THESES OF
DOCTORAL (PhD) DISSERTATION

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Role of the free radical superoxide in symptomless forms of plant disease resistance

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INTRODUCTION AND MAIN AIMS OF DOCTORAL RESEARCH

Although plants can successfully defend themselves against the infection of invading pathogens, this is often accompanied by obvious side-effects, e.g. the death (necrosis) of invaded plant tissues, slower growth, etc. Defense (resistance) against diseases is effective if it is a rapid process, eliminating the pathogen in due time and not overusing resources of the plant organism. In this case the host plant is able to resist infection by a given pathogen without the development of symptoms. Therefore, introduction of crop cultivars that display symptomless resistance to one or more pathogens is of primary importance for plant breeding and crop production.

The development of plant disease resistance is associated with several physiological processes, e.g. accumulation of antimicrobial compounds, plant cell wall fortification, localized plant cell and tissue death (hypersensitive response, HR) and accumulation of reactive oxygen species (ROS). ROS, in particular superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) play a dual role in plant disease resistance: in low concentrations they act as transducers of resistance signals, while in high amounts they can cause cell/tissue death in both the host plant and the pathogen.

The so-called extreme resistance conferred by the *Rx1* gene against *Potato virus X* (PVX) is symptomless, i.e. a highly efficient resistance without a hypersensitive reaction (HR, localized plant cell and tissue death) that is functional in both potato and tobacco (Bendahmane et al., 1999). Previous data suggest that the rapid and early inhibition of virus replication does not allow the development of HR-type symptoms. However, the actual mechanism of antiviral response is still elusive.

Our initial experiments at the Plant Protection Institute, CAR HAS demonstrated a slight but significant increase in superoxide levels in tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) that displays extreme resistance to PVX, as compared to susceptible plants. It seems that a possible cause of the symptomless, extreme resistance to PVX could be the very early superoxide accumulation conferred by the *Rx1* gene that may potentially inhibit virus replication.

Recently, graft-transmissible disease resistance is being also applied in vegetable production, primarily for controlling soilborne diseases (Louws et al., 2010; Al-Mawaali et al., 2012; Guan és Zhao, 2012). However, the exact physiological, biochemical and genetic background of graft-transmissible resistance is largely unknown.

Preliminary investigations at the Plant Protection Institute, CAR, HAS and Faculty of Agriculture, University of Szeged have shown that cherry pepper (*Capsicum annuum* var. cerasiforme) resistant to pepper powdery mildew (*Leveillula taurica*) may transfer disease resistance as a rootstock to susceptible sweet pepper (*C. annuum*) as a scion.
Interestingly, both in cherry pepper cv. Szentesi displaying enhanced, symptomless powdery mildew resistance and in the grafted, originally susceptible cultivar the accumulation of superoxide was significantly higher than in self-rooted susceptible pepper.

Main aims of doctoral research

1. Pinpointing a functional role of early superoxide accumulation in virus-infected tobacco during the so-called extreme (symptomless) resistance conferred by the *Rx1* gene against *Potato virus X* (PVX)?
    - What is the effect of *in planta* reduction of superoxide levels on extreme resistance (i.e. infiltration of antioxidants or crossing resistant tobacco with plants inhibited in ROS production)?
    - Can PVX resistance be conferred in susceptible tobacco by enhancing superoxide production via ROS-producing agents?
    - Characterizing changes in expression patterns of stress (defense) related tobacco genes during symptomless extreme resistance to PVX, as compared to the hypersensitive (HR, localized plant cell and tissue death) resistance to *Tobacco mosaic virus* (TMV).
2. Elucidating the physiological background of graft-transmissible, symptomless resistance of cherry pepper (*Capsicum annuum* var. cerasiforme) to pepper powdery mildew (*Leveillula taurica*).
    - Is the graft-transmissible, symptomless powdery mildew resistance of cherry pepper associated with pathogen inhibition?
    - Relationship of high superoxide levels in powdery mildew resistant plants with disease resistance and plant defense responses.
    - Monitoring inheritance of biochemical resistance markers (gene expression and enzyme activity changes associated with superoxide accumulation) in progeny of powdery mildew resistant self-rooted and grafted pepper.

