Title of reported project:

Induction of symptomless plant disease resistance by reactive oxygen species

Research background and main aims of the project

Plant disease resistance can be activated when a plant resistance (R) gene product recognizes – directly or indirectly – a specific pathogen gene product encoded by an effector gene (gene-for-gene resistance) (Flor, 1971; Jones and Dangl, 2006; Spoel and Dong, 2012). In "gene-for-gene" resistance plant cell and tissue death (necrosis) is usually associated with pathogen arrest at infection sites (hypersensitive response, HR) (Klement, 1982; Goodman and Novacky, 1994). However, plant resistance during an HR is often independent of cell/tissue death in several viral, bacterial and fungal infections (Yu et al., 1998; Bendahmane et al., 1999; Cole et al., 2001; Coll et al., 2010; Künstler et al., 2016). In fact, "gene-for-gene" resistance can be symptomless, e.g. in extreme resistance, a rapid, efficient resistance response of plant hosts operating against viruses like e.g. *Potato virus X* (PVX) (Bendahmane et al., 1999).

Reactive oxygen species (ROS, e.g. superoxide and hydrogen peroxide) have a dual function during plant disease resistance. In low concentrations, ROS transmit resistance signals, while in high amounts they may damage both the host plant and the pathogen (Levine et al., 1994; Torres et al., 2005; Hernández et al., 2016). Our previous research had shown that a macroscopically symptomless resistance can be elicited to *Tobacco mosaic virus* (TMV), if susceptible plants are treated with ROS-generating agents early (2 hours) after inoculation (Bacsó et al., 2011). Furthermore, our preliminary experiments (before starting the present project) suggested that enhanced superoxide accumulation occurs in leaves of a Hungarian cherry pepper cultivar (cv. Szentesi) that is resistant to symptoms of powdery mildew (*Leveillula taurica*) infection and superoxide accumulation / resistance could be transmitted to a susceptible sweet pepper cultivar (cv. Totál) by grafting (Király et al., 2013).

The main aim of this proposal was to investigate whether ROS accumulation can elicit rapid, efficient, symptomless plant disease resistance responses?

- Assessing the currently unknown role of early ROS accumulation in symptomless, extreme resistance to viruses.

- Investigating whether superoxide accumulation indeed plays a pivotal role in symptomless resistance of cherry pepper to powdery mildew?

Results

Contribution of superoxide and other ROS to the symptomless extreme resistance to *Potato virus X* (PVX) determined by the *Rx1* resistance gene in tobacco

In potato a symptomless extreme resistance (ER) to *Potato virus X* (PVX) is known to be conditioned by the *Rx1* and *Rx2* resistance genes (Bendahmane et al., 1999, 2000). It seems that the resistance conditioned by *Rx* genes to PVX occurs so rapidly that the PVX coat protein cannot attain a concentration sufficient to elicit localized cell/tissue death (HR) (Bendahmane et al., 1999). Although the biochemical mechanism of symptomless (extreme) resistance has been unknown, Bendahmane and coworkers (1999) proposed the possible involvement of reactive oxygen species (ROS).

To investigate the role of ROS (superoxide /$O_2^{\cdot-}$/, hydrogen peroxide /H_2O_2/, hydroxyl radical /$OH^{\cdot}$/) in ER we were using transgenic tobacco (*Nicotiana tabacum*, cv. Samsun NN) expressing the *Rx1* resistance gene of potato as a model (Bendahmane et al., 1999).

Verifying expression of the Rx1*resistance gene in transgenic tobacco*

The transgenic tobaccos mentioned above express *Rx1* by its own promoter and display ER to PVX (Bendahmane et al., 1999), as opposed to nontransformed control (wild type) tobaccos which confer rapid replication and systemic spread of PVX, accompanied by occasional mild symptoms (weak yellowing and/or mosaic). First we have verified expression of the *Rx1* transgene in our cv. Samsun NN Rx1 line (a kind gift of Dr. Peter Moffett, University of Sherbrooke, Canada). As expected, monitoring *Rx1* expression at different time points following PVX inoculation (by real time RT-qPCR) no signal was detected in nontransformed plants (using specific primers described by Ahmadvand et al., 2012: St1Rx1RT-FW and St1Rx1RT-REV). Interestingly however, in *Rx1* tobaccos *Rx1* gene expression was also manifested in healthy (uninoculated) and mock-inoculated (i.e. exposed to mechanical stress) plants but the rate of increase in gene expression was lower than in PVX-inoculated tobacco where *Rx1* expression was significantly increased already 6 hours after virus inoculation (HAI) **(Figure 1)**. It seems that the *Rx1* resistance gene may be induced in response to different stresses but it is induced most rapidly during PVX infection, initiating a possible first step in the elicitation of symptomless ER.

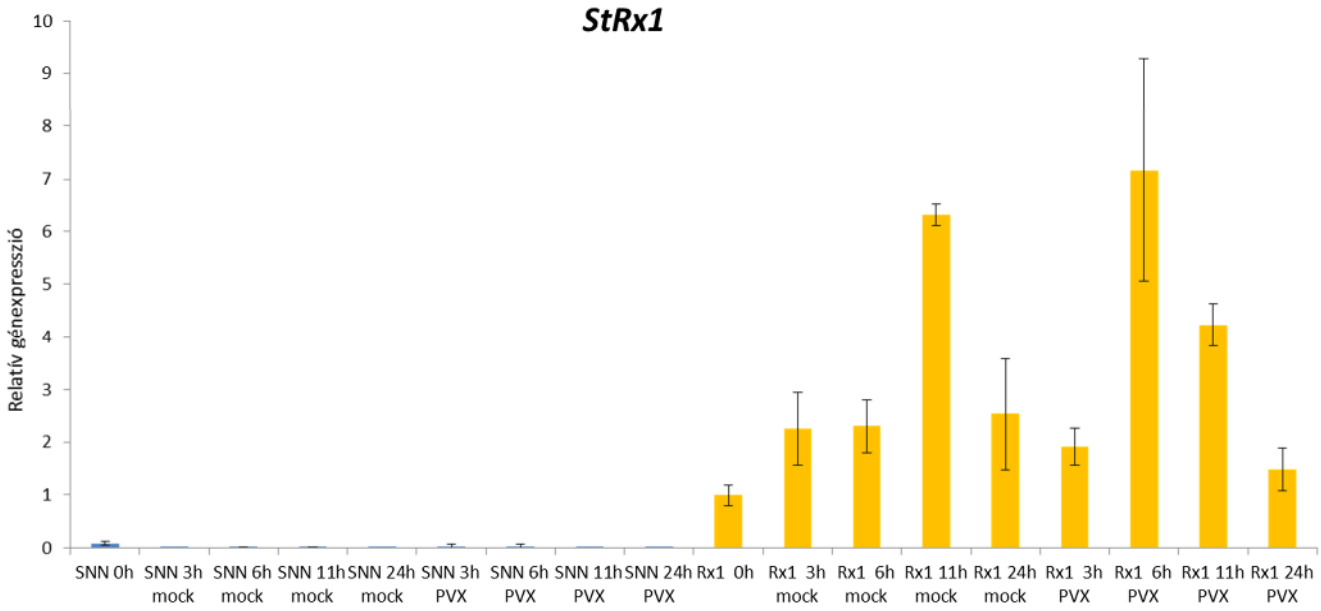


Fig. 1 Verifying expression of the *Rx1* resistance gene in transgenic tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and control plants not containing *Rx1* (*N. tabacum* cv. Samsun *NN*) by quantitative RT-PCR (relative gene expression) at different time points following inoculation of *Potato virus X* (PVX*)* and control („mock") inoculation (3, 6, 11 and 24 hours). 0 h: uninoculated plants. Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference.

Demonstrating the operation of extreme resistance to PVX in transgenic tobacco expressing the Rx1 *gene*

During our investigations we have used the PVX isolate Ny (provided by Dr. Zsolt Polgár, University of Pannonia, Keszthely, Hungary) that is known to elicit *Rx1*-mediated ER in potato (Ahmadvand et al., 2012). We checked whether PVX-Ny can induce ER in *Rx1* tobacco. Inoculation with PVX-Ny suggested that ER is elicited, since no macroscopic symptoms developed in inoculated or systemic leaves. To confirm the presence of ER, we checked PVX-Ny levels within the first 24 hours and 2 and 5 days after inoculation (DAI) in inoculated leaves of both *Rx1* and susceptible wild type tobacco by RT-qPCR using primers homologous to the PVX coat protein (CP) gene (primers PVX5969-FW and PolyT2-REV, provided by Éva Pájtli, Corvinus University, Hungary). Expression of the *PVX CP* gene significantly declined in resistant (*Rx1*) plants from 6 HAI onwards, while in susceptible plants virus titers continuously increased. By 5 DAI PVX levels had been ca. one order of magnitude lower in resistant (*Rx1*) tobaccos, as compared to susceptible plants **(Figure 2)**.

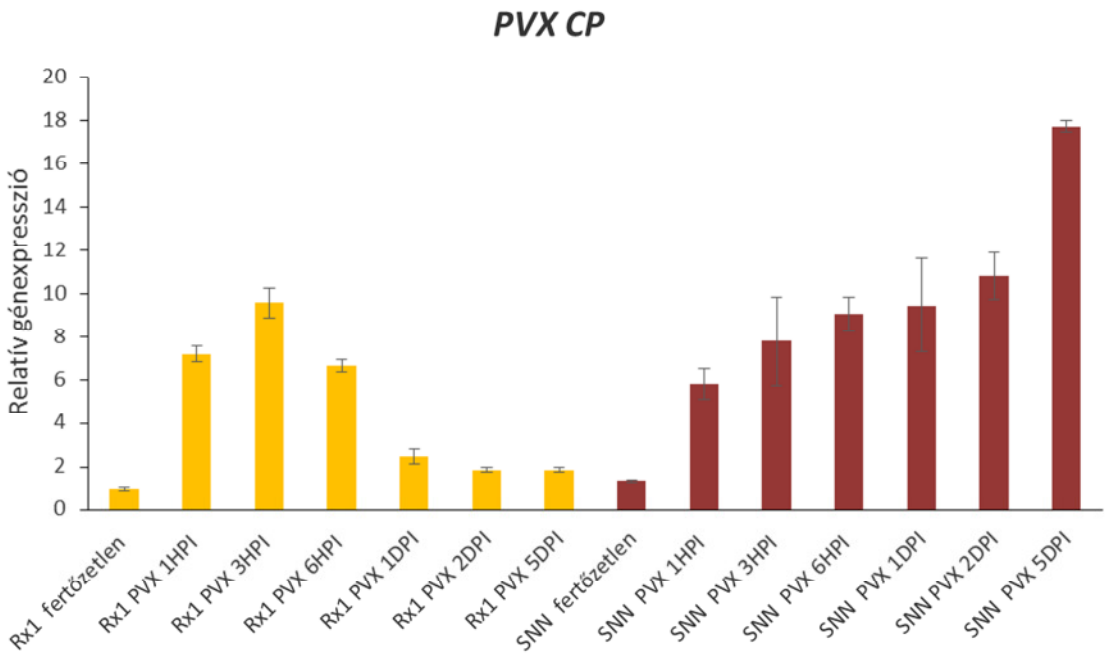


Fig. 2 Accumulation of *Potato virus X* (PVX) in tobacco expressing the extreme resistance gene *Rx1* (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and in susceptible control plants (*N. tabacum* cv. Samsun *NN*), at different time points following PVX inoculation (1, 3 and 6 hours, 1, 2 and 5 days) as assayed by quantitative RT-PCR (*PVX CP* relative gene expression). Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference.

Assaying early accumulation of superoxide by NBT tissue staining in Rx1 *tobacco displaying extreme resistance to PVX*

To gain insight into the possible role of ROS in ER, we monitored superoxide ($O_2^{\cdot-}$) and H_2O_2 accumulation in wild type and *Rx1* tobacco leaves at various timepoints (1, 2, 3, 6 and 48 hours) after PVX inoculation. Nitro blue tetrazolium (NBT) and diamino benzidine (DAB) tissue staining was used to detect superoxide and H_2O_2, respectively. We found that superoxide is closely correlated with ER, showing enhanced accumulation in *Rx1* tobacco already at 1 and 2 HAI, as compared to wild type **(Figure 3)**. In case of H_2O_2 the same difference was not so obvious, only ca. half of virus-infected *Rx1* plants displayed slightly elevated H_2O_2 in comparison to wild type (data not shown).

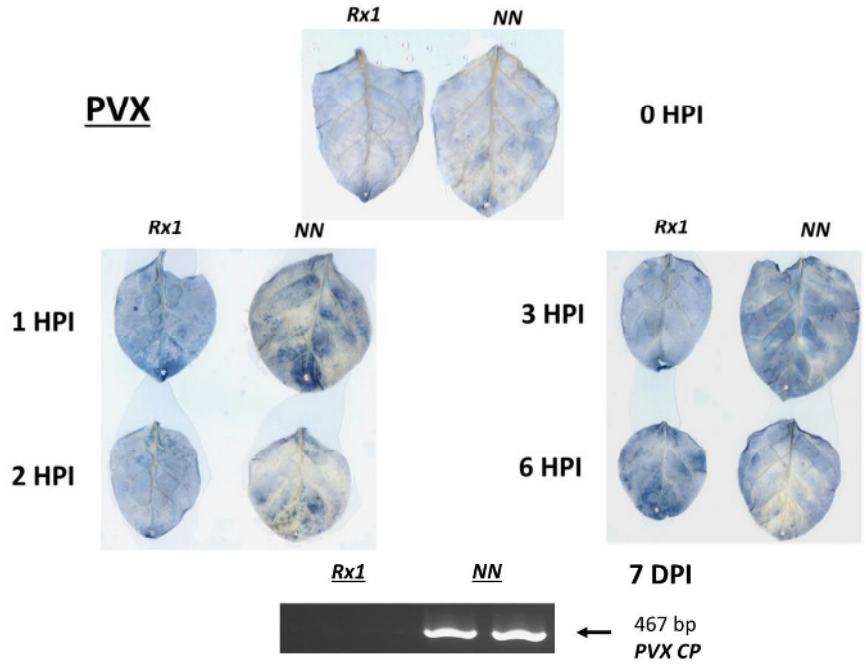


Fig. 3 Accumulation of superoxide ($O_2^{\cdot-}$) – indicated by the blue color – in tobacco expressing the extreme resistance (ER) gene *Rx1* (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and in susceptible control plants (*N. tabacum* cv. Samsun *NN*), at different time points following inoculation by *Potato virus X* (PVX) (1, 2, 3 and 6 hours) (0 HPI = uninoculated plants). Superoxide was detected by NBT (nitro blue tetrazolium) tissue staining. The lower panel demonstrates the operation of extreme resistance to PVX in *Rx1* tobacco as assayed by semiquantitative RT-PCR (see also Figure 2).

In order to detect more subtle differences in superoxide accumulation we have quantified NBT tissue staining results by an image analysis software (Image J, available free at https://imagej.nih.gov/ij/)). These assays indicate that by 48 HAI (2 days after PVX imoculation) superoxide accumulation in *Rx1* tobacco leaves displaying ER declines to levels typical of uninoculated and susceptible plants **(Figure 4)**. Interestingly, however, superoxide accumulates within the first 6 hours of PVX infection during ER and PVX titers begin to sharply decrease only following this time point (compare **Figures 2** and **4)**. These experiments suggest that ROS accumulation, especially $O_2^{\cdot-}$, could directly contribute to the inhibition of the invading virus (PVX) during extreme resistance.

