Final report

Exploring disease susceptibility genes to produce bacterium wilt-resistant potato (RESPOTA)
(NKFIH K132829)

Concise background of the project

Ralstonia solanacearum (*Rs*), the causal agent of bacterial wilt disease in an unusually wide range of host plants, including potato (*Solanum tuberosum*), is one of the most destructive phytopathogens that can dramatically reduce crop yields worldwide. Unraveling the defense mechanisms leading to bacterial wilt resistance is a prerequisite for biotechnological approaches to resistance breeding.

Pathogen resistance in a host plant can principally be improved with two intertwining strategies: (i) by boosting mechanisms for pathogen recognition (such as various receptor *R*-genes) and the subsequently induced defense responses, and/or (ii) by preventing the pathogen from exploiting vulnerable holes within those defense mechanisms. Spontaneous or directed mutations in the host's „susceptibility" genes (or *S*-genes) or modulating their expression can indeed block pathogen invasion and thus restore plant resistance to microbial pathogens.

Three main molecular pathways have been associated with *S*-genes: (i) basic compatibility, which assists in host recognition and penetration; (ii) sustained compatibility, which is required for pathogen proliferation and spread; and (iii) negative regulation of immune signals. The *S*-gene-mediated disease resistance can be pathogen-specific when the impaired pathway is implicated in the pre-penetration, penetration, or post-penetration requirements of a certain pathogen. However, *S*-gene-mediated disease resistance can also be of a broad spectrum when a gene is universally required for the successful pathogenicity of diverse pathogenic microbes.

This concept prompted us to test the effect of a wide range of silenced or mutated (edited) *S*-gene candidates on bacterial wilt resistance specifically for the generation of prototype potato lines and the dissection of the underlying molecular mechanisms. Furthermore, prospective prototypes may also confer resistance to other pathogens, which we are currently testing for the late blight disease (caused by *Phytophthora infestans*).

The main objectives of the project were as follows:

1. Identification of novel *S*-gene targets in potato
2. Discovering new mechanisms in potato for resistance to *Ralstonia solanacearum*
3. Bacterial aspects of the potato-*Ralstonia* interaction

The results and progress of the project will be reviewed here in the order of the above objectives.

1. Identification of novel *S*-gene targets in potato

Based on our data mining and evidence from published studies, several candidate genes were selected for CRISPR/Cas-mediated targeted mutagenesis (**Table 1**) to test their efficacy in conferring resistance to the *Rs* pathogen.

Table 1. Summary of the main results obtained with target genes in this project

Target gene encoding	**Cultivar(s)**	**No. of mutant lines**		**Comment**
		Rs-inoculated	Altered	
Polyphenol oxidase (*Pot32*)	'Désirée', 'Balatoni Rózsa'	2-2	2, 1	Increased susceptibility
miRNA396	'Désirée', 'Botond'	4-4	2, 2	Delayed symptom development
DMR6-like oxygenase 1	'Botond'	4	2	Delayed symptoms in first tests
miRNA159a	'Botond'	3	3	Increased susceptibility in first tests
Sulfate transporter 3;1	*A. thaliana* 'Columbia'	1	1	Very strong resistance in all tests
DND1 (cyclic nucleotide-gated ion channel)	'Désirée'	3	?	No confirmed alteration of resistance
Coryne protein kinase	'Botond'	3	None	No alteration of resistance
WRKY27-1 transcription factor	'Balatoni Rózsa'	6	None	No alteration of resistance
WAT1-related protein	'Balatoni Rózsa'	None	NA	Not applicable
Phytosulfokine receptor	'Balatoni Rózsa'	None	NA	Not applicable

Much of the resistance tests are still in progress, therefore only the results currently under publication (*PPO* and *DND1*) are summarized below (see points 1.1 and 2.2). Work on the microRNA gene *miR396* and the potato ortholog of the *A. thaliana* sulfate transporter (*SULTR*) 3;1 are more promising and will be completed in the near future.

1.1. Polyphenol oxidases (Jose et al., in review)

The browning enzymes, polyphenol oxidases (PPOs) are members of the plant immune system and oxidize phenolic compounds depriving the pathogen of consumable metabolites and leading to the production of quinones, melanins, and cell wall lignification. However, the consequent discoloration is esthetically undesirable and some reports address even the role of PPOs in resistance against pathogens. We aimed to improve the commercial quality of potato by decreasing tuber browning while establishing and validating the relationship between PPO activity and resistance to *Rs*.