MATERIALS AND METHODS

A tobacco line (*Nicotiana tabacum* cv. Samsun) carrying the *Rx1* and *N* resistance genes (Bendahmane et al., 1999) was used along with two tobacco lines (cv. SR1: C8 and F9) overproducing an alfalfa ferritin gene (Deák et al., 1999). Plants were grown under standard greenhouse conditions for 8 weeks. Reciprocal crosses of tobacco lines to obtain F_1 progeny were also conducted in the greenhouse.
Sweet pepper (*Capsicum annuum*) cv. Totál was used along with cherry pepper (*C. annuum* var. *cerasiforme*) cv. Szentesi and cherry pepper cultivars 'Kalocsai A', 'Kalocsai M', 'Globál', 'Koral', 'Garai Fehér', 'Óriás' and grafts of 'Totál' and 'Szentesi' cherry pepper (Totál grafted on Szentesi rootstocks). F_1 progenies from crosses of Totál (♀) x Szentesi (♂) were also used. Plants were grown under standard greenhouse conditions for 14 weeks. Crosses of pepper cultivars to obtain F_1 progeny were also conducted in the greenhouse.
Grafting was carried out on 8 week-old plants. Stems of rootstock and scion plants were cut above cotyledons in a 45 degree angle with a razor blade. Rootstock (cv. Szentesi, with cotyledons) and scion (cv. Totál, above cotyledons) parts were paired with the aid of grafting clips. Two weeks acclimation (in plastic cages, ca. 25 °C and 90% relative humidity) was allowed for the development of graft unions. Grafting efficiency following acclimation was ca. 65 %.

List of pathogens used in the experiments

- *Tobacco mosaic virus* (TMV, U1 strain)
- *Potato virus X* (PVX, Ny isolate) (Ahmadvand et al., 2012)
- Pepper powdery mildew (*Leveillula taurica*)

Viruses were maintained on susceptible tobacco hosts (TMV: *Nicotiana tabacum* cv. Samsun *nn*, PVX: *Nicotiana tabacum* cv. Xanthi *NN*) in the greenhouse (TMV) and in Panasonic MLR-352 growth chambers (PVX). In growth chambers plants were grown under a 16 hour (long day) photoperiod, with a light intensity of 110 µE m^2 s^{-1}. Daytime and night temperatures were 25 ^oC and 22 ^oC, respectively. For virus inoculations, upper leaves of infected, susceptible plants were ground with a mortar and pestle in tap water and carborundum as an abrasive (ca. 1 g leaf / 10 ml tap water). The produced inoculum was used for mechanical virus inoculations. Control (mock) inoculations were done with tap water and carborundum.

A pepper powdery mildew (*L. taurica*) line isolated by us from a production greenhouse in Szentes was maintained on a susceptible pepper host (cv. Totál) in a Panasonic MLR-352 growth chamber, under conditions described above for tobacco. Artificial powdery mildew inoculation was conducted as described by Zheng and coworkers (2013a, b) except that pepper leaves were inoculated with a powdery mildew suspension (25,000 conidia/ml) by a fine paint brush.

Detection of superoxide accumulation

Superoxide radical ($O_2^{\bullet -}$) accumulation was detected by histochemical staining with nitro blue tetrazolium chloride (NBT) (Sigma-Aldrich, Germany). Superoxide reacts with NBT producing dark blue formazane that is detectable with the naked eye. The NBT solution was administered to leaf tissues by vacuum infiltration (Ádám et al., 1989). Infiltrated leaves were placed in a clearing solution for 2 days (Hückelhoven és Kogel, 1998). NBT staining was quantified by the ImageJ computer software (https://imagej.nih.gov/ij/).

Detection of single cell death in pepper leaf tissues

Dead cells in pepper leaf tissues were visualized by a lactophenol Trypan blue staining as described by Pogány et al. (2009).

Conferring PVX susceptibility in resistant tobacco by infiltration of antioxidant enzymes (SOD and CAT)

PVX infected leaf halves of resistant (cv. Samsun *NN Rx1*) and susceptible (cv. Samsun *NN*) control tobaccos were infiltrated with superoxide dismutase (SOD, 3000 U/ml) and catalase (CAT, 5000 U/ml) enzymes dissolved in 10 mM potassium phosphate buffer (pH=7,8). The other (control) leaf halves were infiltrated with 10 mM potassium phosphate buffer (pH=7.8).

Conferring PVX resistance in susceptible tobacco by infiltration of a superoxide (ROS) generating agent

PVX infected leaf halves of susceptible (cv. Samsun *NN*) tobaccos were infiltrated 2 hours after virus inoculation with a superoxide ($O_2^{\cdot -}$) generating agent (66 µM riboflavine, 10 mM L-methionine) as described earlier (Bacsó et al., 2011). The other (control) leaf halves were infiltrated with 10 mM potassium phosphate buffer (pH=7.8).

