Thesis of Doctoral Dissertation

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The role of reactive oxygen species-producing enzyme systems in plant cell disease and resistance in the *Arabidopsis thaliana-Alternaria brassicicola* interaction

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1. PREVIOUS RESEARCH AND MAIN OBJECTIVES

Plants are exposed to biotic and abiotic stresses during their lifetime. Because of their sessile lifestyle, they can not excape from stress, so they have to develop different defensive mechanisms to overcome stress. Under adverse environmental impacts and pathogen attack, plants are producing high reactivity, oxygen free radicals and molecules, the so-called reactive oxygen species (ROS) to protect themselves. Rapidly accumulating reactive oxygen species cause a phenomenon called the oxidative burst (Daudi et al., 2012). ROS can damage pathogens in a number of ways, they cause metabolic inhibition, activate signaling pathways, suppress the nutrient uptake of the pathogens in the necrotic lesions triggered by plant tissue damage (Baker and Orlandi 1995). Inadequate material absorption through plant tissue destruction can be effective against several biotrophic pathogens. However, during the attack of necrotrophic fungi, such as the *Alternaria* species, plant tissue damage caused by the oxidative burst may actually support the colonization by the pathogen because these filamentous fungi are able to get nutrients from dead plant tissues (Glazebrook et al., 2005). Accumulation of plant extracellular reactive oxygen species derives from various enzyme systems, such as NADPH oxidases, cell wall peroxidases, and polyamine oxidases.

The most studied enzyme which is responsible for the extracellular ROS accumulation in plants is the family of NADPH oxidases (RBOHs, respiratory burst oxidase homologues) localized in the plasma membrane (Frederickson Matika and Loake 2013; Liu and He 2016). In addition to this important group of enzymes, the role and significance of cell wall peroxidases and polyamine oxidases against pathogens is not well known yet.

The objectives of my doctoral thesis were as follows:

1. Verify the role of AtPRX33 (*At3g49110*) and AtPRX34 (*At3g49120*) cell wall peroxidases in the extracellular oxidative burst and plant resistance in *Arabidopsis thaliana-Alternaria brassicicola* plant-pathogen interaction. In order to prove their role, we intended to use SALK T-DNA insertion mutant and transgenic plants to investigate the fungal symptoms in absence of cell wall peroxidases.
2. Our aim was to describe regulatory genes that play roles in the activity of PRX33 and PRX34.
3. Adaptation of a TRV-based virus-induced gene silencing system for *Arabidopsis* and using this approach in studying PRX33 and PRX34 functions.

4. Optimization of the electroporation method of *Agrobacterium tumefaciens (Rhizobium radiobacter)* bacteria to make gene technology applications with this organism more efficient.

2. MATERIALS AND METHODS

Used plants and pathogen for infection

Wild type *Arabidopsis thaliana cv*. Columbia (Col-0), SALK T-DNA mutant and transgenic *Arabidopsis* plants were used.

Alternaria brassicicola strain MUCL20297, isolated from cabbage (Thomma *et al.*, 1998), was used.

For the infection, 5-6 weeks plants were grown under 12h light/12h dark cycle and the leaf surfaces were spray-inoculated with 5 x 10^5 conidia/ml suspension.

Mutation and the effect of transgene by qPCR

Quantitative real-time PCR was used to verify the effect of T-DNA insertion in *prx33* SALK T-DNA mutant *Arabidopsis* plants and the diminished gene expression of *PRX33* and *PRX34* gene in transgenic *Arabidopsis*. Relative gene expression was compared with wild type Columbia *Arabidopsis* plants. 6-week-old plants were used for *prx33* mutant, H4 transformants and Columbia plants. Measurements were performed in 3 independent biological replications and for each biological repetition we used 3-3 whole *Arabidopsis* plants rosettes.

Virus-induced gene silencing (VIGS)

For the study of *PRX33* and *PRX34* genes, Tobacco Rattle Virus (TRV)-based gene silencing technique was used, which is an independent method besides the experiments with SALK T-DNA insertion mutants. The mRNA products of the two peroxidase genes (*PRX33, PRX34*) were reduced with virus-induced gene silencing in one plant simultaneously. For this purpose, pTRV1 and pTRV2 virus vectors (Arabidopsis Biological Resource Center, STOCK: CD3-1039) were modified and used.

