THESES OF
DOCTORAL (PhD) DISSERTATION

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Elucidating biochemical and molecular biological mechanisms of certain forms of plant resistance

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INTRODUCTION AND MAIN AIMS OF DOCTORAL RESEARCH

Both plant and animal (human) disease resistance has several different forms. In both cases one can differentiate among innate and acquired resistance. With innate resistance, one can distinguish non-specific (general) and specific forms of resistance. Acquired resistance is executed through immune memory (animals) or stress memory (plants). The different forms of plant disease resistance are summarized in Table 1.

Table 1.

Overview of forms of plant disease resistance (cf. Király et al., 2007)

Forms of plant disease resistance	**Mechanism**
INNATE RESISTANCE	
Non-specific, general resistance	
- Non-host resistance	HR, ROS, BAX-inhibitor, PEN genes
- Basal resistance against bacteria	Flagellin/FLS2 interaction, ROS, antimicrobial compounds
- Race non-specific *mlo* resistance and quantitative resistance to fungi	Cell wall thickening Antimicrobial compounds, ROS
- Resistance to necrosis-inducing stresses	High antioxidant capacity
Specific resistance (cultivar/pathogenic race specificity)	
- Extreme resistance – symptomless gene-for-gene resistance	Unknown
• Rx-resistance against viruses without HR	
• Symptomless reaction to rust pathogens, no visible HR	
- Gene-for-gene resistance *R*-gene/*Avr*-gene interaction associated with HR	ROS, phytoalexins, phenol oxidation, stress proteins
- Resistance to pathogen toxins	Enzymatic detoxification Lack of toxin receptors
- Gene silencing	Recognition and degradation of foreign RNAs with ribonucleases
ACQUIRED RESISTANCE	
After a primary infection an acquired resistance develops against a second infection "Stress memory"	Salicylic acid, antioxidants, Gene silencing, Rhizobacteria

Non-specific innate resistance may be sufficient against a variety of pathogens. Specific resistance means that a given plant *cultivar* displays resistance to only one or a few types of plant *pathogenic strains* (races) but not to others. Therefore, this is a cultivar/race type of specificity. This resistance is often associated with the formation of localized necrosis (hypersensitive reaction, HR) at infection sites, but sometimes it is asymptomatic.

The most typical form of non-specific disease resistance in the plant kingdom is **non-host resistance**. In nature, plant-pathogen interactions usually result in asymptomatic non-host resistance, i.e. every plant individual is resistant, because it is unsuitable to be a host for pathogens. Although this phenomenon has been known for a long time, it is still not clear what inhibits or kills the pathogen in non-host resistant plants (cf. Schulze-Lefert and Panstruga, 2011). Results of several research groups - including those at the Plant Protection Institute - suggest that a primary cause of non-host resistance could be the interaction of so-called reactive oxygen species (ROS) and ROS-neutralizing antioxidants (Ádám et al., 1989; Hafez and Király, 2003; Apel and Hirt, 2004).

Plants respond to different stresses (eg, drought, temperature changes, pathogen infection) in diverse ways including accumulation of reactive oxygen species (ROS) that leads to oxidative stress. Plant oxidative stress results in perturbation of the balance of ROS (prooxidants) and antioxidants in favor of ROS. Reactive oxygen species include oxygen free radicals with an unpaired electron (e.g. $O_2^{\bullet-}$, $OH^{\cdot}$) and molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) that are capable of generating free radicals.

Doke and coworkers (Doke, 1983; Doke és Ohashi, 1988) were the first to draw attention to the role of the ROS superoxide ($O_2^{\bullet-}$) in specific (HR-type) disease resistance. Later, different groups have pointed to the role of several ROS in HR symptomatic non-host resistance (Bestwick et al., 1998; Zurbriggen et al., 2009; Kwak et al., 2009).

Another form of non-specific plant disease resistance is **resistance to necrotic symptoms**. In this case, plants are resistant only to disease symptoms, inhibition or killing of the pathogen does not necessarily take place (Barna et al., 1993; Devadas and Raina, 2002). In this type of resistance salicylic acid (SA) and certain antioxidants play a pivotal role (Barna et al., 1993; Vlot et al., 2009). A starting point of my own experiments was the observation of Cole et al. (2004): resistance to necrotic symptoms following virus infections is different in two interspecific tobacco hybrids derived from the same parents (*Nicotiana glutinosa* x *N.*

clevelandii). One of the interspecific hybrids, *N. edwardsonii* var. Columbia displayed enhanced resistance to symptoms of virus infections caused by *Tobacco mosaic virus* (TMV) and *Tobacco necrosis virus* (TNV) and contained high levels of SA even in a healthy state, as compared to the other hybrid, *N. edwardsonii*.