Gene expression assays, detection of PVX and TMV RNA and pepper powdery mildew (*L. taurica*) DNA

Total plant RNA was extracted from tobacco and pepper leaves (healthy and infected with PVX and TMV viruses and pepper powdery mildew, respectively) with a method involving silicagel membrane minicolumns (Plant Total RNA Extraction Miniprep System, Viogene, and SV Total kit, Promega, USA). Amount and purity of the obtained plant total RNA was verified with a spectrophotometer (NanoDrop ND-1000). For gene expression assays we applied reverse transcription polymerase chain reaction (RT-PCR). First mRNAs were amplified then the cDNA(s) corresponding to a given gene(s) was amplified by PCR according to instructions of the kit manufacturer (KAPA Biosystems, USA). Following RT-PCR, samples were run on 1 % agarose gels stained with GelRed (GelRed Nucleic Acid Gel Stain, 10 000X, Biotium Inc., USA), conferring UV fluorescence of nucleic acids, in order to determine gene expression differences. For reference (constitutive control), expression of a tobacco and pepper actin gene was used. Agarose gels were photographed under UV light (λ = 302 nm) in a gel documentation system (AlphaImager EP). The above mentioned method is suitable for semiquantitative assays of gene expression. For a more sensitive, quantitative assessment of plant gene expression we used the so-called real time quantitative RT-PCR method, according to instructions of the reagent manufacturer (KAPA Biosystems, USA). Following PCR, data were evaluated by a computer software (CFX Manager™ 2.1) provided by the manufacturer of the thermocycler (BIO –RAD CFX 96, Bio-Rad Laboratories, USA) with the aid of an Excel program. For quantification of gene expression, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Schmittgen és Livak, 2008) was applied, expression of the genes mentioned above (tobacco and pepper actin genes) served as internal reference. The gene expression assay methods mentioned above were also used for assessing accumulation of two, tobacco-infecting viruses (PVX and TMV) on the level of viral RNAs.

For assessing levels of *L. taurica*, total genomic DNA was extracted from infected and healthy (control) plants. Plant leaves frozen in liquid nitrogen were ground in a mortar and pestle, DNA extraction was performed from ground tissue samples (REDExtract-N-Amp™ Plant PCR Kit, Sigma Aldrich, Germany). A portion of a specific fungal ITS sequence (*LtLV*) was amplified by semiquantitative PCR (KAPA Taq PCR Kit, KAPA Biosystems, USA). A pepper actin gene was used as a reference. The obtained PCR products were run on 1 % agarose gels

stained with GelRed, conferring UV fluorescence. Agarose gels were photographed under UV light (λ = 302 nm) in a gel documentation system (AlphaImager EP). The above mentioned method is suitable for semiquantitative assays of total genomic (plant and fungal) DNA. For quantification of fungal (powdery mildew) biomass a real time quantitative PCR method was used.

Assays of free and bound salicylic acid in pepper plants

For assays of free and bound (acid hydrolyzable) salicylic acid the method described by Meuwly és Métraux (1993) and Cole et al. (2004) was used. Assays were done by Dr. Gabriella Szalai and her research group (Agriculutre Institute, CAR, HAS, Martonvásár).

Assaying NADPH oxidase enzyme activity in pepper tissues

NADPH oxidase enzyme activity in healthy and powdery mildew-inoculated pepper leaves was assayed spectrophotometrically, essentially as described earlier (Ádám et al., 1997 és Xia et al., 2009).

Determination of glutathione in tobacco infected by TMV and PVX

Glutathione levels were assayed 4 days after inoculation (including mock-inoculated and healthy controls) according the method of Griffith (1980). The 2:30 min kinetics of glutathione accumulation was assayed spectrophotometrically (Shimadzu, UV-160A) at λ = 412 nm.

Assaying glutathione reductase and glutathione S-transferase enzyme activities in tobacco infected by TMV and PVX

Enzyme activities were assayed 4 days after inoculation, including leaf tissues of mock-inoculated and healthy control plants.

Glutathione reductase activity

Glutathione reductase (GR) enzyme activities were assayed by monitoring NADPH consumption with a spectrophotometer (Klapheck et al., 1990).

Glutathione S-transferase activity

Conjugation reactions of reduced glutathione (GSH) are catalyzed by glutathione S-transferase (GST) enzymes. Enzyme activities were assayed by monitoring reaction products of GSH conjugation reactions spectrophotometrically at λ = 340 nm (Mauch and Dudler, 1993).