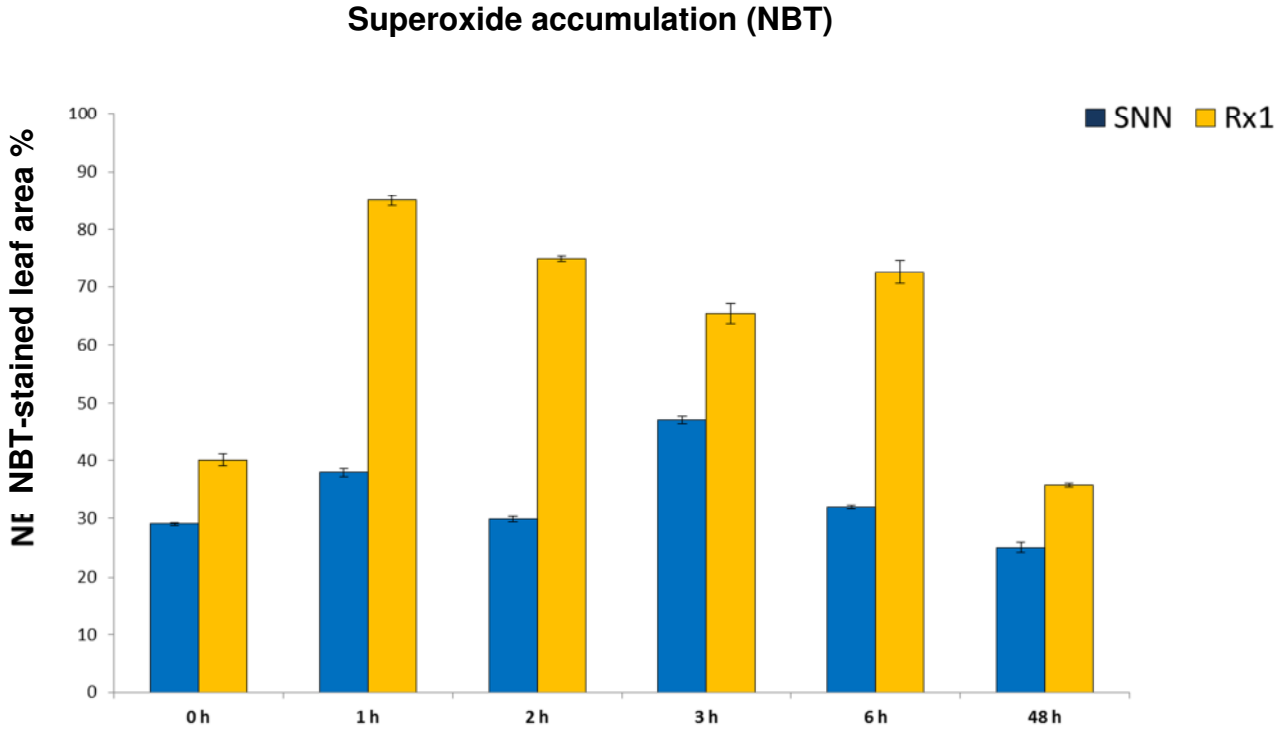


Fig. 4 Assaying superoxide ($O_2^{\cdot-}$) accumulation in tobacco expressing the extreme resistance (ER) gene *Rx1* (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and in susceptible control plants (*N. tabacum* cv. Samsun *NN*), at different time points following inoculation by *Potato virus X* (PVX) (1, 2, 3, 6 and 48 hours) (0 h = uninoculated plants). Superoxide was detected by NBT (nitro blue tetrazolium) tissue staining, quantification of NBT tissue staining was done by the image analysis software Image J.

NBT tissue staining is specific to superoxide in tobacco

In PVX-inoculated *Rx1* tobacco leaves displaying ER the enhanced NBT tissue staining indicating superoxide accumulation at 1 and 2 HAI **(Figures 3** and **4)** was significantly suppressed when leaves were infiltrated not only with NBT but also with superoxide dismutase (SOD, 3000 U/ml) and catalase (CAT, 5000 U/ml) **(Figures 5)**. SOD converts superoxide to H_2O_2 while CAT can degrade H_2O_2. Therefore, we could demonstrate that NBT tissue staining is indeed specific for superoxide in the PVX-infected tobaccos used by us.

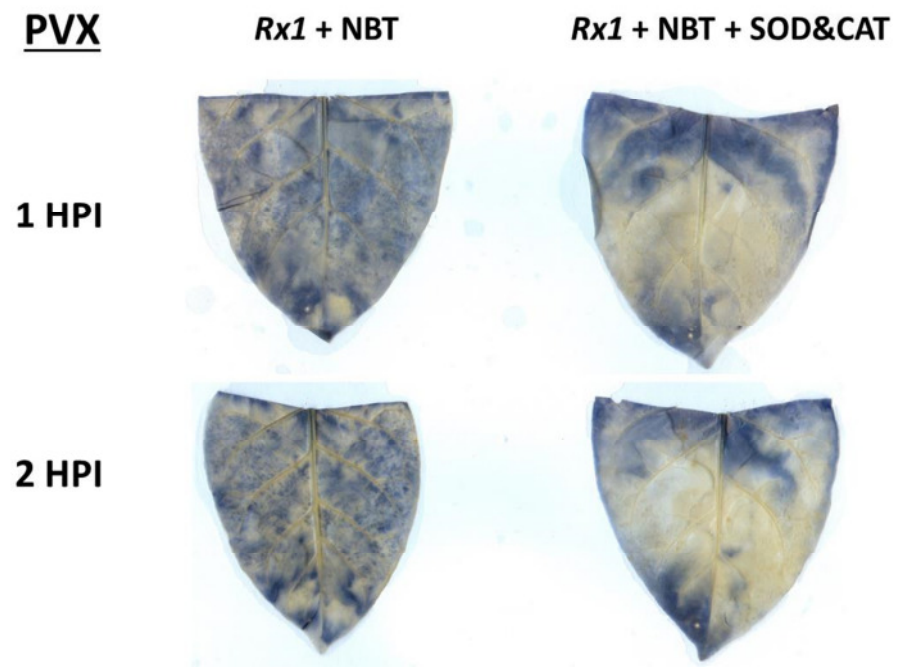


Fig. 5 NBT (nitro blue tetrazolium) tissue staining is specific for superoxide ($O_2^{\cdot-}$) in tobacco displaying extreme resistance (ER) to *Potato virus X* (PVX) (*Nicotiana tabacum* cv. Samsun *NN Rx1*). In order to detect superoxide accumulation 1 and 2 hours after PVX inoculation (HPI) leaves on the left were infiltrated with NBT, while leaves on the right were infiltrated with NBT, superoxide dismutase (SOD, 3000 U/ml) and catalase (CAT, 5000 U/ml).

Effect of infiltrating antioxidant enzymes (SOD and CAT) on the extreme resistance to PVX in Rx1 *tobacco*

If superoxide (ROS) accumulation is indeed a key factor of ER, enhancing antioxidant capacity of the host by e.g. infiltration of antioxidants should reduce/eliminate the resistance to PVX in *Rx1* tobacco. Previously we have shown that infiltration of antioxidant enzymes (superoxide dismutase, SOD and catalase, CAT) can neutralize the effects of high ROS levels in TMV-infected tobacco (Király et al., 2008). Here we found that infiltration of these antioxidants to *Rx1* leaves immediately after PVX inoculation (SOD, 3000 U/ml and CAT, 5000 U/ml) can convert symptomless ER to HR, as indicated by the development of localized necrotic lesions resembling HR between 2 and 7 DAI in ca. 50 % of leaf halves infiltrated with SOD+CAT (no HR appeared in buffer-infiltrated control leaf halves) **(Figures 6** and **7)**.

In order to show that increased PVX levels (i.e. reduced resistance) are indeed associated with the phenotypic conversion of ER to HR in *Rx1* tobacco, we have assessed PVX accumulation in virus-infected and SOD+CAT infiltrated leaves by RT-qPCR with primers homologous to the PVX coat protein (CP) gene. We found that virus accumulation in leaves that display HR type symptoms is at least twice than that in leaves not displaying HR, especially evident at the time of full HR-development (7 DAI) **(Figure 6)**. These experiments demonstrated that ROS degradation by elevated antioxidant levels may significantly suppress *Rx1*-mediated extreme resistance (ER) to PVX, resulting in the development of the slower HR type of resistance that confers a less efficient inhibition of virus replication (see Bendahmane et al., 1999). In summary, our results point to a pivotal role of superoxide (ROS) in the development of the rapid, symptomless ER response.

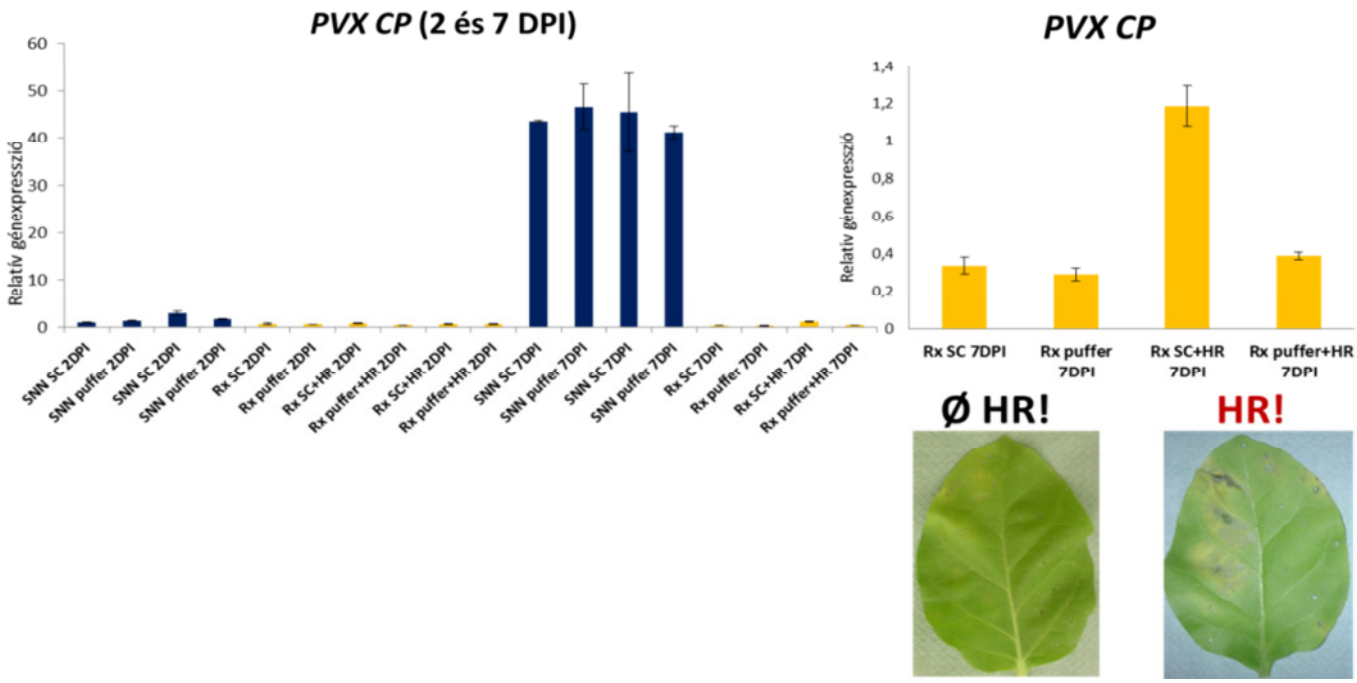


Fig. 6 Effect of antioxidant enzymes on extreme resistance (ER) to *Potato virus X* (PVX) in tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) 2 and 7 days after PVX inoculation (DPI). SC = infiltration of left leaf halves with superoxide dismutase (SOD, 3000 U/ml) and catalase (CAT, 5000 U/ml); buffer = infiltration of right leaf halves with 10 mM potassium phosphate (pH=7,8). Columns designated as „SC" and „buffer" represent PVX accumulation in the left and right halves of inoculated leaves. The last 4 columns of the left panel are enlarged on the right. HR = symptoms resembling hypersensitive, localized necrosis (HR) in left halves of inoculated leaves. Rx and SNN: extreme resistant and susceptible tobacco lines. Accumulation of PVX was monitored by RT-qPCR (*PVX CP* relative gene expression). Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference.

We have also assayed expression levels of a resistance/stress marker tobacco gene (*NtPR-1a*) in the above mentioned plants. The purpose of these experiments was to clarify if *NtPR-1a* expression is a marker of virus resistance during PVX-elicited, *Rx1*-mediated ER or rather a marker of cell/tissue death (HR) when this ER is suppressed by antioxidants. *NtPR-1a* transcript levels were 2-3 times higher in SOD+CAT infiltrated PVX-resistant plants, when visible HR-type symptoms (i.e. suppressed ER) developed, compared to leaves not displaying HR, as assayed at the time of HR-appearance (2 DAI) and a few days later (7 DAI). Negligible gene expression occurred in PVX susceptible (wild type) plants, regardless whether or not leaf halves were infiltrated with SOD+CAT **(Figure 7)**. These results imply that *NtPR-1a* expression is indeed a reliable marker of cell/tissue death (HR) during suppression of PVX-elicited, *Rx1*-mediated ER, but not a marker of intact, symptomless ER. Furthermore, our findings are in line with previous research showing that certain pathogenesis-related (PR) genes/proteins (e.g. PR-1a) are markers of HR-type cell/tissue death that accompanies virus resistance but do not play a functional role in the resistance response *per se* (Cutt et al., 1989; Linthorst et al., 1989).

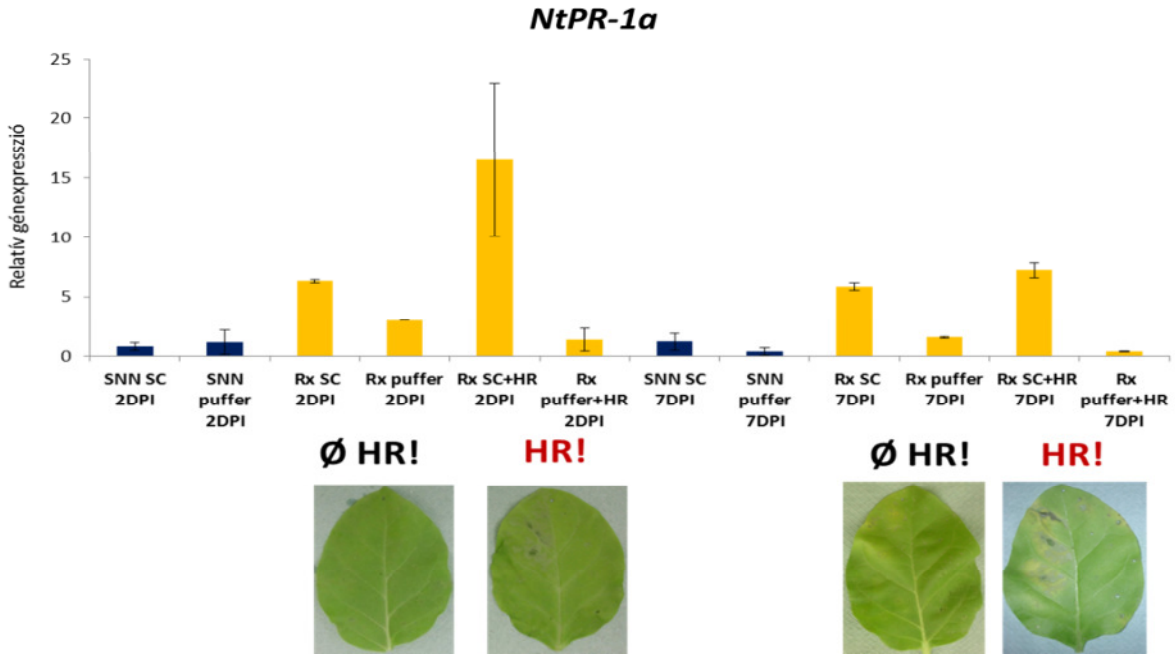


Fig. 7 Effect of antioxidant enzymes on extreme resistance (ER) to *Potato virus X* (PVX) in tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) 2 and 7 days after PVX inoculation (DPI): suppression of ER correlates with the development of HR-type symptoms and enhanced expression of the *NtPR-1a* gene (RT-qPCR, relative gene expression, normalized by using a tobacco actin gene (*NtAct*) as a reference). SC = infiltration of left leaf halves with superoxide dismutase (SOD, 3000 U/ml) and catalase (CAT, 5000 U/ml); buffer = infiltration of right leaf halves with 10 mM potassium phosphate (pH=7,8). Columns designated as „SC" and „buffer" represent *NtPR-1a* expression in the left and right halves of inoculated leaves. HR = symptoms resembling hypersensitive, localized necrosis (HR) in left halves of inoculated leaves. Rx and SNN: extreme resistant and susceptible tobacco lines.