The *PRX33* and *PRX34* (*TRV-GFP-PRX*) gene silencing plasmid was transformed into *Agrobacterium tumefaciens* MOG301 (Hood et al. 1993) by electroporation. For electroporation our own optimized protocol was used. *Agrobacterium* strains containing VIGS plasmids were agroinfiltrated into mGFP-ER green fluorescence protein-expressing *Arabidopsis* plants (Haseloff et al., 1997), which were grown under long day conditions. Agroinfiltration was performed based on the method of Burch-Smith et. al (2006) with minor changes.

The success of gene silencing on plants was verified by UV light and the degree of gene silencing was determined by real-time PCR where mRNA levels of target genes were measured after MOCK and *A. brassicicola* infection.

Determination of gene induction times after *A. brassicicola* infection

In order to find out when the cell wall peroxidases are activated after *A. brassicicola* infection, the transcript level of genes encoding *PRX33, PRX34* cell wall peroxidases were investigated in wild-type plants after infection with *A. brassicicola* at different times, 2, 6, 12, 24 and 48 hours after inoculation. For this examination we used whole rootless plants in 3 independent biological repetitions and 3-4 plants for each repetition. As control, the expression of the genes was measured in uninfected plants. Infected plants were ground with liquid nitrogen, RNA was isolated, cDNA synthesis was performed, and finally mRNA levels of the examined genes were detected by real time PCR.

Pathological tests

To quantify the symptoms of *A. brassicicola* infection, we used lesion assessment and compared the leaf area showing chlorotic and necrotic symptoms to the entire leaf surface. For this purpose, 30-30 middle-aged leaves were cut from all genotypes (*prx33, rbohd*, Col-0, *TRV-GFP-PRX* and *TRV-GFP*) 10 days after inoculation. The experiment was repeated two independent times. We took photographs of the cut leaves and the infected and total leaf surfaces were measured with ImageJ software. The ratio of the two values was calculated, where 1 represented the entire leaf surface.

Detection of hydrogen peroxide under infection was performed using DAB (3,3'-diaminobenzidine) staining. *A. brassicicola*-infected (2 days after inoculation) *Arabidopsis* plants were vacuum infiltrated with DAB dissolved in distilled water (1mg/ml), incubated in light for 2 hours, decolorized with plant clearing solution (80% ethanol, 20% chloroform, 0,15 % trichloroacetic acid) and mounted in 50% glycerol solution. Stained plants were examined with light microscopy.

The fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) was also used to detect hydrogen peroxide. Detached *Arabidopsis* leaves in the middle position (in positions 5 through 8) were vacuum infiltrated with 0.4 mM DCFH-DA in 10 mM sodium phosphate buffer (pH 7.4) as it was described by Bozsó *et al.* (2005). After a 10 minute incubation period in the dark, leaves were photographed under 302-365 nm UV light illumination by an AlphaImager Mini gel documentation system. For each genotype, 30 leaves (in the middle

position) were assayed and for each leaf an average pixel fluorescence intensity was calculated by the AlphaView software of AlphaImager Systems using its Multiplex Band Analysis function.

Determination of *A. brassicicola* biomass

To determine the biomass of *A. brassicicola*, quantitative DNA-based real-time PCR was used to confirm the growth of the fungus in *prx33* mutant and *TRV-GFP-PRX* gene silenced *Arabidopsis* plants compared to the wild type. In this assay DNA was extracted from the infected plants, which contained both plant and fungal DNA. Then, during the real-time PCR, the Ct values of the fungal ITS region were normalized with the Ct values of plant *At4g26410* reference gene.

Relation of gene expression and senescence

Transcript levels of *RbohD, PRX33* and *PRX34* genes from infected *Arabidopsis* Col-0 plants were measured in 3 different leaf ages. Sampling was performed 24 hours following infection. For control Col-0 MOCK-inoculated plants were used. Three independent biological replications were performed on 3 leaf ages, upper-young leaves, middle and lower leaves. For a measurement, 10-10 leaves were utilized from 15 different plants. To measure relative gene expression qPCR was used.