RESULTS AND DISCUSSION

Transgenic „Rx" tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) expresses the *Rx1* resistance gene of potato, conferring extreme resistance (ER) to *Potato virus X* (PVX) (Bendahmane et al., 1999).We have shown that „Rx" tobacco displays symptomless ER also towards a Hungarian strain of PVX (PVX Ny): besides an absence of localized plant cell and tissue death (hypersensitive reaction, HR) PVX titers in extreme resistant tobaccos by the 5th day of infection are only a fraction of those present in susceptible plants. The most likely cause of ER is the rapid and early inhibition of virus replication that does not allow development of HR-type symptoms. However, the actual mechanism of antiviral response is still elusive. Our results show that a reactive oxygen species (ROS) with a role in virus resistance, superoxide ($O_2^{\bullet -}$), accumulates in „Rx" tobacco displaying ER at early time points after PVX inoculation (within 6 hours after inoculation, HAI). Remarkably, PVX titers start to significantly decline only following 6 HAI, showing that a possible cause of symptomless ER against PVX could be the very early accumulation of superoxide conferred by the *Rx1* gene that may inhibit virus replication.

Reduction of superoxide levels in PVX-infected „Rx" tobacco by treatments with antioxidants (superoxide dismutase and catalase) can partially break ER: local necrotic symptoms resembling HR appear and PVX titers significantly increase. Crossing „Rx" tobacco with ferritin overproducing plants (inhibited in production of the hydroxyl radical /$OH^{\cdot}$/) also results in a partially broken ER in the F_1 progeny: during the first 2 days after infection virus levels in inoculated leaves are significantly higher than in „Rx" tobacco and HR-type lesions develop. It seems that a decline in ROS levels due to ferritin overproduction can also confer a reduction of ER during PVX infection. The possible role of superoxide in ER is supported by our results showing that in PVX susceptible tobacco (cv. Samsun NN) treatments with a superoxide generating agent (riboflavin/methionine) can confer partial PVX resistance: localized necrotic symptoms resembling HR appear and PVX titers are significantly lower as compared to untreated plants. Therefore, artificial elevation of superoxide levels in susceptible tobacco cannot confer ER but is capable of conferring a slower, HR-type of resistance that can partially inhibit PVX replication.

Comparing the symptomless, extreme resistance (ER) to PVX to the HR-type of resistance elicited by *Tobacco mosaic virus* (TMV) in „Rx" tobacco we found that during HR virus titers (TMV) are almost an order

of magnitude higher than during symptomless ER. Also, expression of 3 defense/stress-related genes (*NtPR-1a, NtPRB-1b* és *NtGStphi*) and 3 cell death/ROS-regulator genes (*NtBI-1, NtAOX1-2, NtCat1*), along with amounts of a non-enzymatic antioxidant, glutathione, and activities of 2 antioxidant enzymes (glutathione reductase, glutathione S-transferase) are significantly increased during HR. while only slightly changed during ER. The almost undetectable defense processes during symptomless ER seem to be a reliable indicator of the rapid development of virus (PVX) inhibition.

In certain cases plant disease resistance is graft-transmissible, however, the exact biochemical and molecular background of this type of resistance is largely unknown. We found that a sweet pepper cultivar (*Capsicum annuum* cv. Totál) susceptible to pepper powdery mildew (*Leveillula taurica*) becomes resistant when grafted onto a resistant cherry pepper rootstock (*C. annuum* var. *cerasiforme* cv. Szentesi). We have demonstrated that the graft-transmissible, symptomless (without HR) powdery mildew resistance of 'Szentesi' cherry pepper is also effective following artificial infections during controlled laboratory conditions and associated with a significant (50 %) inhibition of pathogen (*L. taurica*) accumulation. In powdery mildew-resistant pepper (self-rooted 'Szentesi' and Szentesi+Totál grafts) a dramatic accumulation of superoxide ($O_2^{\bullet -}$) can be detected already in leaves of healthy, uninfected plants. Activites of NADPH oxidase, the enzyme largely responsible for superoxide production during plant disease resistance correlates well with superoxide accumulation and powdery mildew resistance. Powdery mildew-susceptible 'Totál' sweet pepper becomes resistant and produces large amounts of superoxide when grafted unto either 'Szentesi' or other resistant cherry pepper rootstocks ('Kalocsai A', 'Kalocsai M', 'Garai fehér'). Levels of free salicylic acid (SA) in uninfected leaves of powdery mildew-resistant peppers is ca. twice as high as in susceptible 'Totál' individuals, however, bound SA levels are unchanged. In uninfected leaves of self-rooted 'Szentesi' the extent (density) of spontaneous cell death along the veins is far more intensive than in self-rooted 'Totál'. In uninfected plants expression of a pathogenesis-related gene (*CaPR-1*) in powdery mildew resistant pepper (self-rooted 'Szentesi' and Szentesi+Totál grafts) is a marker of resistance, while elevated expression of *CaPR-2* can be detected only in self-rooted 'Szentesi' cherry pepper. On the other hand, in advanced stages of powdery mildew pathogenesis (45 DPI) the pattern of PR-gene expression is reversed, since in this case very high levels of *CaPR-1* and