Effect of inhibiting ROS-generation on the extreme resistance to PVX in Rx1 *tobacco*

Besides antioxidant (SOD+CAT) infiltration, we tested a different experimental approach to reduce *in planta* ROS-levels and suppress PVX-elicited symptomless ER in *Rx1* tobacco leaves. Overexpression of ferritin, an iron-binding protein in tobacco inhibits generation of a ROS, the hydroxyl radical ($OH^{\cdot}$), due to the unavailability of free Fe (Deák et al., 1999). Crossing tobacco lines overproducing an alfalfa ferritin gene (C8, F9) with *Rx1* tobacco resulted in F_1 generation plants where ferritin overexpression seems to suppress *Rx1*-mediated ER, as indicated by the appearance of local necrotic lesions (HR) by 5 days after PVX inoculation in more than 50 % of Nt cv. SR1 C8 ♂ x Nt cv. Samsun *NN Rx1*♀ and Nt cv. SR1 F9 ♂ x Nt cv. Samsun *NN Rx1*♀ F_1 tobaccos **(Figure 8)**. In order to prove that ER is indeed suppressed in these ferritin-overproducer hosts PVX levels were determined in virus-inoculated leaves by RT-qPCR. We found that ferritin-overproduction confers a significant suppression of *Rx1*-mediated ER. However, suppression of resistance (i.e. an increase in PVX levels) is evident only at 1-2 days after PVX inoculation, while virus levels sharply drop in later stages of PVX infection **(Figure 9)**.

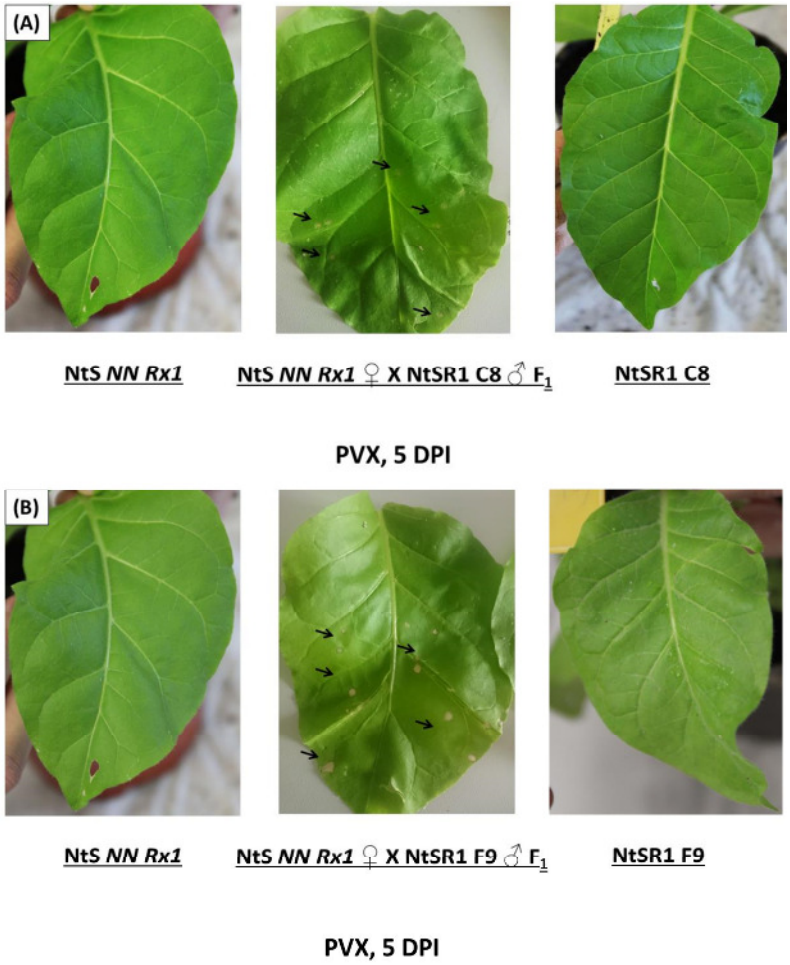
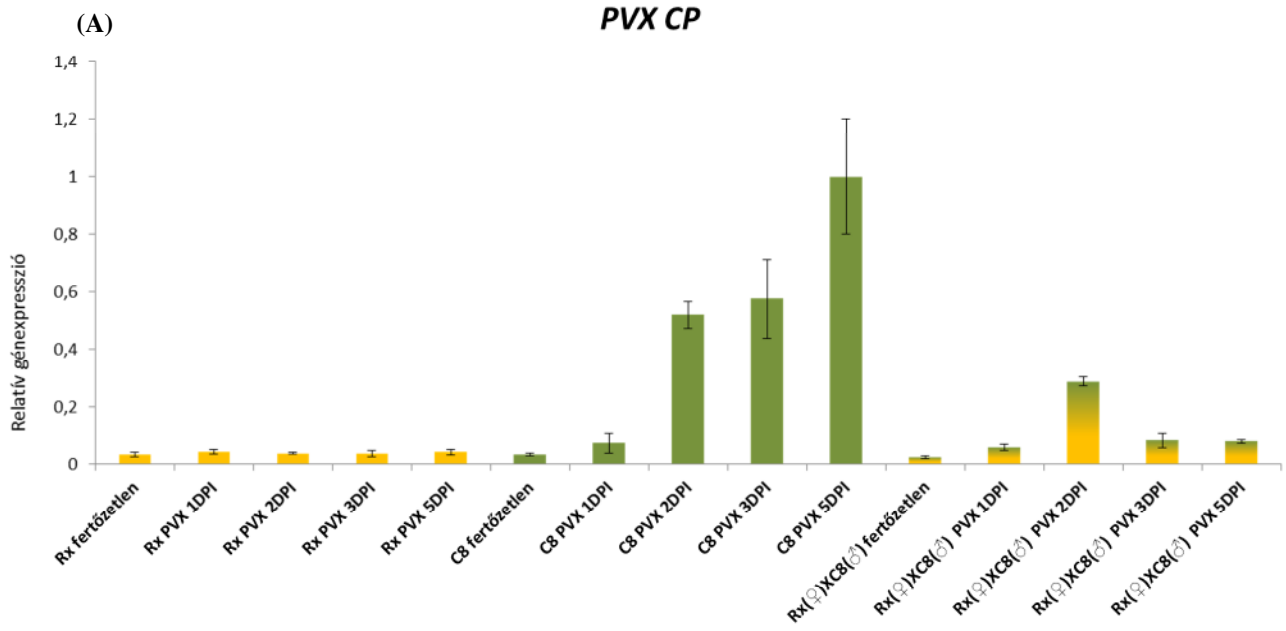


Fig. 8 Partial suppression of extreme resistance (ER) to *Potato virus X* (PVX) in F_1 progeny of crosses of tobacco lines displaying ER (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and two PVX-susceptible, ferritin-overproducing lines (*N. tabacum* cv. SR1; C8 and F9). *Rx1* tobacco was the maternal line (♀) during crosses. In more than 50 % of F_1 plants hypersensitive localized necrosis (HR) appeared in inoculated leaves 5 days after PVX infection (designated by arrows). (A): Crosses of *Rx1* tobacco with the C8 line; (B): Crosses of *Rx1* tobacco with the F9 line.



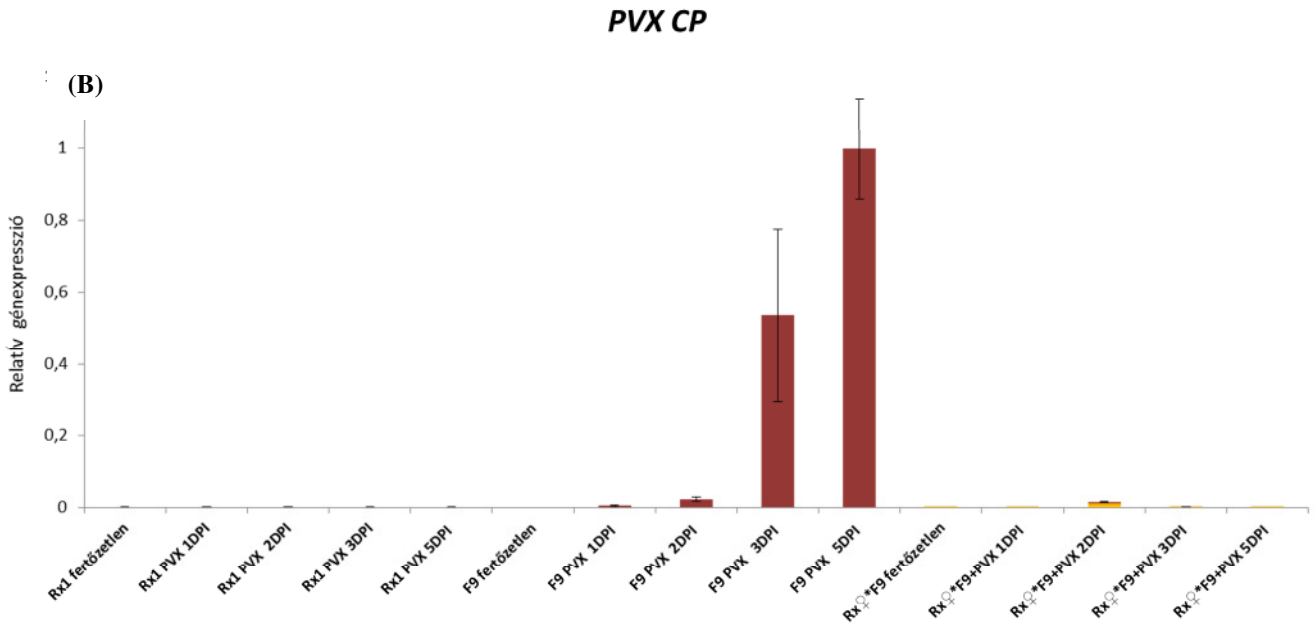


Fig. 9 Partial suppression of extreme resistance (ER) to *Potato virus X* (PVX) in F_1 progeny of crosses of tobacco lines displaying ER (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and two PVX-susceptible, ferritin-overproducing lines (*N. tabacum* cv. SR1; C8 and F9) within 5 days after PVX infection. *Rx1* tobacco was the maternal line (♀) during crosses. Accumulation of PVX in inoculated leaves was monitored by RT-qPCR (*PVX CP* relative gene expression). Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference. **(A)**: Crosses of *Rx1* tobacco with the C8 line; **(B)**: Crosses of *Rx1* tobacco with the F9 line.

We have repeated the above experiments by using reciprocal crosses (Nt cv. Samsun *NN Rx1*♂ X Nt cv. SR1 C8 ♀ and Nt cv. Samsun *NN Rx1*♂ X Nt cv. SR1 F9 ♀) and obtained similar results (i.e. ferritin-overproduction confers suppression of *Rx1*-mediated ER with the occasional appearance of HR-type necrosis). Furthermore, as controls we have also included crosses of "Rx" plants and the wild type SR1 tobacco (no extra ferritin produced) (Nt cv. Samsun *NN Rx1*♂ X Nt cv. SR1 wt ♀) and could show that in this case the suppression of ER was almost non-detectable, as compared to the ferritin-overproducing F_1 progeny, pointing to the specific role of ferritin ($OH^{\cdot}$-deficiency) in suppressing extreme resistance to PVX **(Figure 10)**.

These results indicate that 1/ ROS ($OH^{\cdot}$) could play a key role in virus inhibition during the initial stages (i.e. the first 2 days) of PVX infection, 2/ other, mostly unknown factors besides ROS may also contribute to PVX-elicited, *Rx1*-mediated symptomless extreme resistance.

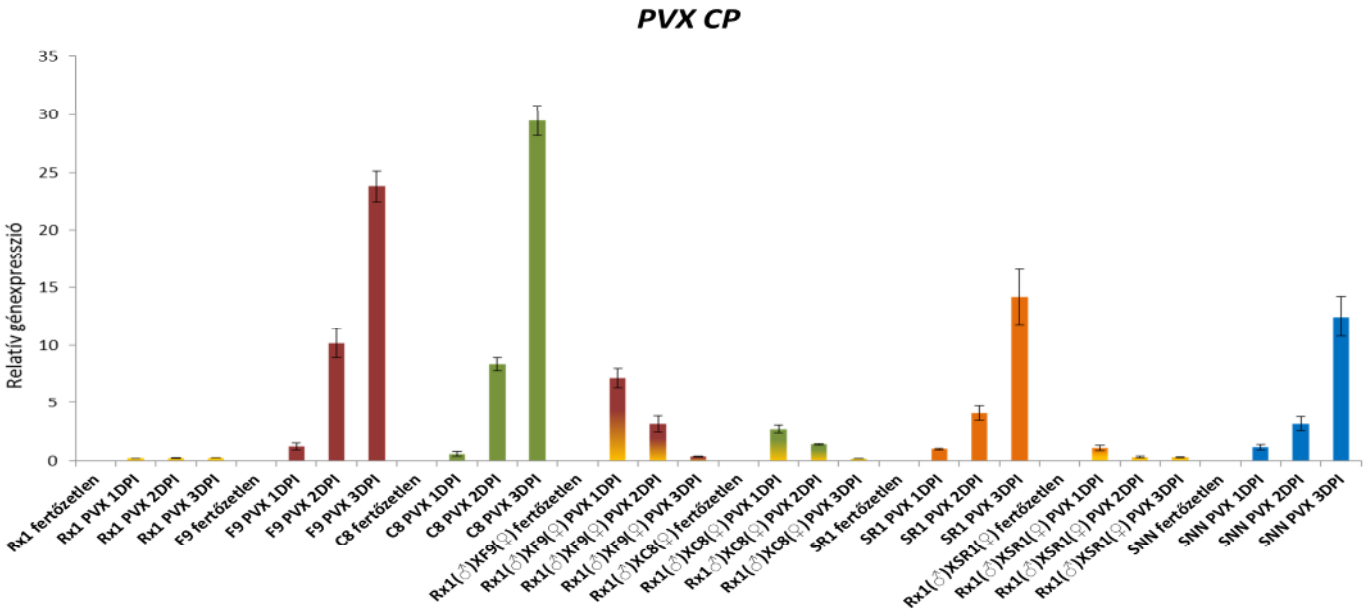


Fig. 10 Partial suppression of extreme resistance (ER) to *Potato virus X* (PVX) in F_1 progeny of crosses of tobacco lines displaying ER (*Nicotiana tabacum* cv. Samsun *NN Rx1*) and two PVX-susceptible, ferritin-overproducing lines (*N. tabacum* cv. SR1; C8 and F9) within 3 days after PVX infection. As a control, *Rx1* tobacco was also crossed with wild type SR1 tobacco. *Rx1* tobacco was the paternal line (♂) during crosses. SNN: PVX-susceptible control tobacco line. Accumulation of PVX in inoculated leaves was monitored by RT-qPCR (*PVX CP* relative gene expression). Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference.

