'Botond' (Bt). Mutations at the two target sites in Bt mutant lines 6, 7, 8, and 9 showed uniform changes across the targets. In DES mutant lines, in Line 3 the entire region between the two targets was deleted in some of the alleles, Line 10 had deletions around the first target, and Line 6 had mutations primarily around

the second target. Gene expression studies revealed consistent reduction in *miR396* expression across all edited lines. Increased expression of LRR receptor-like serine/threonine-protein kinase, multicystatin147, and, in some edited lines, the potassium transporter was higher expressed compared to the control (**Figure 10**).

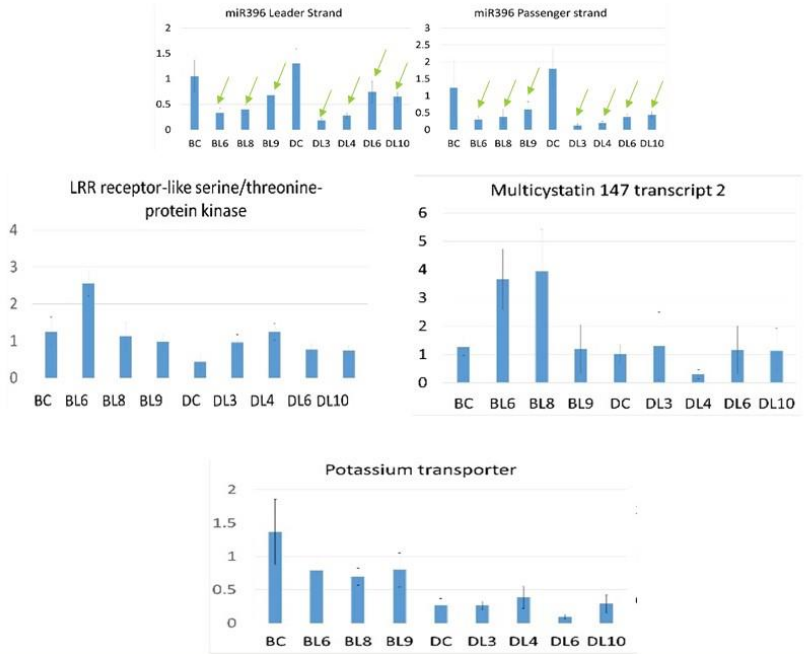

Figure 10. The relative expression of the *miR396* gene and its predicted targets in the *miR396*-edited lines of 'Botond' (BL6, BL8, and BL9) and 'Désirée' (DL3, DL4, DL6, and DL10) along with the respective controls (BC and DC)

Upon infecting miR396-edited Bt Lines 6, 7, 8, and 9 and the control with the virulent *Rs* strain UW551 *in vitro*, Bt Lines 8 and 9 showed delayed symptom development in repeated experiments, although all plants eventually succumbed to the infection. In DES lines, Lines 3 and 10 exhibited delayed symptom development compared to Line 6 and the control in experiments with potted plants (**Figure 11**). CRISPR mutations varied among lines, with larger deletions in target 1 in Lines 3 and 10, and significant deletion around target 2 in Line 6.