One of the recently discovered forms of specific plant disease resistance is **posttranscriptional gene silencing** (PTGS). This defense mechanism had been evolved against "parasitic" nucleic acids like viroids, viruses and transposons. For instance, during virus infections the plant considers viral RNA as foreign. In other words, viral RNA induces gene silencing against itself resulting in the degradation of viral nucleic acids (Baulcombe, 1996; Burgyán, 2007). The silenced gene is transcribed but the messenger RNA is degraded. This mechanism is an unwanted phenomenon during breeding of transgenic plants because the gene of interest can be occasionally silenced. On the other hand, the same mechanism is beneficial when investigating the function of a plant gene by silencing this particular gene.

This dissertation intends to clarify the biochemical and molecular biological mechanisms of two forms of non-specific plant disease resistance: non-host resistance and a type of resistance to necrotic symptoms. In addition, a new case of specific resistance is characterized, where silencing of a resistance gene, effective against a given virus, results not in susceptibility but resistance to another unrelated virus.

Main aims of doctoral research:

1. Clarifying the role of prooxidants (reactive oxygen species, ROS), in particular superoxide, and antioxidants in asymptomatic (without HR) non-host resistance, during incompatible reactions of barley to barley- and wheat powdery mildew.

2. Do antioxidants and salicylic acid play a pivotal role in a type of resistance to necrotic symptoms?

3. Does silencing of a virus resistance gene (*N*) affects infection by another, unrelated virus?

MATERIALS AND METHODS

List of plant material used in the experiments:

Hordeum vulgare cv. Ingrid *Mla*, *Mlo*, *mlo H. vulgare* cv. Botond
Triticum aestivum cv. MV-Emma, *T. aestivum* cv. Buzogány
Cucumis sativus cv. Budai csemege, *C. sativus* cv. Rajnai fürtös
Solanum lycopersicum cv. Kecskeméti 549, cv. Kecskeméti 3F
Nicotiana tabacum cv. Xanthi, *N. tabacum* cv. Xanthi *nahG*
N. benthamiana N. edwardsonii, *N. edwardsonii* var. Columbia
Solanum tuberosum cv. White Lady, *S. tuberosum* cv. Hópehely
Vitis vinifera cv. Nimrang, cv. Kismish vatkana, cv. Bianca

List of pathogens used in the experiments:

Barley powdery mildew (*Blumeria graminis* f.sp. *hordei* race A6)
Wheat powdery mildew (*Blumeria graminis* f.sp. *tritici* Hungarian isolate)
Cucumber powdery mildew (*Podosphaera xanthii*)
Tomato powdery mildew (*Oidium neolycopersici*, BP-P5)
Tobacco powdery mildew (*Golovinomyces orontii*, BP-1TOB)
Grapevine powdery mildew (*Erisyphe necator*)
Late blight pathogen (*Phytophthora infestans*)
Barley leaf rust (*Puccinia hordei*)
Wheat leaf rust (*Puccinia recondita* f. sp. *tritici*)
Crown rust (*Puccinia coronata* f.sp. *avenae*)
Pseudomonas syringae pv. *tomato* DC3000 *P. syringae* pv. *tabaci*
Tobacco mosaic virus (TMV) U1 strain
Tobacco necrosis virus (TNV) E strain

Inoculations with wheat and barley powdery mildews were done in an inoculation tower by spraying conidia on 7 days old, one leaf stage plants. In case of inoculations with tomato, cucumber, tobacco and grapevine powdery mildews, healthy leaves were touched by infected leaves serving as inoculum source.

7 days old, one leaf stage plants were inoculated with wheat leaf, barley leaf and crown rusts. Uredosores were suspended in a starch solution (3.3 g /100 ml tap water).

For inoculations with *Phytophthora infestans* a sporangia suspension was sprayed on plant leaves.

For inoculations with *Pseudomonas syringae* a suspension ($7x10^5$ cfu/ml and $7x10^8$ cfu/ml) was obtained from a 1 day old bacterial culture. The suspension was infiltrated into wounded leaves.

During inoculations with TMV and TNV, leaves serving as virus inoculum source were homogenized with a mortar and pestle with carborundum and tap water (ca. 1 g leaf and 10 ml tap water). This inoculum was rubbed onto healthy leaves.

External application of superoxide dismutase and catalase to barley leaves

For treatments with antioxidants, aqueous solutions of superoxide dismutase and catalase (3000 U/ml and 5000 U/ml, respectively) were infiltrated into detached barley leaves (Király et al., 2008).