The tuber- and root-specific *Pot32 PPO* gene was knocked out in potato cultivars ‘Désirée’ and ‘Balatoni Rózsa’, which resulted in no or significantly decreased browning of their tubers (**Figure 1**) and roots (not shown). The mutant lines showed an increased susceptibility to *Rs*. *PPO*-knockout also resulted in widespread metabolic changes primarily in the roots. Higher levels of phaseic acid and lower levels of vanillic acid possibly play a significant role in the increased susceptibility of the edited lines to *Rs*. Interestingly, reduced PPO activity in the mutant lines was also accompanied by an increased level of hormones jasmonic acid and gibberellic acid.

These results establish the positive relationship between PPO activity in potato and its resistance against *Ralstonia*.

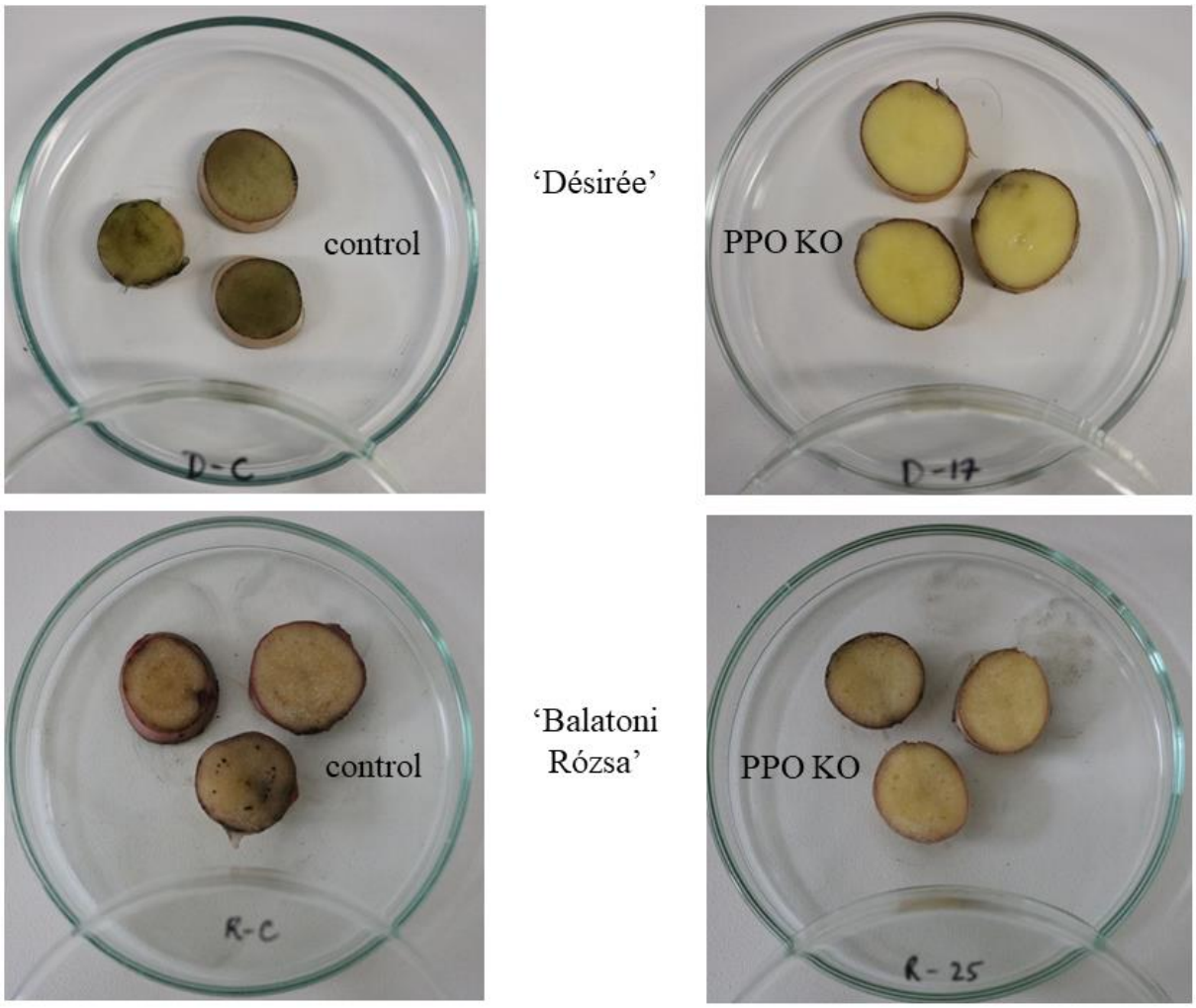

Figure 1. Reduced browning in potato tubers of 'Désirée' and 'Balatoni Rózsa' *PPO*-mutated (PPO KO) lines after a 2-h incubation at 30°C.