CaPR-2 expression correlate with powdery mildew susceptibility. Reduced expression of 2 cell death regulator genes (*CaMlo1* and *CaMlo2*) also proved to be a marker of powdery mildew resistance, both in uninfected and powdery mildew-infected pepper.

In pepper, biochemical markers associated with graft-transmissible powdery mildew resistance (NADPH oxidase activity, high *CaPR1* and *CaPR2*, low *CaMlo1* and *CaMlo2* expression) were detected in ca. half of the progeny of grafted, resistant pepper (Szentesi+Totál). 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 individuals. Three biochemical resistance markers (high *CaPR1*, *CaPR2* and low *CaMlo2* expression) were also inherited in more than half of the F_1 progeny of crossed self-rooted susceptible and resistant pepper [Totál (♀) x Szentesi (♂)]. Inheritance of these 3 resistance markers is likely linked since they were present mostly in the same F_1 progeny individuals.

Our results demonstrate that accumulation of an important plant ROS, superoxide ($O_2^{\bullet -}$) can be a major factor of two, symptomless (without HR-type necrosis) types of resistance: in tobacco, extreme resistance to PVX and in pepper, graft-transmissible resistance to powdery mildew (*L. taurica*). Further research should clarify the exact function of superoxide in these effective, symptomless types of plant disease resistance.

NOVEL SCIENTIFIC RESULTS OF DOCTORAL RESEARCH

- A reactive oxygen species (ROS), superoxide ($O_2^{\bullet -}$), accumulates at early time points after *Potato virus X* (PVX) inoculation (within 6 hours after inoculation) in „Rx" tobacco (*Nicotiana tabacum* cv. Samsun *NN Rx1*) displaying symptomless extreme resistance (ER).
- Reduction of early $O_2^{\cdot -}$ accumulation in PVX-infected „Rx" tobacco by antioxidant treatments (superoxide dismutase and catalase) can partially break ER, while in PVX susceptible tobacco (cv. Samsun NN) treatments with a superoxide generating agent (riboflavin/methionine) confer partial PVX resistance: local necrosis resembling HR appear and PVX titers are significantly lower than in untreated plants.
- Crossing „Rx" tobacco with ferritin overproducing plants (inhibited in $OH^{\cdot}$ production) also results in a partially broken ER in the F_1 progeny. Activities of certain defense-related and ROS-regulator genes (*NtPR-1a*, *NtPRB-1b*, *NtGStphi*, *NtBI-1*, *NtAOX1-2*, *NtCat1*) and antioxidants (glutathione, glutathione reductase, glutathione S-transferase) are significantly increased during HR. while only slightly changed during ER.
- In powdery mildew-resistant pepper (self-rooted 'Szentesi', Szentesi+Totál grafts) $O_2^{\cdot -}$ accumulation can be detected in leaves of both healthy (uninfected) and infected plants. Activites of $O_2^{\bullet -}$ producing NADPH oxidase correlate with $O_2^{\bullet -}$ levels and graft-transmissible powdery mildew resistance.
- In uninfected plants expression of a PR gene (*CaPR-1*) in powdery mildew resistant pepper ('Szentesi' and Szentesi+Totál grafts) is a marker of resistance, while elevated *CaPR-2* expression can be detected only in 'Szentesi'. In late stages of pathogenesis (45 DPI) *CaPR-1* and *CaPR-2* expression correlate with susceptibility. Reduced expression of 2 cell death regulator genes (*CaMlo1* and *CaMlo2*) is also a marker of resistance, both in uninfected and powdery mildew-infected pepper.
- In pepper, biochemical markers of graft-transmissible powdery mildew resistance (NADPH oxidase activity, high *CaPR1* and *CaPR2*, low *CaMlo1* and *CaMlo2* expression) can be detected in ca. half of the progeny of grafted, resistant pepper (Szentesi+Totál). Three markers (high *CaPR1*, *CaPR2* and low *CaMlo2* expression) are also inherited

in ca. half of the F_1 progeny of susceptible and resistant pepper [Totál (♀) x Szentesi (♂)], mostly in the same plants (linked inheritance).

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