Conferring PVX resistance in susceptible tobacco (cv. Samsun NN*) by infiltration of a superoxide (ROS) generating agent*

To further demonstrate the role of ROS in PVX-elicited symptomless ER of *Rx1* tobacco, we have attempted to elicit PVX resistance in susceptible tobacco leaves by infiltrating agents (chemicals) that generate ROS. Conferring at least a partial PVX resistance by infiltration of ROS-generating agents into susceptible plants would also suggest a function of ROS (e.g. superoxide, $O_2^{\cdot-}$) in *Rx1*-mediated resistance (ER) to PVX. Previously we have shown that infiltration of a superoxide-generating riboflavin-methionine mixture into TMV-susceptible tobacco leaves 2 hours after virus inoculation confers symptomless virus resistance (Bacsó et al., 2011). Here we found that infiltration of riboflavin (66 µm) and methionine (10 mM) into leaves of PVX susceptible (wild type) tobacco 2 hours after inoculation with PVX seems to convert susceptibility to resistance, as indicated by the appearance of local necrotic lesions resembling HR by 5 DAI in more than 50 % of leaf halves infiltrated with riboflavin-methionine (no HR appeared in buffer-infiltrated controls) **(Figure 11)**. This could indicate that a partial PVX-resistance (i.e. HR) has been induced in susceptible tobacco. To validate this assumption, we have assayed PVX accumulation in riboflavin-methionine-infiltrated, PVX-infected leaves by RT-qPCR and found that this treatment confers significantly reduced PVX titers, as compared to buffer-infiltrated controls at 3 and 7 DAI **(Figure 12)**.

Taken together, the above results indicate that artificially increased superoxide levels in PVX-susceptible tobacco likely cannot induce extreme resistance (ER), only the slower HR (localized necrosis) but are capable of partially suppressing PVX replication. Nevertheless, this finding supports the possible role of superoxide in extreme resistance.

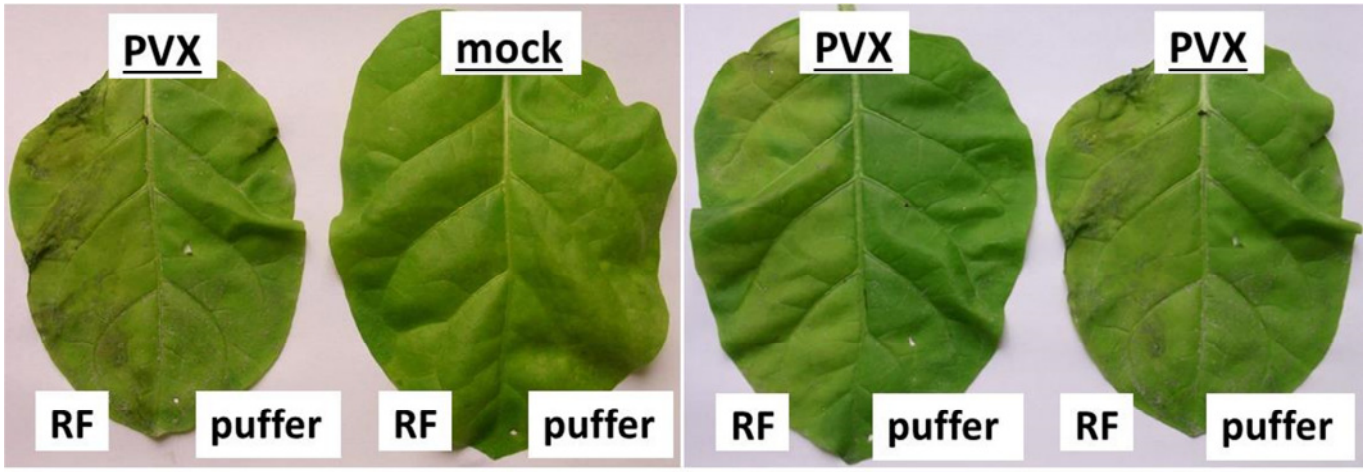


Fig. 11 Conferring resistance to *Potato virus X* (PVX) in susceptible tobacco (*Nicotiana tabacum* cv. Samsun *NN*) by infiltration of an agent (66 µM riboflavin, 10 mM L-methionine) that generates superoxide ($O_2^{\cdot-}$) into leaf halves (RF) (buffer = buffer-infiltrated leaf halves). In most plants local necrotic lesions resembling a hypersensitive response (HR) appeared in virus-inoculated, riboflavin-infiltrated leaf halves by 5 days after PVX infection. „mock" = control-inoculated leaves (mechanical stress).

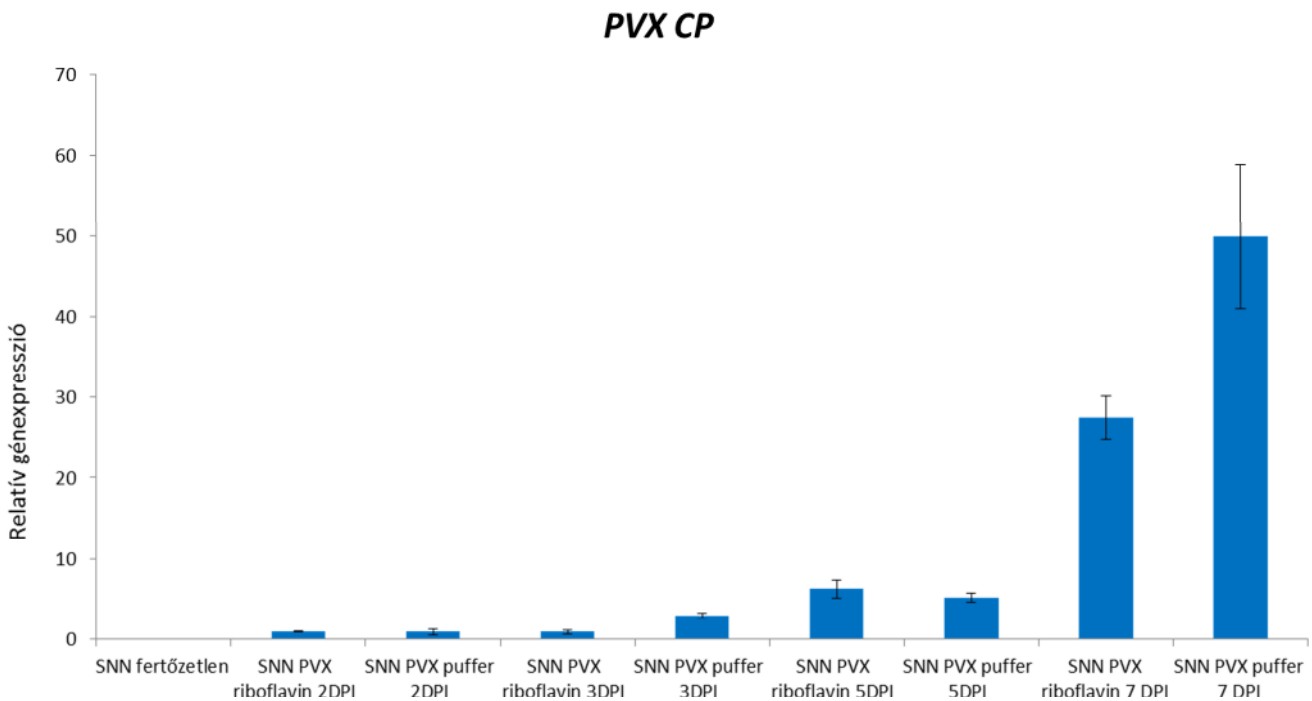


Fig. 12 Conferring resistance to *Potato virus X* (PVX) in susceptible tobacco (*Nicotiana tabacum* cv. Samsun *NN*) by infiltration of an agent (66 µM riboflavin, 10 mM L-methionine) that generates superoxide ($O_2^{\cdot-}$) into leaf halves (riboflavin) (buffer = buffer-infiltrated leaf halves) within 7 days after PVX infection. Accumulation of PVX in inoculated leaves was monitored by RT-qPCR (*PVX CP* relative gene expression). Gene expression was normalized by using a tobacco actin gene (*NtAct*) as a reference.

Comparing symptomless extreme resistance to PVX and HR-type (local lesions) resistance to *Tobacco mosaic virus* (TMV) in tobacco - expression/activities of defense-related genes, NADPH-oxidase and antioxidants

To demonstrate that differential expression of defense/stress-related (e.g. PR and antioxidant) genes and changes in activities of NADPH-oxidase and antioxidants mark the development of extreme resistance (ER) vs. HR, we compared these responses in tobacco (cv. Samsun NN) inoculated with PVX and *Tobacco mosaic virus* (TMV, U1 strain), respectively. The Samsun NN tobacco used by us express both the *Rx1* and *N* resistance genes, conferring ER to PVX and the slower HR-type of resistance to TMV. Extreme resistance elicited by PVX was clearly detectable already at 6 HAI (by real time RT-qPCR) and fully developed by the first day of PVX infection (1 DAI) confirming our earlier results, while in TMV-elicited HR virus titers were significantly increasing even at 2 DAI, when visible necrotic lesions develop **(Figure 13)**.

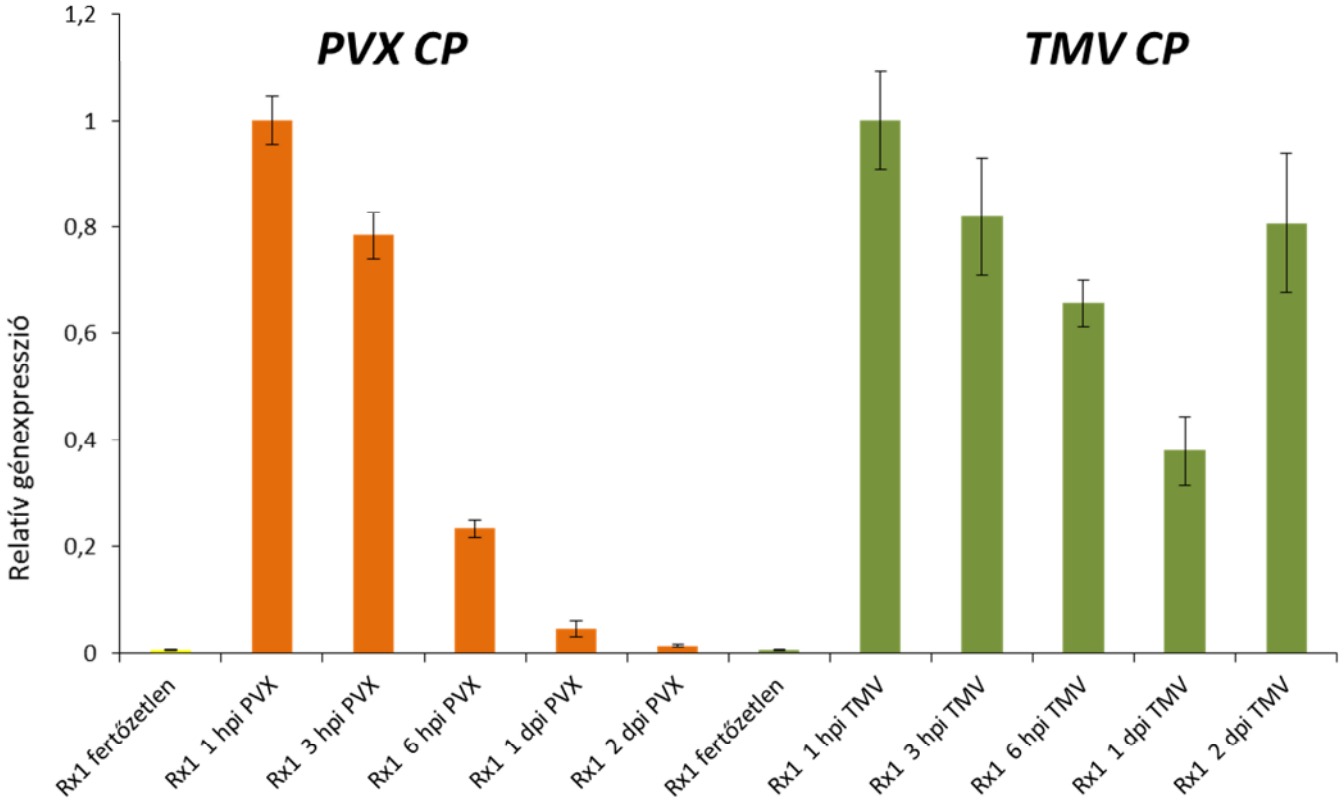


Fig. 13 Accumulation of *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) in tobacco expressing the *Rx1*extreme resistance gene and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1*) at different time points after virus inoculation (1, 3 and 6 hours, 1 and 2 days). Gene expression was assayed by RT-qPCR (*PVX CP* and *TMV CP* relative gene expression) and normalized by using a tobacco actin gene (*NtAct*) as a reference.

Expression of defense-related (PR) and cell death/ROS regulator genes during symptomless extreme resistance (ER) and HR

Only a minimal activity of three defense-related (pathogenesis-related, PR) genes (*NtPR-1a, NtPRB-1b, NtGSTphi*=glutathione S-transferase) occurred in PVX-inoculated plants exhibiting ER, as opposed to plants showing TMV-elicited HR where gene expression markedly increased. We have also included three cell death and ROS-regulator genes (*NtBI-1*=BAX inhibitor-1, *NtAOX1-2*=alternative oxidase, *NgCat1*=catalase) in this analysis. Although an early, usually slight and transient induction of the above mentioned genes signaled the onset of resistance, gene expression either did not change or decreased following the development of ER (from 6 HAI onwards). On the other hand, beginning from the first day after TMV inoculation (DAI), expression of these genes has significantly increased, concomitant with the development of visible local necrotic lesions (HR) **(Figures 14** and **15)**.

Our results demonstrated that PVX-elicited ER is accompanied by an almost negligible induction of defense/stress-related (PR) and cell death/ROS-regulator genes, as compared to TMV-elicited HR, a further indication that symptomless ER is a rapid, early defense response, as compared to cell/tissue death-associated HR.