2. New mechanisms in potato for disease resistance

2.1. In Rs-*resistant germplasm resources* (published by Jose et al. 2022)

Resistance to *Rs* in the field has been reported only in a few potato landraces and cultivars. Our *in vitro* inoculation bioassays (see point 3.2 below) confirmed that the cultivars 'Calalo Gaspar' (CG) and 'Cruza 148' (CR) are resistant to *Rs* infection. Comparative transcriptome analyses of CG and CR roots, as well as of the roots of an *Rs*-susceptible cultivar, 'Désirée' (DES), were carried out two days after *Rs* inoculation (2 dpi), in parallel with their respective non-inoculated controls. In CR and DES, the upregulation of chitin interactions and cell wall-related genes was detected. The phenylpropanoid biosynthesis and glutathione metabolism pathways were induced in CR as confirmed by high levels of lignification (**Figure 2**). At 6 dpi, the extent of lignification was not changed in CG (**Figure 2D**), whereas it was enhanced over the whole stele in CR (**Figure 2E**) and even more strikingly in the xylem of DES (**Figure 2F**). The limited lignification induction in DES may not have been sufficient to prevent *Rs* invasion (**Figure 2F**). At the same time point, *Rs* infection greatly increased the concentrations of chlorogenic acid and quercetin derivatives in CG roots as detected via ultra-performance liquid chromatography-tandem mass spectrometry. Characteristic increases in the expression of MAP kinase signaling pathway genes and the concentrations of jasmonic, salicylic, abscisic, and indoleacetic acids were measured in DES roots. These results indicate different *Rs* defense mechanisms in the two *Rs*-resistant potato cultivars and a distinct response to *Rs* inoculation in the susceptible cultivar.

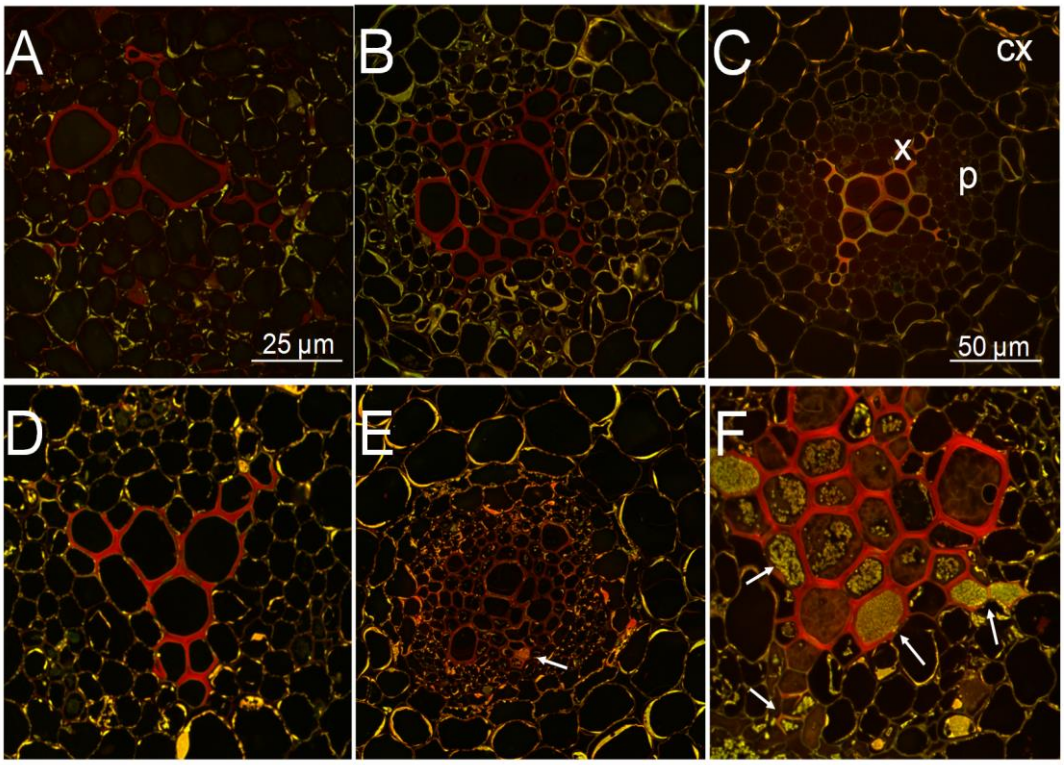

Figure 2. Root cross-sections from control (A-C) and *Rs*-inoculated (D-F, 6 dpi) potato plants stained with safranin for lignins.
(A) and (D) 'Calalo Gaspar', (B) and (E) 'Cruza 148', (C) and (F) 'Désirée' control. cx, cortex; p; parenchyma; x, xylem; arrows, *Rs* bacteria.