Optimization of *Agrobacterium* electroporation

Agrobacterium tumefaciens MOG301 (Hood et. al 1993) was used for the optimization of electroporation. Various amounts of plasmid DNA were added to a quantity of electrocompetent bacterial suspension to determine the lowest plasmid amount that is still sufficient for transformation. The electroporations were performed with 1, 5, 10, 25, 75 and 100 ng of pTRV2 (Arabidopsis Biological Resource Center, STOCK: CD3-1043).

To achieve the most efficient electroporation parameters we used different setup modes. Three voltages, 1, 2 and 2.5 kV / cm were tested with 200 Ohm resistance and 25 μF. In addition, the resistance was varied and the effect of 400 and 800 Ohm resistances was also investigated at a voltage of 2.5 kV / cm and 25 μF.

To increase the electroporation efficiency, multiple electroporations were carried out according to Mahmood et al. (2008). Once, twice or three times our samples were electroporated at 2.5 kV / cm at 200 Ohm resistance and 25 μF.

We compared the effect of two types of media, the SOC and LB liquid media.

Electroporated samples were incubated for 1, 2 and 3 hours at 27 ° C, testing the necessity of longer incubation time.

For each set-up mode, we used a negative control, without template. Two other strains of *Agrobacterium*, LBA4404 (Hoekema et al. 1983) and EHA105 (Hood et al., 1993) were also transformed by using the most suitable conditions. Estimated colonies were counted and transformation efficiency was calculated from the colony forming unit (CFU) / μg plasmid DNA data.

3. RESULTS

Effect of T-DNA insertion and transgene in *prx33* and H4 plants

Based on our results, the *prx33* mutant is a knock down mutant, in which the *PRX33* cell wall peroxidase gene expression is very low compared to the wild type.

AsFBP1.1 or H4 *Arabidopsis* (Bindschedler et al. 2006) is expressing the *Phaseolus vulgaris* peroxidase gene in antisense orientation. Based on our own findings, H4 transgenic *Arabidopsis* was not considered the most appropriate for our studies as the transcript level of the *PRX34* gene did not decrease statistically significantly compared to the wild type.

Silencing of *PRX33* and *PRX34* genes by VIGS method

We successfully constructed *PRX33* and *PRX34* silencing constructs and gene-silenced *Arabidopsis* plants by VIGS method. The effect of VIGS was verified by measuring mRNA levels in plants under control conditions and 24 hours after *A. brassicicola* infection. Due to gene silencing, the mRNAs of *PRX33* and *PRX34* genes were significantly lower in all conditions than in the *TRV-GFP* control plants.

Gene inductions under *A. brassicicola* infection

Our results showed that the two cell wall peroxidase genes were already activated at an early stage of the infection (6 hours after inoculation), and the gene expressions reached their maximum at 24 hours post inoculation. At 48 hours transcription of *PRX33/PRX34* genes was reduced.

Evaluation of *A. brassicicola* symptoms

Arabidopsis prx33, rbohd mutant plants and *TRV-GFP-PRX* gene-silenced *Arabidopsis* plants as well as their controls (Columbia, *TRV-GFP*) were infected with *A. brassicicola* and the symptoms were evaluated on the 10th day after infection. On *prx33* plants we found fewer necrotic lesions and less cell death than on the wild type. On *TRV-GFP-PRX* gene-silenced *Arabidopsis* (similarly to the *prx33* mutant), (*TRV-GFP*), apparently less symptoms were observed after the infection compared to its control. Results showed that reduced *PRX33*/*PRX34* gene activity led to reduced tissue necroses.

Detection of hydrogen peroxide production after *A. brassicicola* infection in *Arabidopsis* plants

It was found by DAB staining, that PRX33 cell wall peroxidase contributes to the production of extracellular hydrogen peroxide. In *prx33* plants a diminished level of DAB staining was detected. The number of stained cells was also decreased compared to wild type Columbia plants recorded 2 days after inoculation. Similar results were observed in *TRV-GFP-PRX* gene-silenced *Arabidopsis* plants, where less stained cells were found compared to the corresponding control plants.