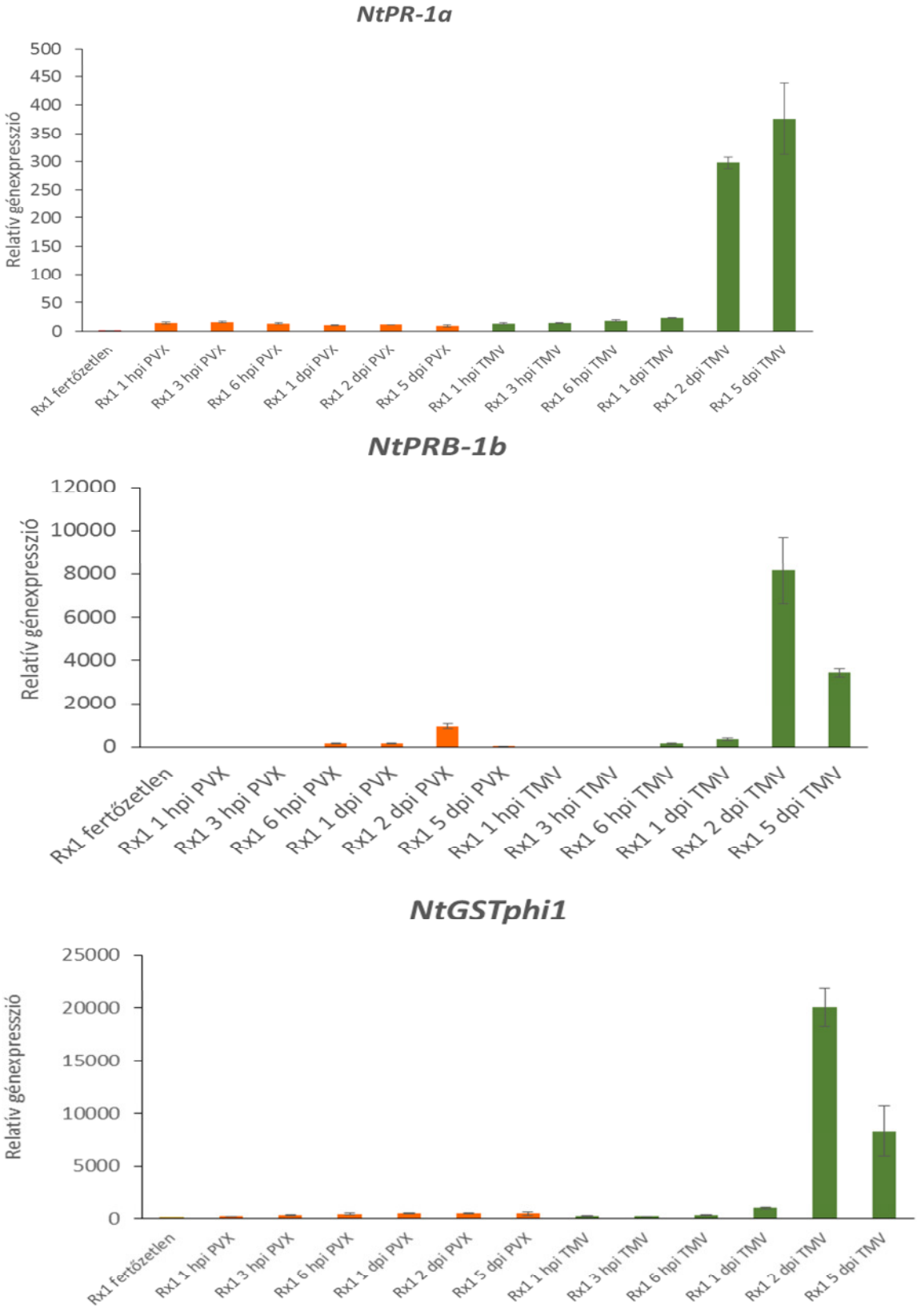


Fig. 14 Expression of defense/stress-related genes (*NtPR-1a*, *NtPRB-1b* és *NtGSTphi1*) in tobacco inoculated with *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1*) at different time points after virus inoculation (1, 3 and 6 hours, 1, 2 and 5 days). Gene expression was assayed by RT-qPCR (relative gene expression) and normalized by using a tobacco actin gene (*NtAct*) as a reference. Results were also normalized to gene expression values of control-inoculated („mock") leaves.

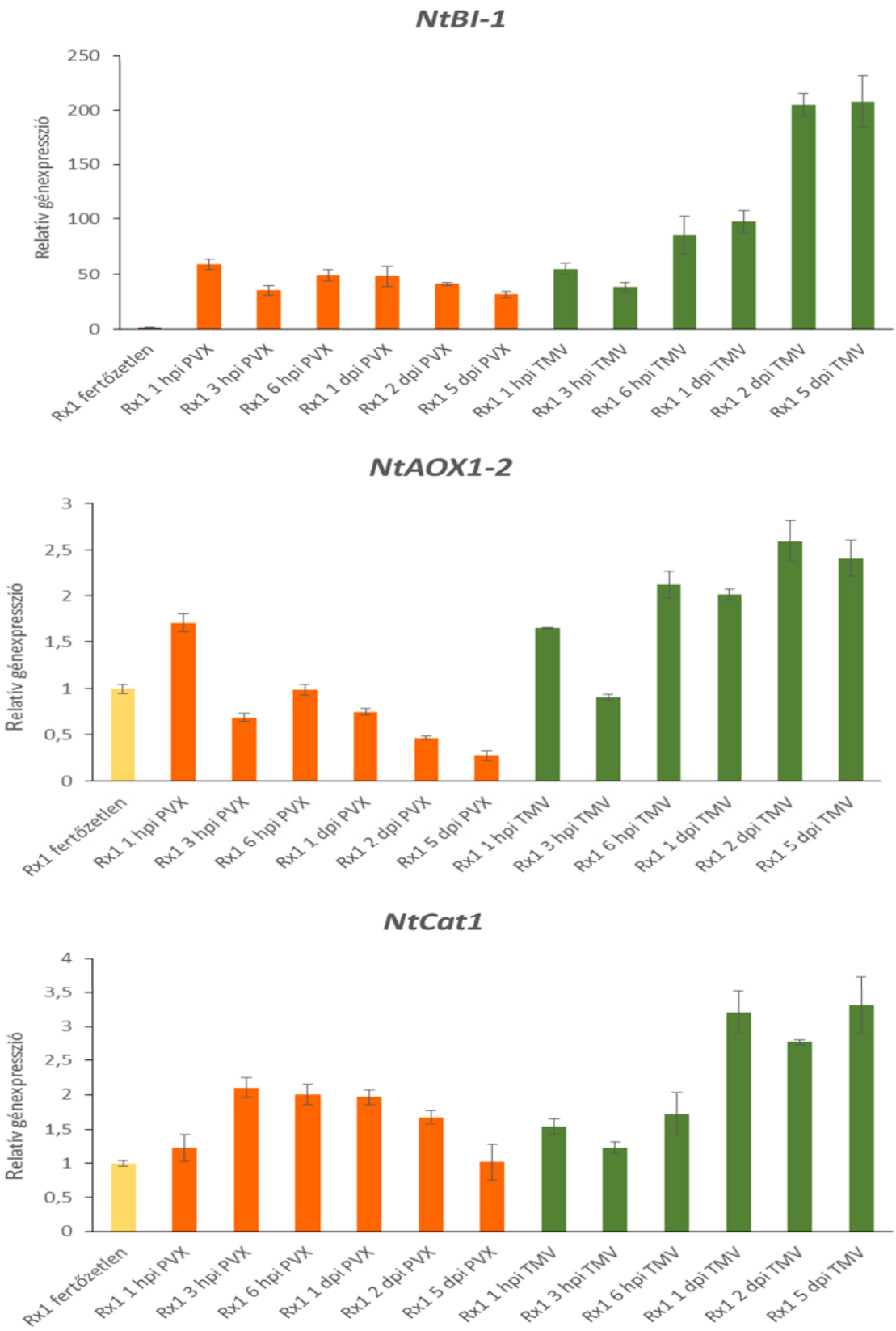


Fig. 15 Expression of cell death and ROS-regulator genes (*NtBI-1*, *NtAOX1-2* and *NtCat1*) in tobacco inoculated with *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1*) at different time points after virus inoculation (1, 3 and 6 hours, 1, 2 and 5 days). Gene expression was assayed by RT-qPCR (relative gene expression) and normalized by using a tobacco actin gene (*NtAct*) as a reference. Results were also normalized to gene expression values of control-inoculated („mock") leaves.

Activites of NADPH-oxidase and antioxidants during symptomless extreme resistance (ER) and HR

In order to obtain a closer picture of the possible role/mechanism of early superoxide accumulation during extreme resistance (ER) we monitored expression of *NtRBOHD*, the tobacco gene encoding a NADPH oxidase involved in superoxide production during the HR-type of resistance to e.g. TMV (Király et al., 2008). We found that by 24 HAI much higher (ca. twice) expression-levels of *NtRBOHD* were retained in PVX-infected *Rx1* tobacco, as compared to susceptible plants (data not shown). The early induction of *NtRBOHD* expression points to the role of NADPH oxidase-generated superoxide in ER development. For further confirmation we assayed NADPH oxidase enzymatic activity in PVX-infected resistant (*Rx1*) and susceptible tobaccos and TMV-infected *Rx1* tobaccos (the latter display HR-type of resistance to TMV). An early (from 0.5 and 2 HAI), significant induction of NADPH oxidase activity was detected in PVX-infected extreme resistant tobacco (in line with superoxide accumulation, see Figures 3 and 4) as compared to plants susceptible to PVX and plants displaying HR in response to TMV. These results **(Figure 16)** indeed suggest an instrumental role of NADPH oxidases – and NADPH oxidase-generated superoxide – in the development of symptomless extreme resistance to PVX.

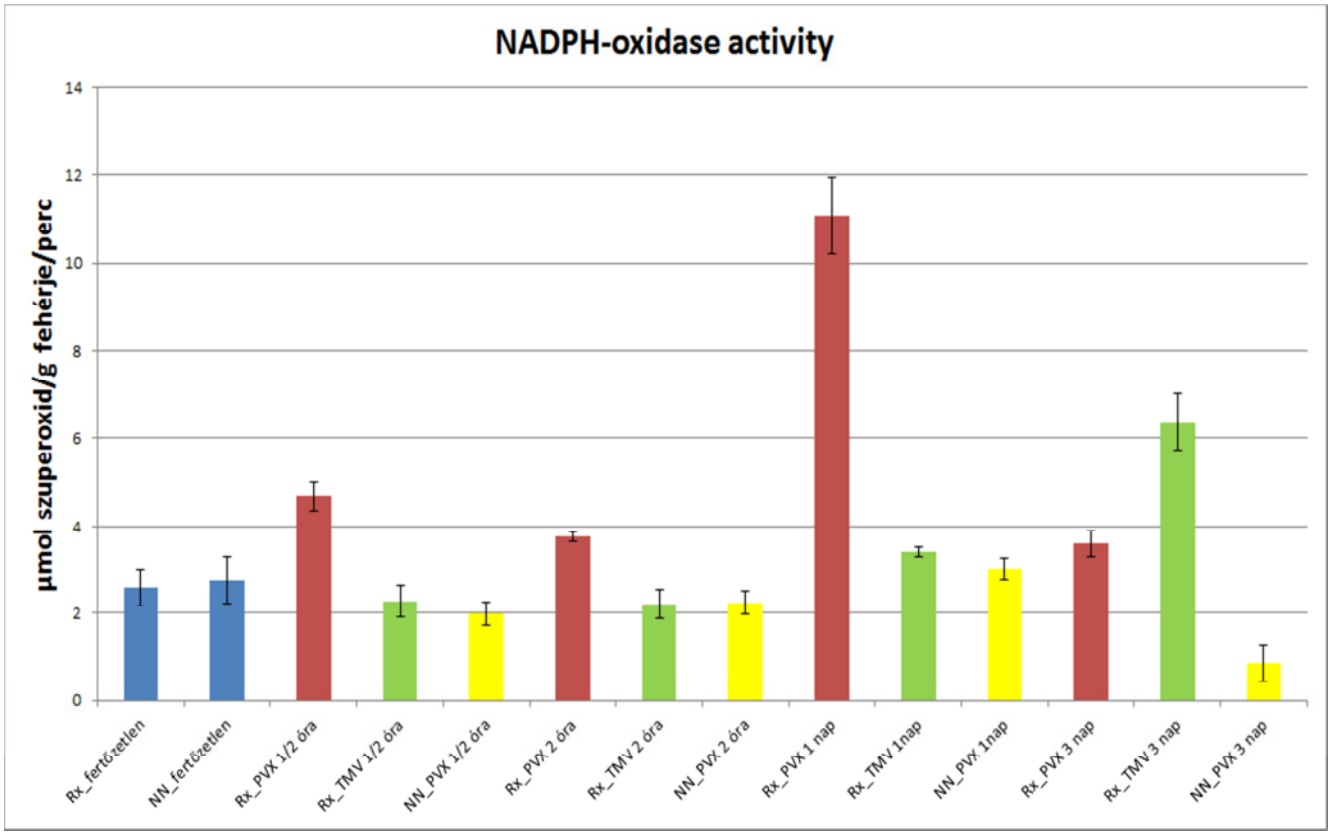


Fig. 16 Enzymatic activity of NADPH-oxidase in tobacco inoculated with *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1* „Rx-PVX" and „Rx-TMV") and in PVX-susceptible tobacco (*N. tabacum* cv. Samsun *NN* „NN-PVX") at different time points after virus inoculation (0.5, and 2 hours, 1 and 3 days).

To further characterize pathophysiological differences between symptomless (ER) and cell death-associated (HR) virus resistance, accumulation/activity patterns of antioxidants were also assayed. In case of TMV-induced HR, amounts of the non-enzymatic antioxidant and signaling agent, glutathione (in particular, the reduced form, GSH) and activities of glutathione-associated antioxidant enzymes (glutathione reductase and glutathione S-transferase) has markedly increased 4 DAI, while no such changes occurred during PVX-induced ER (**Figures 17** and **18**).

Overall, our results demonstrated that the extreme resistance (ER) elicited by PVX is accompanied by a low activity of antioxidants pointing to symptomless ER as a rapid, efficient, early defense response, as compared to cell/tissue death-associated HR.

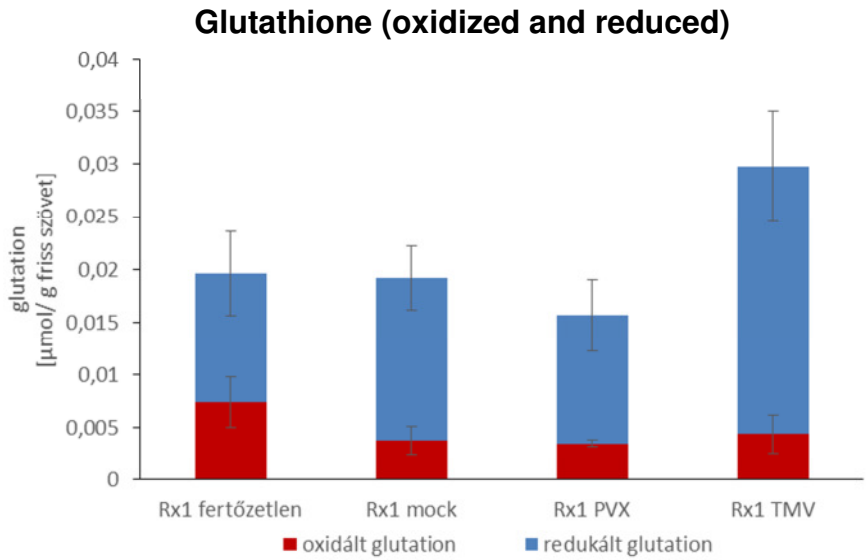


Fig. 17 Accumulation of glutathione and ratio of oxidized and reduced glutathione in tobacco inoculated with *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1*) 4 days after virus inoculation. „mock" = control-inoculated leaves (mechanical stress).