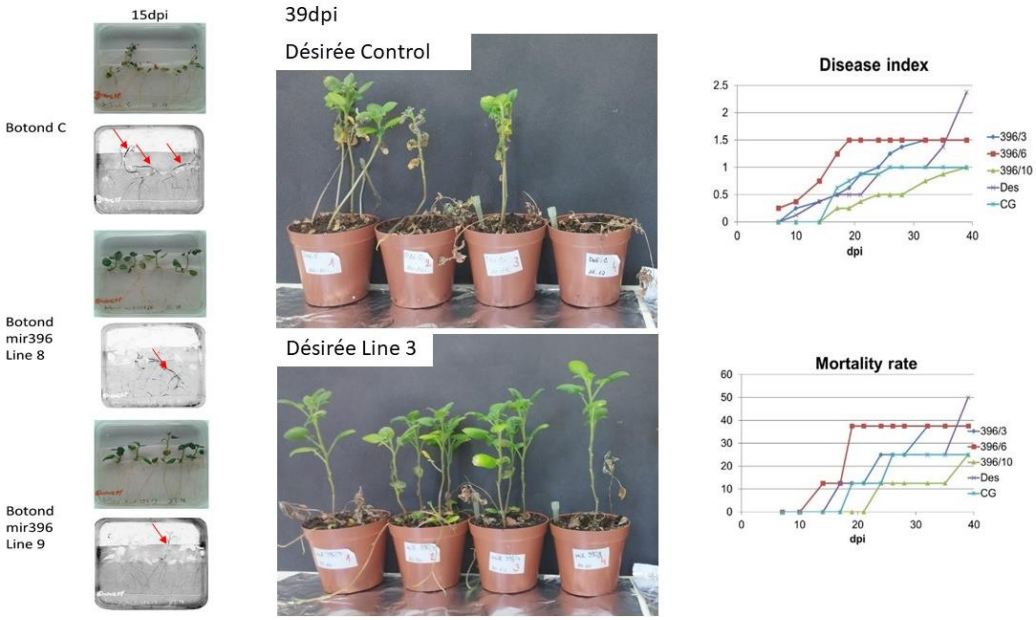

Figure 11. Visual evaluation of response to *Rs* infection (15 dpi) in the *in vitro* inoculation bioassay under visible light for the observation of wilting (upper row) and UV light (lower row) for the detection of *GFP*-expressing *Rs* bacteria (Column 1). 'Botond' control and 'Botond' mutant *miR396* Line 8 and Line 9, of 'Désirée' Control and edited Line 3 (Column 2), quantified disease index and mortality rate for 'Désirée' control (DES) and *miR396* edited lines (396/3, 396/6,396/10) together with *Rs*-resistant variety 'CG' (Column 3). (Z. Bozsó, NÖVI)

Previous studies (Zhao *et al.*, 2015) indicated downregulation of *miR396* in resistant peanut varieties exposed to *Rs* infection, suggesting a role in SA-mediated resistance responses. Consequently, enhanced resistance was anticipated in miR396 mutant plants, and delayed symptoms were observed specifically in Bt Lines 8 and 9 and DES Lines 3 and 10 (exhibiting larger deletions at the CRISPR target site).

In gene-edited 'Botond' and 'Désirée' potato lines, increased susceptibility to *Pi* was observed, with rapid disease progression compared to unedited controls (**Figure 12**). This suggests a crucial role for the miR396 gene in regulating defense against *Pi*.

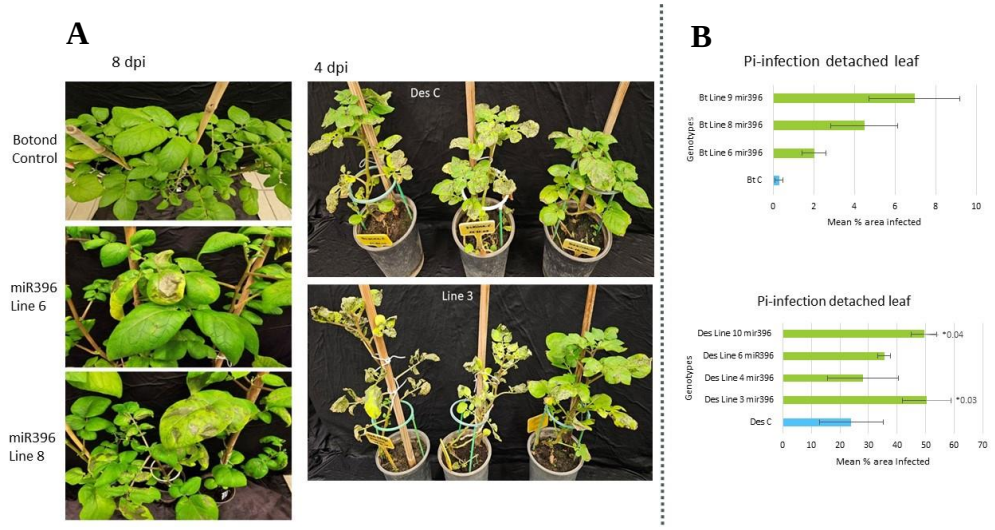

Figure 12. Visual evaluation of response to *Pi* infection in Botond control and edited Botond *mir396* Lines 6, and 8 (Column 1) and 'Désirée' control and edited Désirée *mir396* Line 3 (Column 2) at 8 dpi and 4 dpi respectively in the whole plant bioassay for the observation of necrotic spots (A) % area infected among the Botond edited *mir396* Lines 6, 8, and 9 with control (Top) and Désirée edited Line 3, 4,6 and 10 with the control (Bottom) (B).