2.2. In DND1-*silenced plants* (Bánfalvi et al., in preparation)

DEFENSE NO DEATH 1 (DND1) is a cyclic nucleotide-gated ion channel protein. It was shown that silencing of the *DND1* gene in potato leads to resistance to major fungal diseases like late blight, powdery mildew, and gray mold. Our initial experimental data indicated the role of DND1 in *Rs* resistance, too. However, *DND1* silencing can also reduce plant growth and cause leaf necrosis. To learn the molecular events behind the pleiotropic effect of *DND1* downregulation in potato, metabolite and transcriptome analysis were performed on three *DND1*-silenced lines of the cultivar 'Désirée'. A massive increase in the salicylic acid content of leaves was detected. Concentrations of jasmonic acid and chlorogenic acid and their derivatives were also elevated. The expression of 1866 genes was altered in the same way in all three *DND1*-silenced lines including genes involved in synthesizing secondary metabolites. Activation of several leaf rust, late blight, and other disease resistance genes as well as induction of pathogenesis-related genes was detected (**Table 2**) which, in conjunction with the elevated level of secondary metabolites, may contribute to the disease resistance of *DND1*-silenced lines. WRKY and NAC transcription factor families were upregulated, whereas basic helix–loop–helix (bHLH) expression was downregulated indicating their central role in the transcriptome changes. These results suggest that the maintenance of a constitutive defense state leads as a cost to the reduced growth of *DND1*-silenced potato plants.

Table 2. Some differentially expressed resistance genes in the leaves of three *DND1*-silenced lines (DND1-5, DND1-8, and DND1-17) compared to the control 'Désirée' leaves

DND1-5	DND1-8	DND1-17	Gene function	Upregulated
5.340709	5.313257	4.738854	TMV resistance protein N	
1.714354	1.893802	1.687573	TMV resistance protein N	
1.670808	1.897231	1.711697	TMV resistance protein N	
1.809483	1.658407	1.67387	TMV resistance protein N	
1.656592	1.713184	1.572405	TMV resistance protein N	
1.73163	1.73163	1.204582	TMV resistance protein N	
1.377183	1.282032	1.348671	TMV resistance protein N	
1.217233	1.258855	1.322936	TMV resistance protein N	
				Downregulated
-0.58349	-0.58763	-0.62739	Hypersensitive-induced response protein 1; HIR1	
-0.76825	-0.80305	-0.86986	Downy mildew resistance 6; DMR6	
-0.92729	-0.94126	-0.95678	Pathogenesis-related leaf protein 4; P4	

Color intensities indicate quantitative differences in the log2-foldchanges of gene expression

3. Bacterial aspects of the potato-*Ralstonia* interaction*

For testing the *Rs* resistance of the developed potato prototypes a reliable bioassay is required both *in vitro* as well as *in vivo*. To this end, an *Rs* strain expressing the *GFP* reporter gene was first developed for the convenient monitoring of bacterial movement within the infected host. Next, the conditions of bacterial inoculation were set in culture plates and soil followed by temporal monitoring of symptom development.

3.1. Generation of test bacterial strain

The *Rs* strain UW551 (race3, biovar2; phylotype IIB, sequevar 1), a wild-type isolate from geranium, is highly virulent on potatoes and is one of the standards for bacterial wilt studies. This strain was transformed via electroporation with the pDSK-GFPuv plasmid (**Figure 3**), containing the mutant green fluorescent protein (GFP) reporter gene *GFPuv* (optimized for fast and bright fluorescence when excited by UV light) under the control of the constitutive chloroplast *psbA* (photosystem II) gene promoter. Colonies of UW551 transformants, containing the plasmid pDSK-GFPuv, were selected on CPG medium (casamino acid 1 g · L^{-1}, peptone 10 g·L^{-1}, glucose 5 g·L^{-1}, agar 17 g·L^{-1}, pH 6.5) supplemented with 30 mg·L^{-1} kanamycin and identified by fluorescence under long-wavelength UV light.