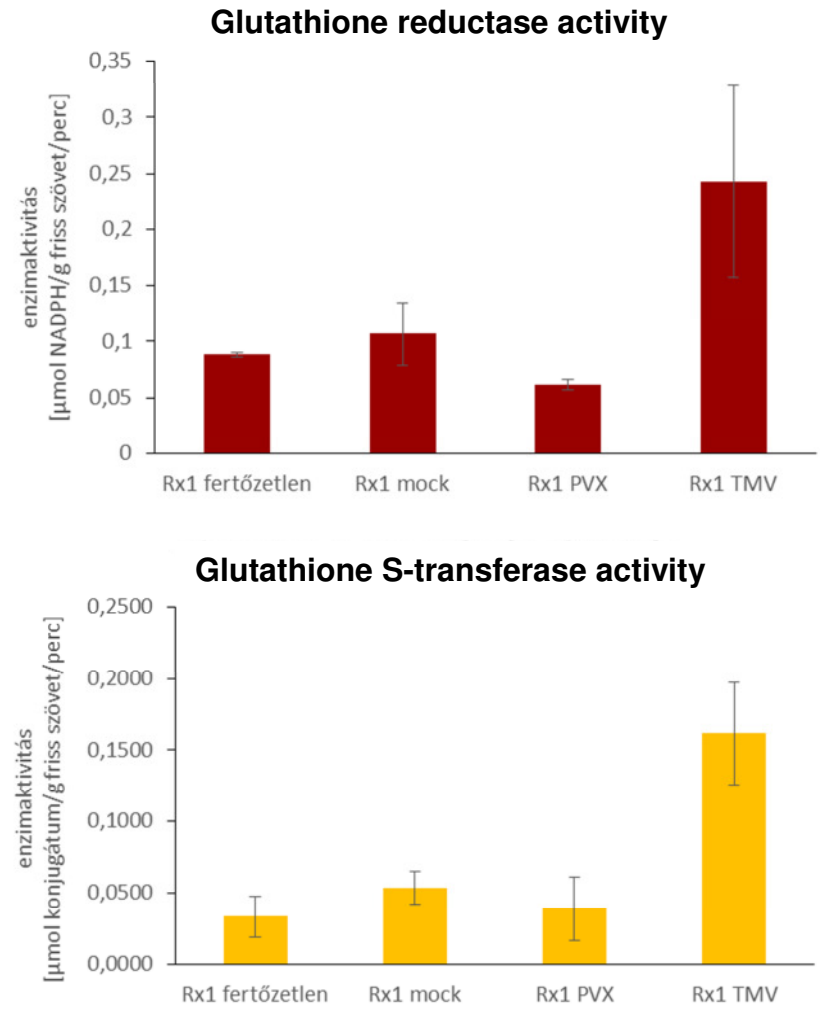


Fig. 18 Enzymatic activities of glutathione-reductase (GR) and glutathione S-transferase (GST) in tobacco inoculated with *Potato virus X* (PVX) and *Tobacco mosaic virus* (TMV) and resistant to PVX and TMV (*Nicotiana tabacum* cv. Samsun *NN Rx1*) 4 days after virus inoculation. „mock" = control-inoculated leaves (mechanical stress).

Symptomless resistance of cherry pepper to its powdery mildew (*Leveillula taurica*) – pathophysiological background, the role of superoxide (ROS) accumulation

Our preliminary experiments involving provocation tests have shown that the Hungarian cherry pepper (*Capsicum annuum* var. cerasiforme) cultivar ´Szentesi´ exhibits a symptomless resistance to pepper powdery mildew (PM). In fact, we have observed a strong, robust resistance of cv. Szentesi to PM symptoms in commercial greenhouses in Szentes (Southwestern Hungary) for the sixth consecutive growing season since 2012. On the other hand, the sweet pepper (*Capsicum annuum*) cv. Totál was extremely susceptible to PM, as shown for most other sweet pepper cultivars. During these provocation tests we have also found that the PM resistance characteristic of cherry pepper cv. Szentesi can be transmitted to the susceptible sweet pepper cv. Totál by grafting. Fruit yield in these Szentesi + Totál grafts was similar to that of healthy control self-rooted 'Totál'.

One of the main aims of this project was to investigate whether accumulation of the ROS superoxide ($O_2^{\cdot-}$) indeed plays a pivotal role in the graft-transmissible, symptomless resistance of cherry pepper to its powdery mildew?

Graft-transmissible symptomless resistance of cherry pepper to powdery mildew is associated with inhibition of the pathogen (Leveillula taurica)

In order to see if the graft-transmissible resistance of cherry pepper to powdery mildew is effective under controlled laboratory conditons and associated with inhibition of the PM pathogen (*Leveillula taurica*), a PM isolate was collected from one of the commercial greenhouse sites mentioned above from susceptible sweet pepper cv. Totál plants. Re-infection of healthy plants (self-rooted cv. Totál, cv. Szentesi and Szentesi + Totál grafts) under laboratory conditions essentially as described by Zheng et al. (2013) and microscopic observation of fungal structures confirmed presence of the pepper PM pathogen, *L. taurica*. 45 days after inoculation (DAI) self-rooted 'Totál' displayed typical PM symptoms (chlorosis on adaxial leaf surfaces, powdery mildew on abaxial leaf surfaces), 'Szentesi' did not display any visible PM symptoms, while Szentesi + Totál grafts displayed only occasional mild symptoms on lower (senesced) leaves, similarly as observed in provocation tests **(Figure 19A)**.

For further confirmation of identity of the pathogen, detection assays were conducted by qPCR with specific primers based on *L. taurica* ITS sequences (Zheng et al., 2013). Due to occasional genomic DNA contamination, these assays were also repeated by using total RNA templates derived from PM-infected plants (i.e. RT-qPCR). At 45 DAI PM-infected resistant pepper plants (self-rooted 'Szentesi' and Szentesi + 'Totál' grafts) contained less than half the amount of fungal DNA (RNA) than susceptible plants ('Totál') **(Figure 19B)**, demonstrating that cv. Szentesi and Szentesi + 'Totál' grafts indeed display a true resistance to *L. taurica*, being resistant to both PM symptoms and pathogen accumulation.

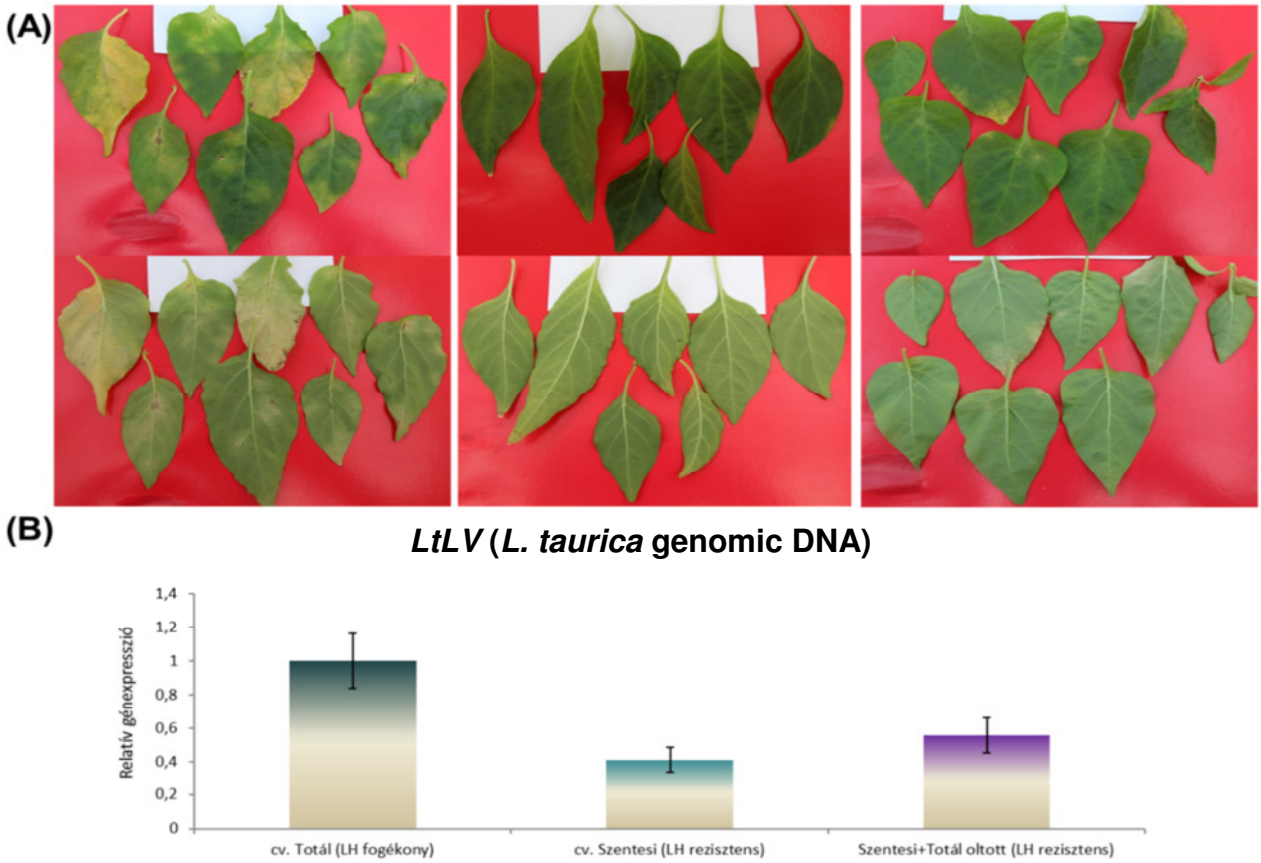


Fig. 19 Resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) is graft-transmissible: resistance induced in susceptible sweet pepper (*C. annuum* cv. Totál) grafted on resistant cherry pepper cv. Szentesi. Typical symptoms of powdery mildew infection in pepper leaves 45 days after inoculation (DAI) **(A)**. Upper panels: chlorotic flecks on adaxial leaf surfaces; lower panels: powdery mildew on abaxial leaf surfaces. Left, middle and right panels: leaves of susceptible (cv. Totál) and resistant (cv. Szentesi and Szentesi + Totál grafts) pepper, respectively. Quantification of PM levels in inoculated pepper leaves at 45 DAI by qPCR using the LtLV primer pair **(B)**. Gene expression was normalized by using a pepper actin gene (*CaAct*) as a reference.

Involvement of NADPH oxidase-generated superoxide (ROS) accumulation in the graft-transmissible symptomless resistance of cherry pepper to powdery mildew

To assess the possible role of the ROS superoxide in the graft-transmissible PM-resistance of cherry pepper, superoxide accumulation in healthy and PM-inoculated (45 DAI) pepper leaves was detected by histochemical staining with NBT. In healthy pepper, negligible amounts of superoxide were present in PM-susceptible 'Totál' leaves, while a pronounced superoxide accumulation was apparent in leaves of PM-resistant 'Szentesi' and Szentesi + Totál grafts (**Figure 20A** upper panels). Interestingly, in PM-inoculated pepper, overall superoxide levels increased slightly, i.e. still very little superoxide accumulated in PM-susceptible 'Totál' leaves, while high superoxide levels were essentially retained in PM-resistant leaves (**Figure 20B** upper panels). These findings suggest that superoxide accumulation is a marker and a possible functional component of this graft-transmissible PM-resistance in pepper.

Since pathogenesis-related, plasma membrane-derived superoxide accumulation in plants has been primarily associated with the activity of NADPH oxidases (see e.g. Marino et al., 2012; Kadota et al., 2015), we thought that the elevated superoxide accumulation associated with the graft-transmissible PM-resistance of pepper could be also a consequence of NADPH oxidase activity. To test this hypothesis, NADPH oxidase enzymatic activity was assayed in healthy and PM-inoculated pepper leaves at 45 DAI. In healthy pepper, a basal NADPH oxidase activity was present in PM-susceptible 'Totál', while a significantly higher activity was apparent in PM-resistant 'Szentesi' and Szentesi + Totál grafts (**Figure 20A** lower panels). In PM-inoculated pepper, overall NADPH oxidase activities increased but high activities were retained in PM-resistant leaves ('Szentesi' and Szentesi + Totál grafts) as compared to PM-susceptible 'Totál' (**Figure 20B** lower panels).

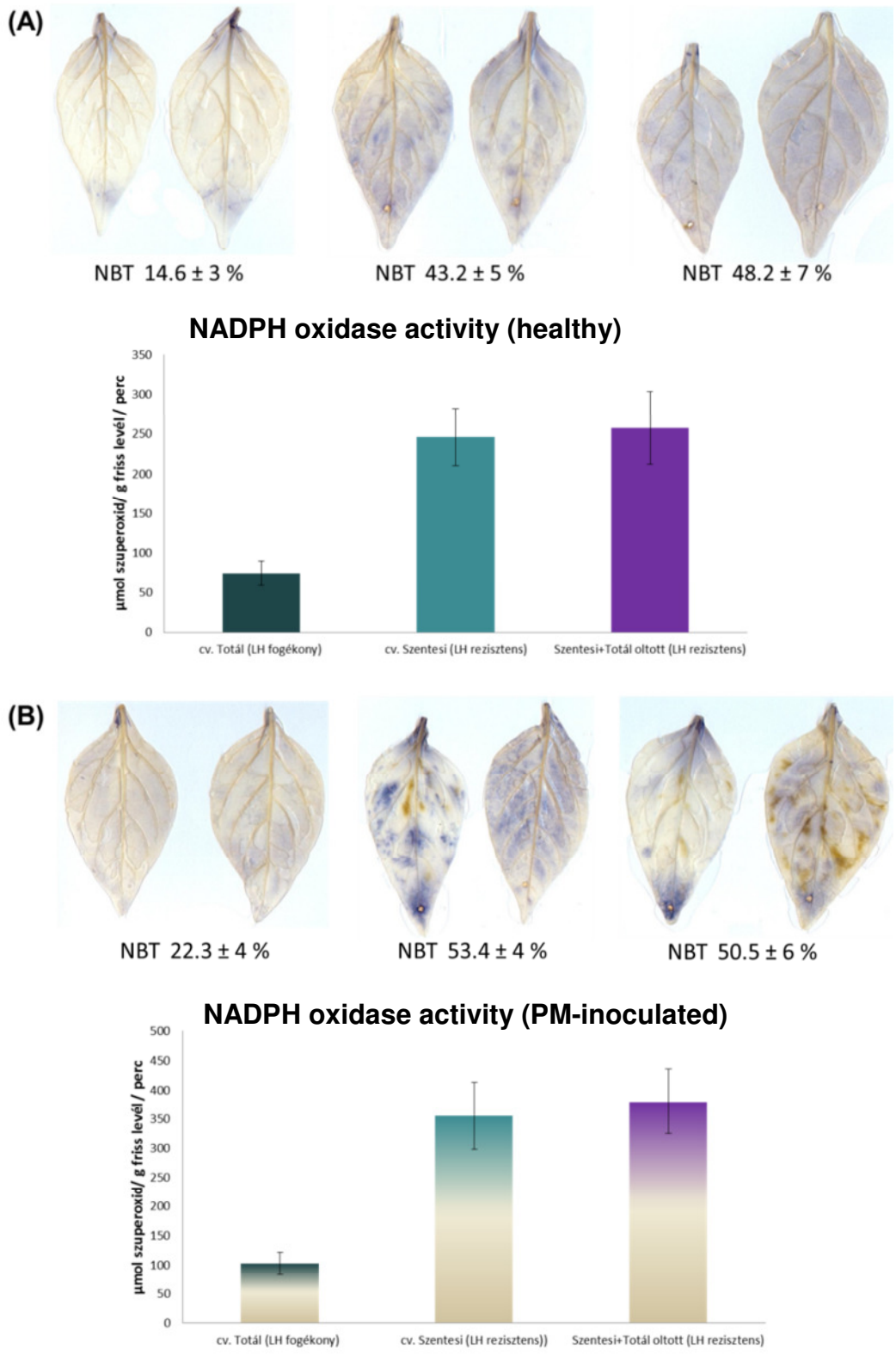


Fig. 20 Graft-transmissible resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) is associated with superoxide ($O_2^{\cdot-}$) accumulation and elevated NADPH oxidase activity in both healthy **(A)** and PM-inoculated (45 DAI) **(B)** plants. Upper panels: superoxide accumulation in healthy/PM-inoculated pepper leaves as visualized by nitro blue tetrazolium chloride (NBT) tissue staining (quantification by the image analysis software Image J). Left, middle and right panels: leaves of susceptible (cv. Totál) and resistant (cv. Szentesi and Szentesi + Totál grafts) plants, respectively. Lower panels: enzymatic activity of NADPH oxidase in healthy/PM-inoculated pepper leaves.