Using DCFH-DA fluorescence dye, the amount of hydrogen peroxide was determined 2 days after *Alternaria* infection. In the *prx33* mutant and *TRV-GFP-PRX* gene-silenced plants a lower fluorescence signal was measured compared to the controls, indicating lower hydrogen peroxide formation.

Evaluation of the amount of *A. brassicicola* biomass

Fungal genomic DNA specific real time PCR assay was performed to quantify the amount of fungal biomass in colonized *prx33* mutant and *TRV-PRX-GFP* gene-silenced plants 10 day after infection with *A. brassicicola*. As a result of reduced gene function, the relative biomass of *A. brassicicola* was lower in *prx33* and *TRV-GFP-PRX* plants compared to the controls, therefore inhibition of fungal colonization occured.

Relation between *PRX* gene activity and the age of leaves

The basal expression (mock) of the three examined genes (*PRX33*, *PRX34* and *RBOHD*) was the highest in the regime of older leaves and the lowest in the youngest leaves. However, after infection with *A. brassicicola* non-senescent middle leaves showed the highest induction in *PRX33*, *PRX34* and *RBOHD* gene activity.

Genes interacting with *PRX33* and *PRX34*

In the group of 10 pre-selected SALK T-DNA mutant *Arabidopsis* lines 6 genotypes were selected which showed altered susceptibility to *A. brassicicola*. These were *ein2*, *nia2*, *nho1*, *jar1*, *mpk6* and *npr1* mutants. In these six mutants the transcript levels of the *PRX33* and *PRX34* genes were compared to the wild-type under MOCK and *A. brassicicola*-infected conditions. Mutants with impaired NHO1 and JAR1 activity exhibited perturbed *PRX33* and *PRX34* gene expression in contrast with wild type plants. In another approach, genes were

selected whose protein products were reported to establish a physical interaction with RBOHD (Geisler-Lee *et al.,* 2007; Jones *et al.*, 2014). In the case of two selected mutants, a significant difference was observed in the symptoms of *A. brassicicola* compared to the wild type. In these selected mutants the mutation affected the same locus, *At2g26330*, which encodes ERECTA (Quantitative Resistance to Plectosphaerella 1). Symptoms on the two *erecta* mutants were the same as the symptoms of the *rbohd* mutant 7 days after inoculation with *A.brassicicola*. Considerably enhanced cell death was observed on these leaves.

Optimization of *Agrobacterium* electroporation

We developed a refined *Agrobacterium* electroporation method that significantly reduces the electroporation time and costs compared to previous protocols without compromising in efficiency and reliability.

Our results show that the number of transformant cells was increased with higher resistance but after 400 ohms the increase was not significant anymore. The voltage of 2 kV / cm also improves the efficiency compared with 1.5 kV / cm.

We tested the effect of multiple pulses on transformation efficiency of MOG301 *A. tumefaciens* bacteria. The results imply that a second electroporation did not increase the number of transformant cells significantly.

Between two media, the composition of SOC medium is more appropriate for the growth of bacteria after electroporation than the LB medium.

The longer incubation time resulted more transformant colonies, but even 1 hour incubation time provided sufficient amount of transformant cell. Shorter incubation time can be enough for routine transformation work.

NEW SCIENTIFIC RESULTS

1. We have demonstrated that genes encoding PRX33 and PRX34 cell wall peroxidases are activated by *A. brassicicola* necrotrophic fungal infection in *Arabidopsis thaliana* plants and their expression reach the top at 24 hours after infection.
2. Due to decreased *PRX33* / *PRX34* gene activity, plants are more resistant to *A. brassicicola* infection. Functional *PRX33/PRX34* genes apparently increase the susceptibility of the plant to the infection of *A. brassicicola.*
3. During colonization of *A. brassicicola*, plants with low *PRX33* and *PRX34* activity produce less hydrogen peroxide than infected wild-type plants do.
4. For *PRX33/PRX34* cell-wall peroxidase and *RBOHD* NADPH oxidase encoding genes we could reveal altered gene activity in different developmental leaf stages under *A. brassicicola* infection and uninfected circumstances.
5. *JAR1* and *NHO1* genes have regulatory roles in the activity of *PRX33* and *PRX34* cell-wall peroxidase genes. We could describe the protein coding *ERECTA* gene as an interacting partner in the activity of RBOHD NADPH oxidase.
6. For *Agrobacterium tumefaciens* transformation we have developed an optimized electroporation protocol.