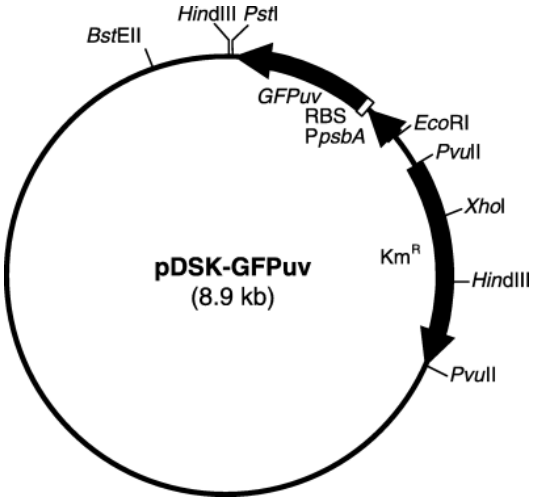

Figure 3. Schematic diagram of the plasmid pDSK-GFPuv which can express the green fluorescent protein variant GFPuv at high levels directed by the constitutive promoter *psbA* (*PpsbA*) and a ribosomal binding site (RBS) from *T7 gene10* (Wang et al., 2007).

3.2. Rs *inoculation and symptom development (bioassay)*

In vitro (plate)

Seven to ten days old rooted plantlets were transferred into rectangular Petri dishes containing RM medium (MS medium without vitamins) only in the lower half of the plates, which was covered with a folded sterile filter paper to separate the roots from the medium. The Petri dishes, containing four to six plants, were placed in a vertical position and incubated at a constant 24°C with a 16-h photoperiod at a light intensity of 45-95 µmol·m^{-2}·s^{-1} for an additional 10-12 days before inoculation. In general, 20 plantlets per accession were tested for *Rs* resistance.

* Parts of this work have been published in Jose et al. (2022).

The test bacteria were suspended at a concentration of 5-7 × 10^8 cells · mL^{-1} ($O.D._{600}$ = 0.8) in sterile deionized water. For inoculations, the plant roots were wounded by sterile scalpels approximately 1 cm above their tip and inoculated via pipette with 350 µL of bacterial suspension per plant. The rate of infection was observed at 1, 5, 7, 9, 12, 15, 19, and 21 days post-inoculation (dpi) in all the accessions. Photos were taken in visible light to assess the evolution of disease symptoms, as well as under UV light to monitor the spread of GFP-expressing bacteria within the plants (**Figure 4**).

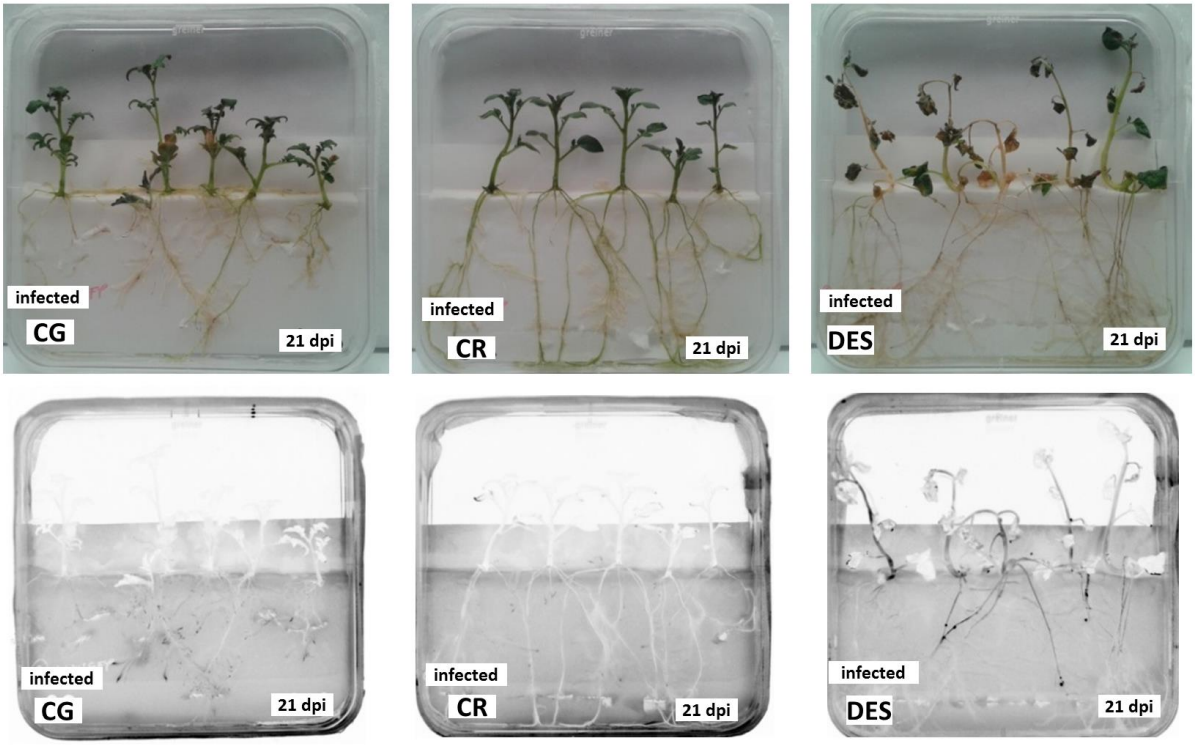

Figure 4. Visual evaluation of resistance to *Rs* inoculation (21 dpi) in the *in vitro* bioassay under visible light for the observation of wilting (upper row) and UV light for the detection of GFP-expressing *Rs* bacteria (lower row). CG, 'Calalo Gaspar'; CR, 'Cruza 148'; DES, 'Désirée'.