Overall, these results suggest that elevated superoxide levels and NADPH oxidase activity are both markers and possible functional components of the graft-transmissible PM-resistance of pepper.

PM resistance and superoxide accumulation/NADPH oxidase activity are linked traits that are graft-transmissible from different cherry pepper rootstocks

To verify if PM resistance and superoxide accumulation/NADPH oxidase activity are indeed linked traits and graft-transmissible from different cherry pepper rootstocks, we have grafted susceptible sweet pepper cv. Totál unto several cherry pepper cultivars displaying different degrees of PM resistance. Cultivars ´Kalocsai A´ and ´Kalocsai M´ are highly resistant to PM, ´Garai fehér´ and ´Óriás´ are moderately PM resistant, while ´Globál´ and ´Korál´ are PM susceptible. When cv. Totál was grafted on the above mentioned cherry pepper cultivars, we found that superoxide accumulation was always correlated with PM resistance during provocation tests under greenhouse conditions **(Table 1)**. In fact, these two traits are graft transmissible in concert to susceptible sweet pepper.

Table 1 Powdery mildew resistance and superoxide accumulation in sweet pepper (*Capsicum annuum*) grafted on different cherry pepper (*C. annuum* var. cerasiforme) rootstocks

Cherry pepper rootstock (scion: cv. Totál)	**Resistant (#)**	**Susceptible (#)**	**Superoxide [1]**
Szentesi	15	0	48.2 ± 7 %
Kalocsai A	13	2	45.7 ± 5 %
Kalocsai M	12	3	39.3 ± 4 %
Globál	0	15	13.4 ± 3 %
Koral	0	15	11.2 ± 3 %
Garai fehér	10	5	35.2 ± 5 %
Óriási cseresznye	2	13	15.6 ± 4 %

[1] Detected by nitroblue tetrazolium (NBT) staining. Quantification by the ImageJ software (% NBT stained leaf area).

We further showed that high NADPH oxidase activity is also closely associated with PM resistance in the above-mentioned healthy and PM-infected grafted plants. In response to PM-infection under laboratory conditions (45 DAI), overall NADPH oxidase activities increased but the significantly higher activities were retained in leaves of PM-resistant grafts **(Figure 21)**.

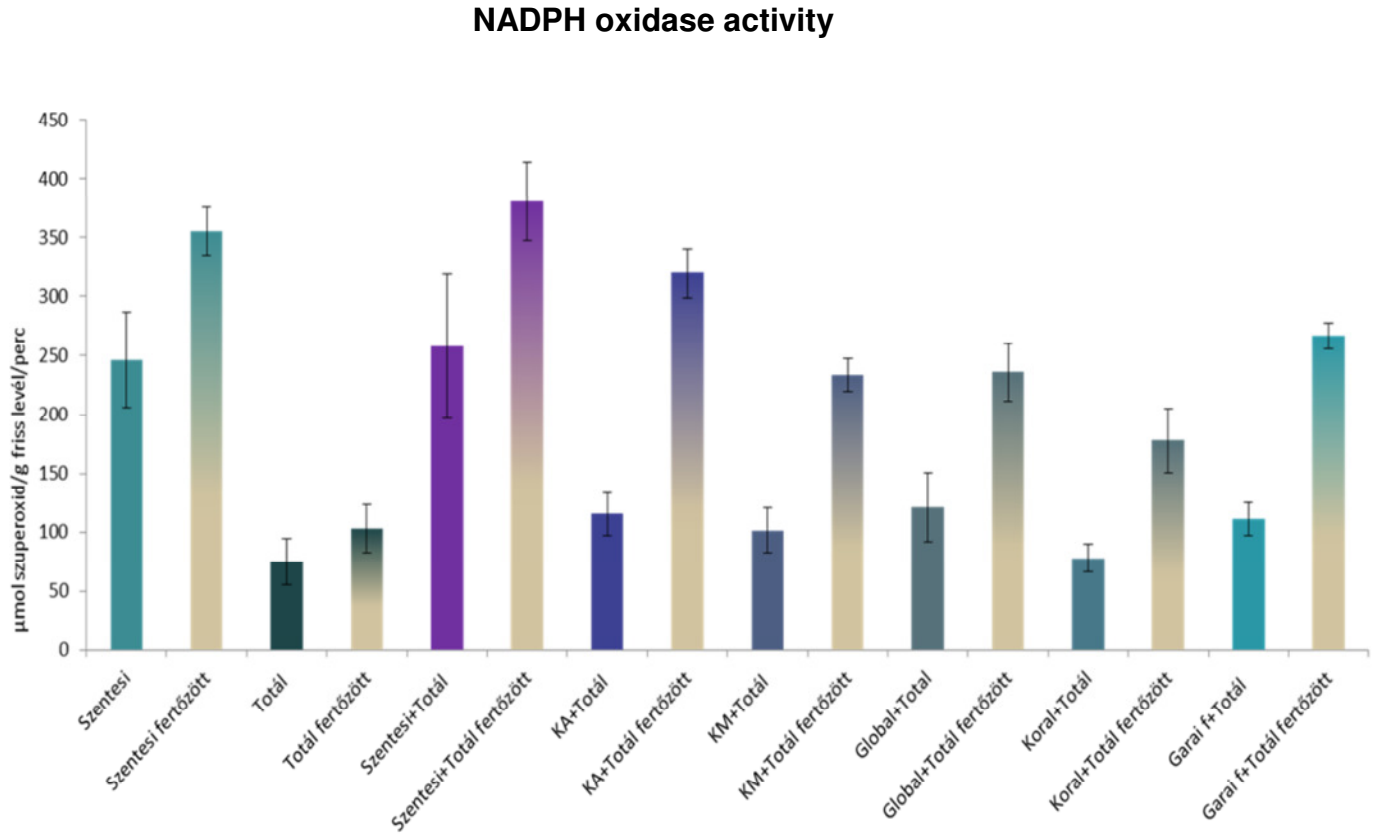


Fig. 21 NADPH oxidase activity of sweet pepper (*Capsicum annuum* cv. Totál) grafted on different cherry pepper (*C. annuum* var. *cerasiforme*) rootstocks, either healthy (-) or inoculated with pepper powdery mildew (PM) (*Leveillula taurica*). 'Szentesi', 'Totál': self-tooted plants; Szentesi+Totál ,Kalocsai A+Totál, etc.: grafted plants. NADPH oxidase in healthy/PM-inoculated pepper leaves was assayed 45 DAI.

Defense responses associated with graft-transmissible symptomless resistance of cherry pepper cv. Szentesi to powdery mildew – salicylic acid accumulation and spontaneous cell death

ROS production in plants is closely associated with levels of a defense-related hormone, salicylic acid (SA), an important component of plant disease resistance (Chen et al., 1993; Neuenschwander et al., 1995). In order to further elucidate the mechanisms of graft-transmissible PM resistance in cherry pepper cv. Szentesi and reveal any relationship between superoxide accumulation and SA, we have monitored levels of salicylic acid (SA) in healthy pepper plants. Assays of free and bound (glycosylated) SA by high performance liquid chromatography (HPLC) revealed that in leaves of PM-resistant pepper (cv. Szentesi and Szentesi + Totál grafts) levels of free SA are ca. twice as high as in PM-sensitive plants. No difference occurred in levels of bound (glycosylated) SA **(Figure 22)**.

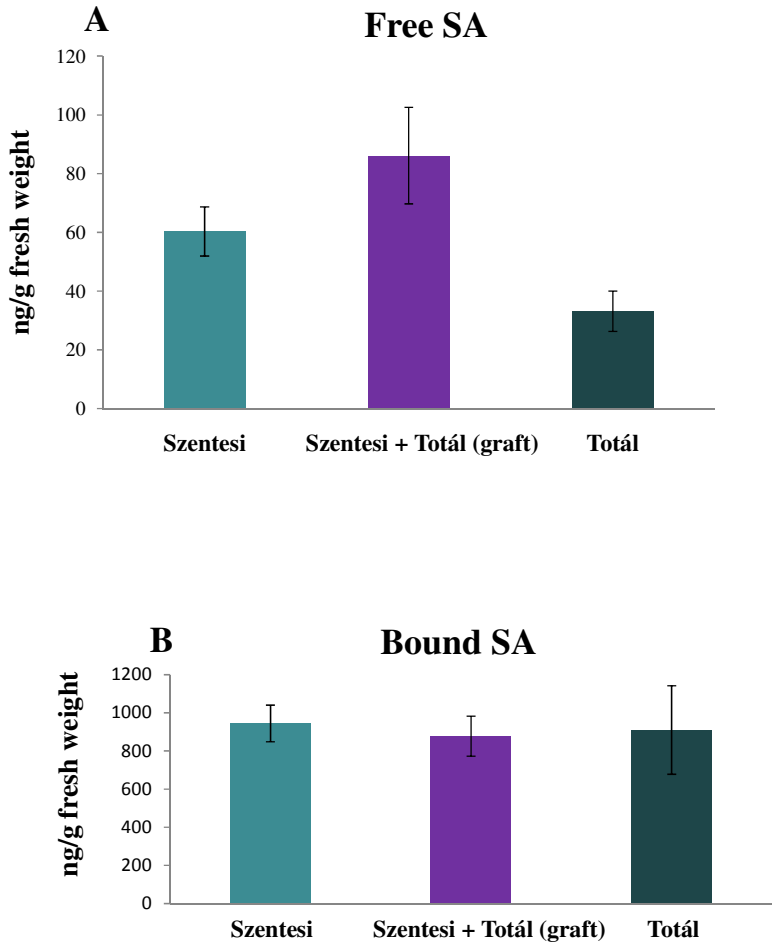


Fig. 22 Salicylic acid (SA) accumulation is a marker of graft-transmissible resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) in healthy, uninfected plants. Levels of free **(A)** and bound (glycosylated) **(B)** SA in leaves of healthy pepper plants assayed by HPLC in PM-resistant cherry pepper cv. Szentesi and sweet pepper cv. Totál grafted on cv. Szentesi rootstocks and in PM-susceptible sweet pepper cv. Totál.

Spontaneous cell death ("micro HR") had been associated with higher than normal SA and ROS levels and acquired disease resistance in dicotyledonous plants (Alvarez et al., 1998). Since even healthy (uninfected) PM-resistant cv. Szentesi pepper contains elevated levels of superoxide and SA we wanted to check if this is coupled to a "micro HR"? We could show that spontaneous cell death is far more intense in healthy (uninfected) leaf tissues of PM-resistant cv. Szentesi than in PM-susceptible cv. Totál (Trypan blue staining) (**Figure 23**), as previously demonstrated for barley *mlo* mutants with symptomless PM-resistance (Büschges et al., 1997). Interestingly, intracellular (vacuolar) browning associated with spontaneous cell death was also more intense in PM-resistant cv. Szentesi **(Figure 23)**. This possibly reflects the presence of calcium-oxalate crystals in vacuoles (Weryszko-Chmielewska and Michałojć, 2009) and the fact that cv. Szentesi can efficiently accumulate calcium (Lantos, 2011), an important regulator of NADPH oxidase-mediated superoxide production and disease resistance (Dubiella et al., 2013; Arfaoui et al., 2018). To verify that cv. Szentesi contains higher levels of calcium, as compared to cv. Totál, calcium contents in healthy pepper fruits were assayed by plasma emission (ICP) spectrometry at the University of Debrecen, Hungary. In ripe (red colored) fruits of healthy cv. Szentesi, ca. 30 % more calcium was detectable than in corresponding fruit samples of cv. Totál (ca. 280 vs. 210 mg/kg, respectively).

In summary, our results show that in cherry pepper cv. Szentesi accumulation of salicylic acid and spontaneous cell death in leaves is correlated with high calcium levels that likely contribute to an enhanced PM-resistance.

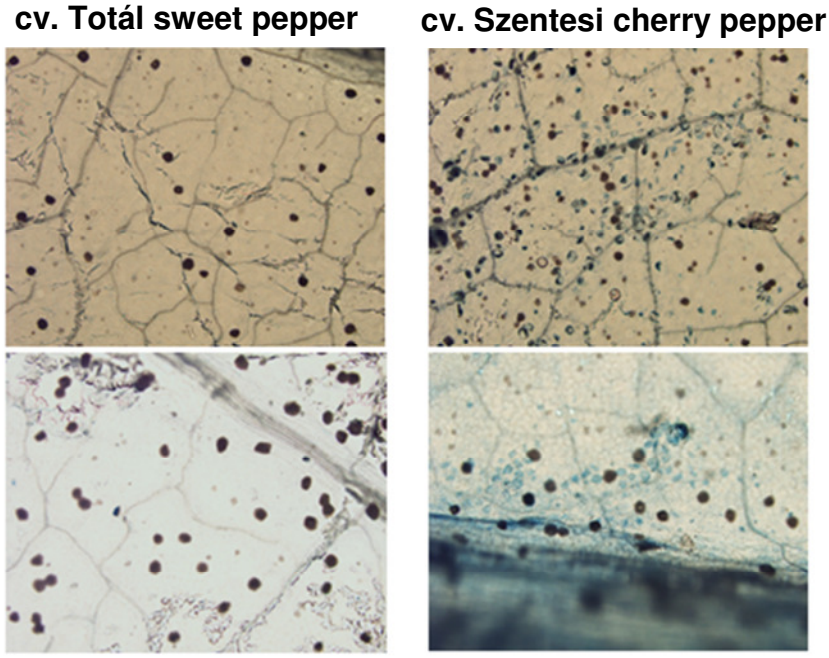


Fig. 23 Enhanced spontaneous cell death ("micro HR") is a marker of graft-transmissible resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) in leaves of healthy, uninfected plants. Spontaneous cell death (assayed by Trypan blue tissue staining) in leaves of healthy, PM-resistant (self-rooted cherry pepper cv. Szentesi) and PM-susceptible (self-rooted sweet pepper cv. Totál) pepper. Brown spots likely indicate the accumulation of calcium-oxalate crystals in vacuoles of plant cells (see Weryszko-Chmielewska and Michałojć,2009).