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4. CONCLUSIONS

Our knowledge about the role of PRX33 and PRX34 cell wall peroxidases are scarce, though many studies indicate their importance. In our work we examined the two class III peroxidases PRX33 and PRX34 and NADPH oxidase RBOHD in the same *Arabidopsis-Alternaria* pathosystem.

We presented microscopic images on the altered pattern of ROS accumulation which showed less hydrogen peroxide accumulation in *Arabidopsis* plants following inoculation with *A. brassicicola* when *PRX33* and *PRX34* transcript levels were suppressed. In contrast with the ROS-producer NADPH oxidase RBOHD, which regulates the spread of cell death in pathogen-infected *Arabidopsis* tissues and the lack of functional *RBOHD* gene triggers enhanced necrotic lesions, the diminished expression of PRX33 and PRX34 cell wall peroxidases generate resistance against *Alternaria*. Active PRX33 and PRX34 cell wall peroxidases contribute to the accumulation of hydrogen peroxide and promote the colonization process of the necrotrophic fungus *A. brassicicola*.

Senescence plays an important role in the activity of *PRX33* and *PRX34* genes. Different transcript levels of genes encoding extracellular ROS-producing proteins were found in younger, middle or older leaved. It is concluded that the activity of these genes depends on the age of the leaves also affecting virulence of the fungal pathogen. From several resistance-related genes *JAR1, NHO1* and *ERECTA* were selected, indicating their involvement in the PRX33, PRX34 and RBOHD signaling networks.

Within the frames of this research we also developed an electroporation protocol which makes the transformation of *Agrobacteruim tumefaciens* cells more efficient and faster.

5. SCIENTIFIC PUBLICATIONS RESULTING FROM THE DOCTORAL RESEARCH

Papers in journals with impact factors

Kámán-Tóth, E., Dankó, T., Gullner, G., Bozsó, Z., Palkovics, L., & Pogány, M. (2018). Contribution of cell wall peroxidase-and NADPH oxidase-derived reactive oxygen species to *Alternaria brassicicola*-induced oxidative burst in *Arabidopsis*. *Molecular Plant Pathology*. (in press) **IF 4,18**

Kámán-Tóth, E., Pogány, M., Dankó, T., Szatmári, Á., & Bozsó, Z. (2018). A simplified and efficient *Agrobacterium tumefaciens* electroporation method. *3 Biotech*, *8*(3), 148. **IF 1,49**

Pogány, M., Dankó, T., Kámán-Tóth, E., Schwarczinger, I. & Bozsó, Z. (2015). Regulatory Proteolysis in *Arabidopsis*-Pathogen Interactions. *International Journal of Molecular Sciences* 16:(10) pp. 23177-23194. **IF 3,25**

Papers in journals without impact factors

Kámán-Tóth Evelin, Palkovics László, Pogány Miklós (2016). *Alternaria* fajok által okozott kórfolyamatok élettana és molekuláris biológiája növényekben. *Növényvédelem* 52:(11) pp. 557-566.

Conference proceedings

Tóth E, Nagy VA. Bozsó Z, Pogány M (2015) Reaktív oxigén fajtákat termelő enzimrendszerek és az etilén kölcsönhatása *Arabidopsis* sejtek kórfolyamataiban. 61. Növényvédelmi Tudományos Napok Konferenciakiadványa, Budapest, p.51.

Tóth E, Nagy VA, Bozsó Z, Pogány M (2014) Reaktív oxigén fajtákat termelő alternatív enzimrendszerek szerepének vizsgálata *Arabidopsis* sejtek megbetegedésében és a rezisztenciában. 60. Növényvédelmi Tudományos Napok Konferenciakiadványa, Budapest, p. 111.

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