To verify this *in vitro* inoculation assay, a preliminary screening of nine reportedly *Rs*-tolerant potato accessions was performed in parallel with the *Rs*-susceptible commercial cultivar 'Désirée' (DES) as a control. Two cultivars, 'Calalo Gaspar' (CG) and 'Cruza 148' (CR), survived at 100%, even at 21 dpi, compared to 32% survival of the DES control. The two *Rs*-resistant cultivars remained green with minimal bacterial penetration, whereas the susceptible control wilted with the *Rs* bacteria distributed throughout the plants (**Figure 4**), and the non-inoculated control plants grew vigorously (data not shown).
The above results demonstrate that this *in vitro* bioassay can be applied to testing *Rs* resistance in potato.

In vivo (soil)
While the above *in vitro* test can be useful for the initial screening of candidate plants, a more realistic assay would be advantageous to evaluate and select promising lines before testing in

the field. Therefore, potted ‘Désirée’ plants were inoculated with the same *Rs* test bacteria as above by dropping 10 mL of UW551 suspension in the surrounding soil. Several parameters were tested: (i) bacterial concentration, (ii) incubation time according to symptom progression, and (iii) simultaneous soil loosening as an aid for better penetration and more even distribution of the inoculum (**Figure 5**).

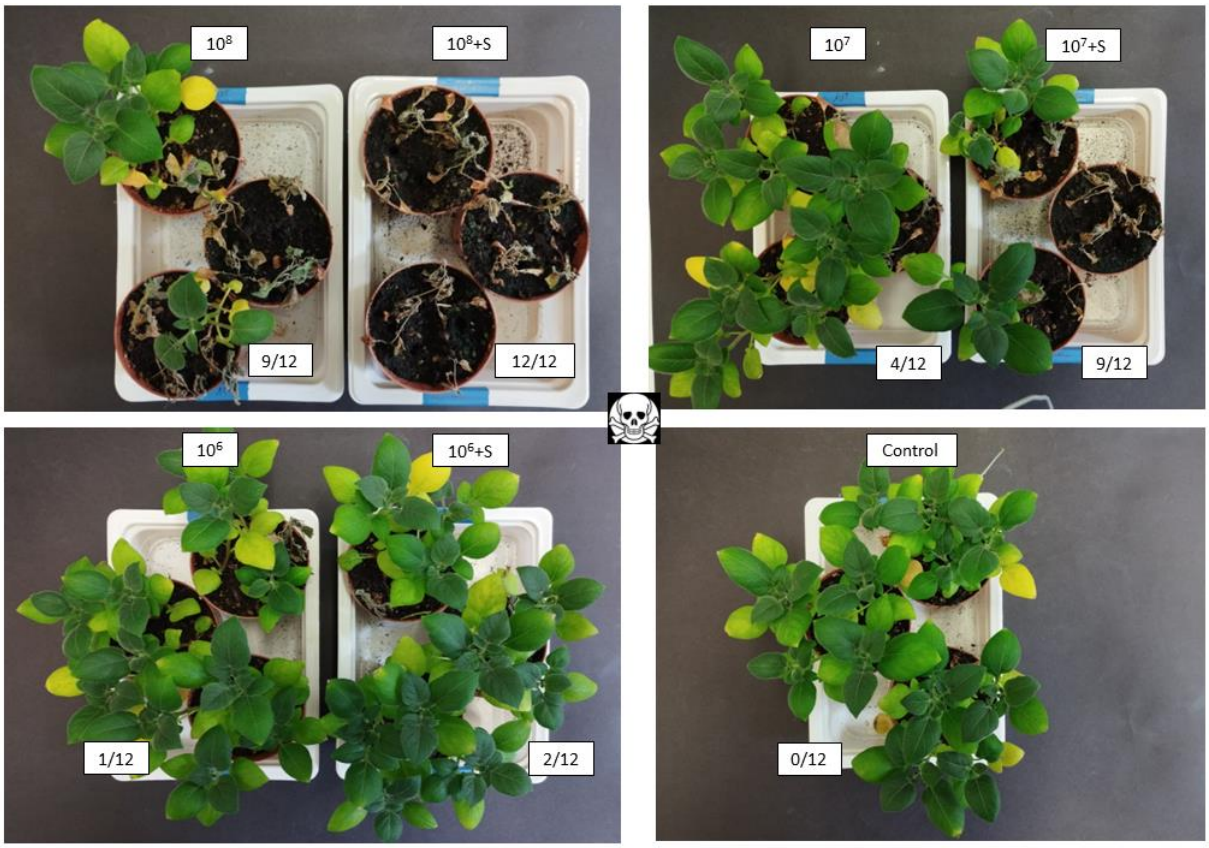

Figure 5. Visual evaluation of the effect of *Rs* inoculation (24 dpi) in the *in vivo* pot assay using three bacterial inoculum concentrations (10^6, 10^7, and 10^8 cells · mL^{-1} and a non-inoculated control) with (+S) or without soil disintegration in ‘Désirée’ plants. Death rates (☠) of 12 plants per treatment are shown below each tray (0-12/12).

The obtained results indicate a major and linear effect of the inoculum concentration and a similar influence of soil manipulation on the stringency of the assay. According to the data, 10^7 and 10^8 cells · mL^{-1} concentrations can be used as selective disease pressure in susceptible plants, which can be further enhanced by the soil treatment in putative resistant plants. While the first symptoms can be observed as early as 2 weeks after inoculation, their full development and plant wilting should be recorded after 3-4 weeks.

Reference

Wang K, Kang L, Anand A, Lazarovits G, Mysore KS (2007) Monitoring *in planta* bacterial infection at both cellular and whole-plant levels using the green fluorescent protein variant GFPuv. New Phytology 174: 212–223. DOI: 10.1111/j.1469-8137.2007.01999.x