While miR396 is generally considered a negative regulator of plant immunity, it enhances resistance to some pathogens (Fahlgren *et al.*, 2007). Our study revealed that miR396 editing increased susceptibility to *Pi* but improved resistance to the hemibiotrophic *Rs*. This differential response may be due to miR396's effect on specific target genes, such as NBS-LRR receptor-like kinases and multicystatins (Dievart and Clark, 2004; Kaschani *et al.*, 2010) . Increases in multicystatin expression, which is a protease inhibitor linked to pathogen defense, interestingly resulted in greater susceptibility to *Pi*. Literature sources suggest that while protease inhibitors are effective against herbivores and certain pathogens, the defence against other invaders might require hypersensitive response where plant proteases are of greater importance.

3.4. The *Arabidopsis thaliana* sulphate transporters and resistance response to *Rs*

The *AtSultr3;1* sulphate transporter mutant plants showed a notable increase in resistance to *Rs* (GMI1000) when compared to the Col-0 (Columbia ecotype) control. In parallel, the *AtSultr1;2* mutant also underwent *Rs* infection and also revealed enhanced resistance relative to the Col-0 control, but less compared to *AtSultr3;1*. The elevated resistance manifested through a significantly diminished incidence of wilted plants resulting in lower disease index and mortality rate compared to the Col-0 control (**Figure 13**). Importantly, this consistent pattern was observed across multiple experimental repetitions.

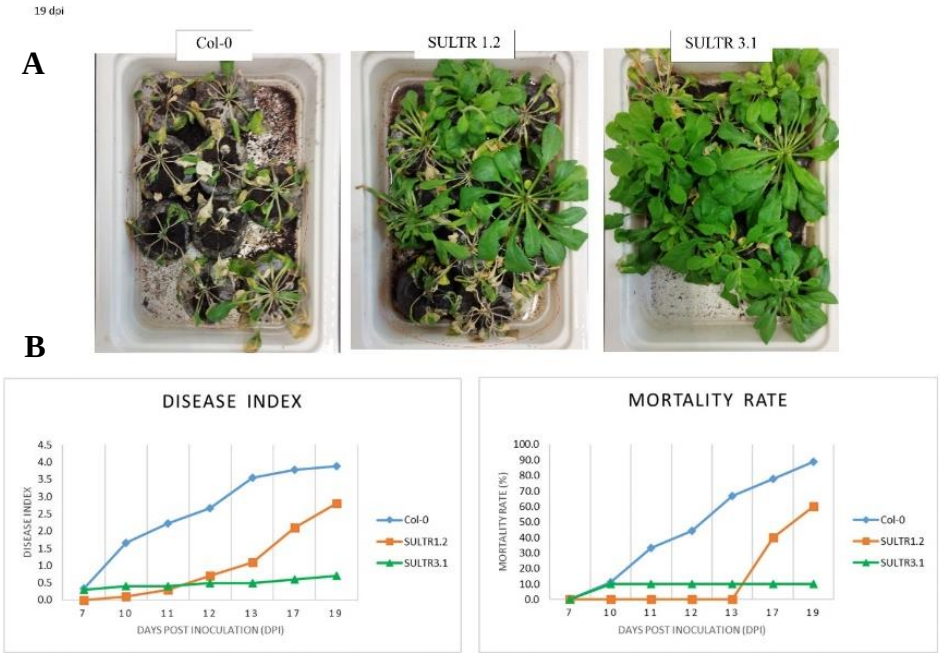

Figure 13. Visual evaluation of response to *Rs* infection at 19 dpi Jiffy soil inoculation for the observation of wilting in *Arabidopsis thaliana* Col-0 ecotype and sulphate transporter mutant lines in *Arabidopsis* Col-0 background SULTR1;2 and SULTR3;1. Images captured by Z. Bozsó. (A). Disease index and mortality rate calculated based on the no. of wilted plants and the severity of wilting among the genotypes (B)

Elevated level of suberisation in the two mutants, particularly in the vicinity of the vascular bundles was observed in the longitudinal sections of the roots using fluorescence microscopy. (**Figure 14**).