Defense responses associated with graft-transmissible symptomless resistance of cherry pepper cv. Szentesi to powdery mildew – expression of pathogenesis-related and cell death regulator genes

In planta-produced superoxide during infections can be converted to hydrogen peroxide inducing - along with SA - e.g. pathogenesis-related (PR) gene/protein expression, processes which lead to further plant defense responses and resistance to pathogenic infections (see e.g. in Van Loon et al., 2006; Torres, 2010; Lehmann et al., 2015). In pepper, PR-1 and PR-2 proteins and genes (*CaPR-1*, *CaPR-2*) contribute to resistance to bacterial and oomycete pathogens (Sarowar et al., 2005; Silvar et al., 2008).

We found that expression of the *CaPR-1* gene (determined by RT-qPCR) was several times higher in leaves of healthy, uninfected PM-resistant pepper than in PM-sensitive plants. On the other hand, high expression levels of the *CaPR-2* (glucanase) gene did not entirely correlate with PM-resistance, being detectable only in PM-resistant cv. Szentesi plants but neither in PM-resistant Szentesi + Totál grafts nor in susceptible controls (cv. Totál) **(Figure 24).** Nevertheless, it seems that enhanced expression of two defense-related genes (*CaPR-1*, *CaPR-2* /glucanase/) can predict PM-resistance in healthy plants.

In order to clarify if enhanced PR gene expression may have a role in maintaining PM resistance, we have monitored expression of *CaPR-1* and *CaPR-2* also in PM-infected plants. However, we found that during advanced stages of PM-pathogenesis (45 DAI) expression of *CaPR-1* and *CaPR-2* is by far the highest in PM-susceptible 'Totál' but significantly lower in PM-resistant plants (cv. Szentesi and Szentesi + Totál grafts) **(Figure 24)**. These results indicate that PR gene expression in pepper does not have a functional role in maintaining resistance to PM, although enhanced expression of *CaPR-1* and *CaPR-2* is a marker of PM-resistance in healthy pepper plants.

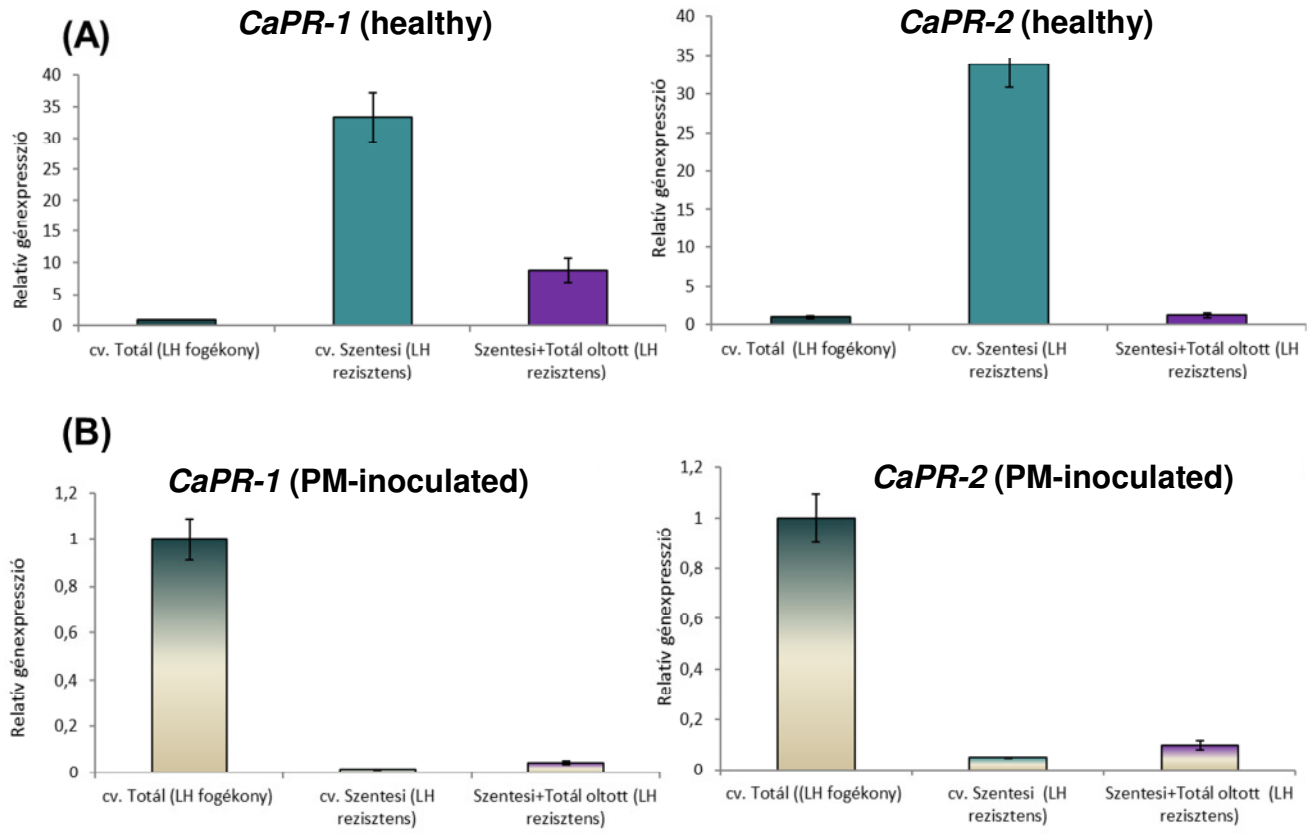


Fig. 24 Enhanced expression of pathogenesis-related genes (*CaPR-1* and *CaPR-2*) is a marker of graft-transmissible resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) in healthy **(A)** but not in PM-inoculated (45 DAI) **(B)** plant leaves. Expression of *CaPR-1* and *CaPR-2* as assayed by RT-qPCR in healthy/PM-inoculated leaves of susceptible (cv. Totál) and resistant (cv. Szentesi and Szentesi + Totál grafts) pepper, respectively. Gene expression was normalized by using a pepper actin gene (*CaAct*) as a reference.

We have been looking for additional molecular/biochemical markers of PM-resistance in pepper. Our attention turned towards genes that encode MLO, a cell death suppressor protein conferring susceptibility to PM pathogens in various hosts, including pepper. It has been shown that in pepper, two MLO-encoding genes (*CaMlo1* and *CaMlo2*) may confer PM-susceptibility (Zheng et al., 2013). Interestingly, it is also known that a lack of MLO-expression in barley mutants (*mlo*) confers spontaneous cell death and symptomless PM-resistance (Büschges et al., 1997). We found that cell death is far more intense in healthy leaf tissues of the PM-resistant cv. Szentesi, as opposed to the PM-susceptible cv. Totál (see Figure 23). This implies that a reduced (or zero) expression of either *CaMlo1* and/or *CaMlo2* might be associated with symptomless PM resistance. Our gene expression assays indeed demonstrated that expression of both *CaMlo1* and *CaMlo2* is significantly lower in both healthy and PM-infected resistant pepper (cv. Szentesi and Szentesi + Totál grafts), as opposed to PM susceptible cv. Totál plants **(Figure 25)**.

Therefore, a reduced expression of pepper genes encoding the cell death suppressor MLO (*CaMlo1*, *CaMlo2*), along with enhanced expression of pathogenesis-related (PR) genes (*CaPR-1*, *CaPR-2*) are molecular/biochemical markers that can predict PM-resistance in pepper.

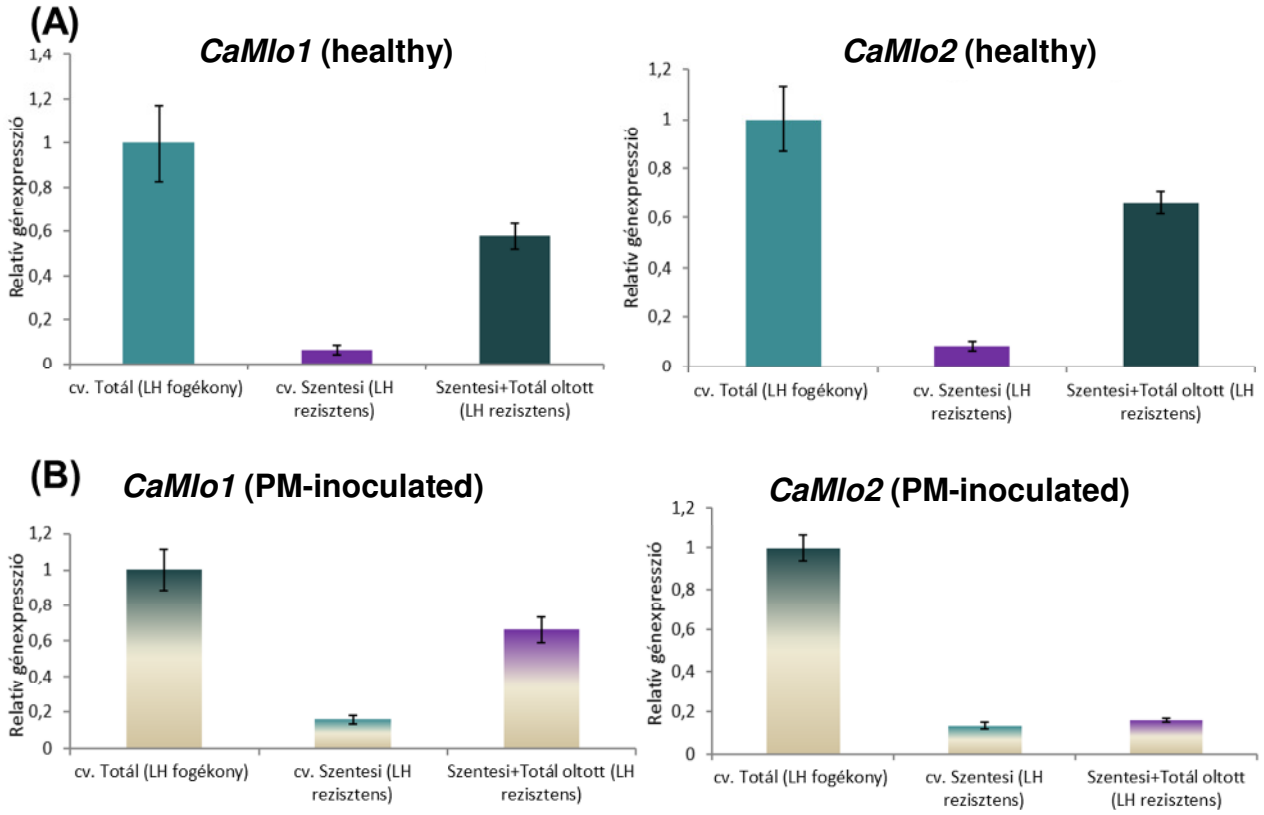


Fig. 25 Reduced expression of the *CaMlo1* and *CaMlo2* genes is a marker of graft-transmissible resistance of cherry pepper (*Capsicum annuum* var. *cerasiforme* cv. Szentesi) to pepper powdery mildew (PM) (*Leveillula taurica*) in healthy **(A)** and in PM-inoculated (45 DAI) **(B)** plant leaves. Expression of *CaMlo1* and *CaMlo2* as assayed by RT-qPCR in healthy/PM-inoculated leaves of susceptible (cv. Totál) and resistant (cv. Szentesi and Szentesi + Totál grafts) pepper, respectively. Gene expression was normalized by using a pepper actin gene (*CaAct*) as a reference.

Inheritance of biochemical markers of graft-transmissible resistance to powdery mildew in progeny of grafted, resistant pepper

We continued our experiments to monitor the inheritance of some of the above mentioned resistance markers (enhanced NADPH oxidase activity, enhanced expression of *CaPR-1*, *CaPR-2*, reduced expression of *CaMlo1*, *CaMlo2*) in the progeny of grafted resistant plants. We assayed markers in the progeny of two grafted plants (15 and 13 progeny individuals, respectively) and detected markers in more than half of the progeny. Inheritance of four markers (NADPH oxidase activity, high *CaPR1*, *CaPR2* and low *CaMlo2* expression) is likely linked since they were present mostly in the same plants (10 progeny individuals). Out of the 10 plants expressing the four markers mentioned above, four plants displayed all markers (including low *CaMlo1* expression).

It seems that in pepper most of the biochemical markers associated with graft-transmissible PM resistance are efficiently inherited in the progeny of grafted, resistant plants. To our knowledge our results provide the first evidence for the inheritance of biochemical markers of graft-transmissible PM resistance.

Inheritance of biochemical markers of graft-transmissible resistance to powdery mildew in progeny of crosses of self-rooted resistant and susceptible pepper

We also wanted to clarify whether the five biochemical PM-resistance markers mentioned above (NADPH oxidase activity, high *CaPR-1*, *CaPR-2* and low *CaMlo1*, *CaMlo2* expression) can be inherited in progeny of crosses of self-rooted resistant and susceptible pepper? To investigate this phenomenon, we crossed PM-resistant self-rooted 'Szentesi' cherry pepper to PM-susceptible 'Totál' sweet pepper [Totál (♀) x Szentesi (♂)].We monitored markers in 12 individuals of the F_1 progeny.

Resistance markers were manifested in more than half of F_1 individuals, except for enhanced NADPH oxidase activity and low *CaMlo1* expression which were detected in only a smaller portion of the progeny (2/12 and 3/12 F_1 plant). Inheritance of three markers (high *CaPR-1, CaPR-2* and low *CaMlo2* expression) is likely linked since they were present mostly in the same plants (6 F_1 individuals). Currently we are in the process of monitoring inheritance of these resistance markers in F_1 progenies of reciprocal crosses [Szentesi (♀) x Totál (♂)].

It seems that in pepper most of the biochemical markers associated with graft-transmissible PM resistance is efficiently inherited in the F_1 progeny of crosses of PM-resistant self-rooted 'Szentesi' cherry pepper and PM-susceptible 'Totál' sweet pepper.

Future research should clarify if inheritance patterns of PM-resistance are similar to those of the above-mentioned biochemical resistance markers.

Future perspectives of the research project

We have shown that accumulation of superoxide ($O_2^{\cdot-}$) and other ROS are pivotal factors of two symptomless (without HR-type localized noecrosis) forms of plant disease resistance: 1/ extreme resistance to a virus (PVX) in tobacco, 2/ graft-transmissible, symptomless resistance to powdery mildew in pepper.

Our results point to the fact that the outcome of plant defenses largely depends on the speed of host responses, including early ROS accumulation, at pathogen infection sites. Rapid host reactions may result in early pathogen elimination without any oxidative stress (i.e. a symptomless/extreme resistance). On the other hand a slower host response allows a certain degree of pathogen multiplication and movement, resulting in side effects of oxidative stress and programmed death of affected plant cells before conferring pathogen arrest (hypersensitive response, HR). Therefore, monitoring and characterizing symptomless plant disease resistance responses is of primary importance (currently gaining momentum in both basic and applied research) and should be a part of contemporary plant breeding programs. This could include e.g. 1/ identifying plant genes that determine symptomless (extreme) resistance (e.g. *Rx* orthologs), 2/ monitoring correlation of high ROS levels and symptomless (extreme) resistance in various plant-pathogen interactions, 3/ elucidating biochemical mechanims of (symptomless) graft-transmissible resistance, including the role of ROS.

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