Deviations from the original workplan during the project: A Hungarian *Rs* strain (1454/2016, available from the National Food Chain Safety Office, Plant Health National Reference Laboratory, Pécs) was originally planned to work with in the experiments. However, this and other Hungarian field isolates have not been characterized in detail, nor subjected to genome sequencing. Therefore, we chose the standard strain UW551 (see point 3.1 above) for use throughout the project.

List of scientific publications*

International and reviewed journals with impact factor (IF)

Jose J, **Éva C**, **Bozsó Z**, Hamow KÁ, Fekete Z, Fábián A, **Bánfalvi Z**, **Sági L** (2022) Global transcriptome and targeted metabolite analyses of roots reveal different defence mechanisms against *Ralstonia solanacearum* infection in two resistant potato cultivars. Frontiers in Plant Science 13: 1065419 (D1, IF: 6.627) DOI: 10.3389/fpls.2022.1065419

Jose J, Hamow KÁ, **Éva C**, Moncsek B, Kyrpa T, **Reinoso LG**, **Bozsó Z**, Bakonyi J, **Balázs E**, **Sági L** (2023) CRISPR-mediated knockout of *PPO* genes in potato causes differential resistance responses against bacterial wilt and late blight with widespread metabolic and hormonal changes. BMC Plant Biology, in review (D1, IF: 5.3)

Bánfalvi Z, Kalapos B, Hamow KÁ, **Jose J**, **Éva C**, Odgerel K, Karsai-Rektenwald F, Villányi V, **Sági L** Transcriptomic and metabolite changes in leaves of *DND1*-silenced potato plants. manuscript to be submitted in January 2024, Scientific Reports (D1, IF: 4.6)

Dissertation to be submitted for the degree of PhD

Jeny Jose (2024) Analysing disease susceptibility genes to generate potato plants resistant to bacterium wilt. Hungarian University of Agriculture and Life Sciences, Gödöllő (local defense: January 2024, public defense: May 2024)

International conferences (posters, presentations, abstracts in proceedings)

Éva C, **Jose J**, **Bozsó Z**, Moncsek B, Hamow KÁ, **Reinoso LG**, **Balázs E**, **Sági L** (2021) Edition of potato for reduced PPO activity confers resistance to *Ralstonia solanacearum*. Oral presentation at the 2nd PlantEd Conference on Plant Genome Editing, 20-22 September 2021, Lecce (Italy), Institute of Sciences of Food Production (ISPA–CNR) p. 38

Jose J, **Éva C**, **Bánfalvi Z**, **Bozsó Z**, Hamow KÁ, **Balázs E**, **Sági L** (2021) Molecular and metabolomic analysis of resistant potato varieties as a way forward to generate resistance to *Ralstonia solanacearum*. Oral presentation at the 2nd PlantEd Conference on Plant Genome Editing, 20-22 September 2021, Lecce (Italy), Institute of Sciences of Food Production (ISPA–CNR) p. 39

* All the listed publications have acknowledged support from the NKFIH K132829 project.