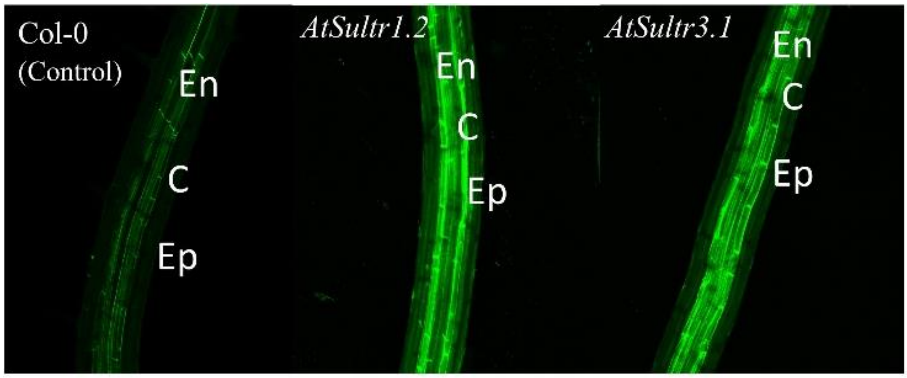

Figure 14. Confocal microscopy images to observe the level of suberisation in 7-day old roots of Col-0 ecotype (control) and the two sulphate transporter mutants (*AtSultr1;2* and *AtSultr3;1*). More intense Fluorol Yellow fluorescence is indicative of increased suberisation, particularly in the endodermis (En). C, cortex; Ep, epidermis

The plant cell wall serves as structural support and a barrier against pathogens, with suberin playing a critical role in defense. Suberin production increases in response to various stresses, including pathogen attack and sulphur deficiency (Barberon *et al.*, 2016). The suberised root endodermis acts as a protective layer, crucial for nutrient uptake and preventing pathogen invasion (Kashyap *et al.*, 2021). In potatoes, suberin contributes to pathogen resistance (Lulai and Corsini, 1998). For vascular pathogens like *Rs*, a consolidated endodermis in sulphate transporter mutants can block infection. At 7 days post-infection, *Rs* colonized the roots of both *AtSultr3;1* mutants and controls, but was limited in the cortex of mutants (**Figure 15A**). In shoots, *Rs* was absent in the pith of *AtSultr3;1* mutant but fully colonized the xylem in controls, demonstrating the enhanced resistance in the mutant (**Figure 15B**). This highlights the importance of cell wall dynamics in plant defense against pathogens.

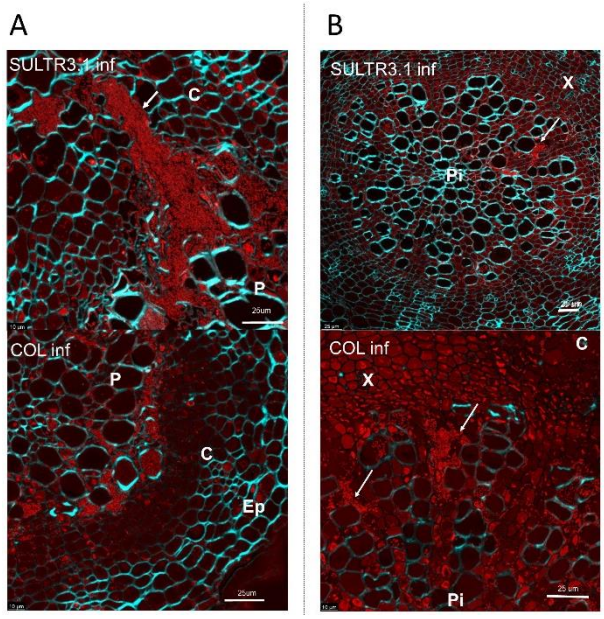

Figure 15. Confocal microscopy images of *A. thaliana* root cross-sections (A) and shoot cross section (B) from *Rs*-infected plants of the *AtSultr3;1* mutant (top) and Columbia (COL) control (bottom). Samples from 4-week-old, *in vitro* plants were stained at 7 dpi with Calcofluor white for cell walls and Fast Green for contrast. Ep, epidermis; C, cortex; P, phloem; X, xylem; Pi, pith; arrows, *Rs* bacteria

SULTR1;2 and *SULTR3;1* are sulphate transporter genes with distinct functions: *SULTR1;2* is active in the root's outer layers, while *SULTR3;1* is expressed in shoots, in the plastids and chloroplasts. *SULTR3;6*, a related sulphate transporter was formerly established as a susceptibility gene in rice to the bacterial leaf streak (Cernadas *et al.*, 2014). Our data suggests that the *SULTR3;1* gene, identified as a candidate susceptibility gene from transcriptomic analysis of *Rs*-resistant lines, interacts with *Rs* effectors to increase potato susceptibility to the bacterial pathogen. The supported resistance in *AtSultr3;1* mutant indicates that targeted editing of *SULTR3;1* could confer *Rs* resistance in potatoes