Jose J, **Éva C**, **Bánfalvi Z**, **Bozsó Z**, Hamow KÁ, **Balázs E**, **Sági L** (2021) Transcriptome and targeted metabolite analysis of resistant potato varieties as a way forward to generate resistance to *Ralstonia solanacearum*. Oral presentation at the International Conference on Plant Genomes in a Changing Environment, 18-20 October 2021, Wellcome Genome Campus, Hinxton (UK) (online)

Jose J, **Éva C**, **Bánfalvi Z**, **Bozsó Z**, **Balázs E**, **Sági L** (2022) Harnessing S-gene candidates for conferring resistance against *Ralstonia solanacearum* in potato. Oral presentation at the 3rd PlantEd Conference on Genome Editing in Plants, 5-7 September 2022, Dusseldorf (Germany), Book of Abstracts p. 36

Éva C, **Jose J**, **Bozsó Z**, Bakonyi J, Kyrpa T, Hamow KÁ, **Balázs E**, **Sági L** (2023) Studying potato resistance and susceptibility factors against pathogens with the use of genome editing. Oral presentation at the 4th PlantEd Conference, 18-20 September 2023, Porto (Portugal), Book of Abstracts p. 43

National conferences (posters, presentations, abstracts in proceedings)

2 oral presentations and 2 posters on the same topics as above

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People affiliated with the project are highlighted in **bold**.

Summary

We have collected and edited putative susceptibility (*S*-)genes by CRISPR/Cas-mediated mutagenesis to generate bacterial (*Ralstonia solanacaerum*) wilt-resistant prototypes in potato and to reveal the underlying defense mechanism(s). Besides the model cultivar 'Désirée', the targeted mutagenesis procedure was adapted to two commercial and popular Hungarian potatoes ('Balatoni Rózsa' and 'Botond'). In total, 10 *S*-genes have been tested so far: at least five of them exerted altered *Ralstonia*-reaction phenotypes, two-three genes were not effective, and with two genes no mutants were obtained. A comprehensive transcriptomic and metabolomic analysis of prototype plants with two edited *S*-genes (*PPO* and *DND1*) indicated widely different defense reactions, including the activation of plant hormone biosynthetic pathways and typical R-genes as well as pathogenesis-related *PR*-genes, respectively. For the evaluation of the obtained potato lines, bacterial inoculation assays were developed both *in vitro* (plates) and *in vivo* (soil), using a standard *Ralstonia solanacearum* strain engineered for convenient monitoring with the *GFP* reporter gene.

The prototype plants can be further investigated in the field and the accumulated experience can be applied in a broader assortment of commercial potato cultivars.

Összefoglalás (in Hungarian)

Lehetséges fogékonysági (*S*-)géneket gyűjtöttünk össze és szerkesztettünk CRISPR/Cas-technikával, hogy baktériumos (*Ralstonia solanacaerum*) hervadásra rezisztens burgonya prototípusokat állítsunk elő, és feltárjuk a mögöttes védekezési mechanizmusokat. A 'Désirée' modellfaján kívül két népszerű hazai burgonyafajtára ('Balatoni Rózsa' és 'Botond') adaptáltuk a célzott mutagenezis módszerét. Összesen 10 *S*-gént vizsgáltunk eddig: ebből legalább öt gén szerkesztése eredményezett megváltozott *Ralstonia*-reakciójú fenotípust, két-három gén nem bizonyult hatékonynak, és két génnel nem kaptunk mutánsokat. A prototípus növények átfogó transzkriptomikai és metabolomikai elemzése két szerkesztett *S*-gén (*PPO* és *DND1*) esetében igen eltérő védekezési válaszokat mutatott: a növényi hormonszintézis reakcióutak aktivációját, illetve a klasszikus rezisztenciagének, valamint a patogenezishez köthető *PR*-gének indukcióját. Az előállított burgonya alapanyagok értékeléséhez bakteriális bioteszteket is fejlesztettünk *in vitro* (lemezen) és *in vivo* (talajban) egyaránt, amelyekben a könnyű nyomon követéshez a *GFP* riporter génnel módosítottunk egy standard *Ralstonia solanacearum* törzset.

A prototípus növényeket szántóföldi körülmények között lehet tovább vizsgálni, és az eddig felhalmozott tapasztalatok egy szélesebb burgonya fajtakörben is alkalmazhatóak.