Pathogens, especially (hemi)biotrophs, depend on host sulphur metabolism for virulence. Sulphur deprivation triggers plant defence responses suggesting that manipulating sulphur transport genes can enhance resistance (Criollo-Arteaga *et al.*, 2021; Kies and Hammer, 2022). Studies also

highlight that the corky biosynthetic pathway is crucial for pathogen resistance, as demonstrated in peanuts and tomatoes (Kashyap *et al.*, 2021). Suberin, a key cell wall component, plays an essential role in this defence mechanism, even if direct evidence in plants is limited (Shi *et al.*, 2023). Overall, plant defence against pathogens involves multiple strategies, including sulphur metabolism and corky biosynthesis.

4. CONCLUSIONS AND RECOMMENDATIONS

Our research aimed to enhance potato resistance against major pathogens *Ralstonia solanacearum* (*Rs*) and *Phytophthora infestans* (*Pi*) using genome editing techniques.

We initially investigated various *Rs*-resistant potato cultivars, revealing key differences in resistance mechanisms and metabolic responses. Notably, resistant cultivars like 'Calalo Gaspar' (CG) and ‘Cruza 148’(CR) exhibited increased chlorogenic acid levels and unique phenolic profiles, while susceptible cultivars like 'Désirée' (DES) showed insufficient lignification and persistent bacterial infection. This data not only characterizes cultivars but also offers insights for future research, contributing to identifying susceptibility genes for targeted interventions.

Based on strong supportive literature of polyphenol oxidases (PPO) and microRNA396 (miR396) being involved in *Rs* and *Pi* resistance, we knocked out the tuber and root-specific *PPO* gene (in DES and ´Balatoni Rózsa’ (BR) genotype) and *miR396* gene (in ´Botond’ (Bt) and DES genotype) using modular CRISPR/Cas9 systems.

PPO editing led to reduced enzyme activity and increased susceptibility to *Rs*, despite elevated antimicrobial metabolites. However, the *PPO* mutants did not show significant differences in *Pi* resistance, challenging previous findings.

For miR396, edited lines displayed delayed symptom development for *Rs* but increased susceptibility to *Pi*, suggesting a complex role of miR396 in pathogen response. Notably, NBS-LRR disease resistance gene and multicystatin were higher expressed in the edited lines relative to the control which may have influenced the distinct resistance responses observed against *Rs* and *Pi*. It is a novel finding and an interesting aspect to be explored further.

Additionally, transcriptomic analysis of CG, CR, and DES highlighted sulphate transporters as potential susceptibility genes. Arabidopsis mutants for the transporters *AtSULTR3;1* and *AtSULTR1;2* showed increased resistance to *Rs* and thicker, suberised cell walls, pointing to their significant role in resistance. Ongoing research aims to explore these mechanisms further and apply findings to potato for improved disease resistance.

Our study has uncovered some novel findings, elucidated vital concepts regarding *Rs* and *Pi* resistance and requires further research to come forth with ultimate resistance utilising CRISPR/Cas knockouts of susceptibility genes.

5. NEW SCIENTIFIC RESULTS

- We characterised and identified the common and different defence responses between the two known *Rs* resistant varieties CG and CR including glutathione metabolism, phenylpropanoid pathways and lignification.
- We have generated several transgenic lines, namely, *PPO* knockout lines of ´Désirée' and 'Balatoni Rózsa', and *miR396* knockout lines of ´Désirée' and ´Botond'.
- We established that PPO is positively related to resistance to *Rs* and that the loss of PPO activity increases the susceptibility of plants despite increase of beneficial metabolites. We could not support the claim of *PPO* mutants being more resistant to *Pi*.
- We established that *miR396* mutant plants are delayed in symptom development to *Rs* and increased in susceptibility to *Pi* and that miR396 targets, the *MULTICYSTATIN147* and an *NBS-LRR* gene, have a role in these responses.
- We found *Rs* resistance in *SULTR* mutants in *Arabidopsis* making them good susceptibility gene candidates with increased suberisation along the root cortex and thick cell walls.

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