Heat shock treatment of barley leaves

Intact barley leaves were immersed into warm water (49 ºC) for 45 seconds. Infection was conducted after leaves have dried.

Detection of superoxide accumulation by a biochemical method

The superoxide radical reacts with nitro blue tetrazolium (NBT) resulting in generation of dark blue formazan. NBT was introduced into leaves by vacuum infiltration (Ádám et al., 1989). Leaves were illuminated for 20 minutes and subsequently placed into a destaining solution. The temporal appearance of blue color was monitored.

Gene expression assays

Total RNA from infected leaves was extracted by a silicagel membrane minicolumn protocol. For synthesis and amplification of cDNAs representing the gene of interest, total RNA was subjected to nucleic acid amplification preceded by reverse transcription (RT-PCR). For detection of gene expression differences, samples were separated on 1 % agarose gels. References (constitutive controls) of gene expression were a tobacco actin gene and a barley ubiquitin gene. For quantitative detection of gene expression we used a real time quantitative RT-PCR procedure. In order to quantify differences in gene expression we employed the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Internal references were the genes mentioned before.

Assays of free and bound salicylic acid

To assay levels of free and bound (acid hydrolysable) salicylic acid we used the method published by Meuwly and Métraux (1993) and Cole et al. (2004). Salicylic acid assays were done by high performance liquid chromatography (HPLC) with the aid of a deactivated, reverse phase column and a fluorometric detector.

Paraquat tratments

25 μM and 50 μM paraquat solutions were prepared in tap water. Solutions were infiltrated into leaves, infiltrated areas were marked. Resistance to paraquat was judged by assessing the area and spreading of tissue necrosis. For negative controls leaves were infiltrated with tap water.

Detection of plant pathogenic viruses

Detection of TMV and TNV by enzyme linked immunosorbent assay (ELISA)

Assessment of virus titers (detection of viral coat protein with ELISA) was done based on the method of Clark and Adams (1977) and Tóbiás et al. (1982). For TMV and TNV detection, kits from Bioreba (Reinach, Switzerland) and Loewe (Sauerlach, Germany) were used, including antibodies generated against serotypes TMV U1 and TNV-E, respectively.

Detection of genes encoding TMV and TNV coat proteins by reverse transcription polymerase chain reaction (RT-PCR)

For the detection of plant pathogenic viruses total RNA from infected leaves was extracted by a silicagel membrane minicolumn protocol. For synthesis and amplification of cDNAs representing the virus gene of interest, total RNA was subjected to nucleic acid amplification preceded by reverse transcription (RT-PCR). For cDNA synthesis reverse primers based on the sequence of the virus gene of interest were used. The cDNA obtained was subjected to PCR with virus gene specific primers.

Detection of plant pathogenic bacteria (*Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *tabaci*)

To determine bacterial numbers leaf discs (0.9 cm in diameter) cut from leaves of infected plants were homogenized in a 10 mM potassium phosphate buffer (pH 7.0) and transferred onto solid King's B medium in serial dilutions. Bacterial numbers were calculated based on the number of appearing colonies (Ott et al. 2006).

Assays of NADPH oxidase enzymatic activities

To detect NADPH oxidase enzymatic activities first a cell membrane fraction was isolated from infected leaves (Xia et al. 2009). During a photometric assay 50 µl suspended precipitate was added to the reaction mixture (50 mM HEPES pH 6.8, 0.2 mM NADPH, 0.3 mM NBT) (Ádám et al. 1997).

RESULTS AND DISCUSSION

1. Clarifying a core mechanism of non-host and host resistance

I have determined the temporal accumulation of superoxide ($O_2^{\bullet-}$) in twenty one host/pathogen combinations. These combinations included susceptible, host resistant and so-called non-host resistant plants. Superoxide accumulation was tested by nitro blue tetrazolium (NBT) staining.

- In case of the tested susceptible host/pathogen interactions no superoxide ($O_2^{\bullet-}$) accumulation can be detected. In host resistant plants (with HR-type symptoms) this ROS accumulates 48 hours after inoculation. However, in non-host resistant plants, $O_2^{\bullet-}$ accumulates always earlier (i.e. 24 hours after inoculation). This early superoxide accumulation is associated with the symptomless (HR^-) phenotype of non-host resistant interactions. Furthermore, early $O_2^{\bullet-}$ accumulation is closely related to the enhanced activation of NADPH oxidase, the enzyme that plays a pivotal role in superoxide generation during plant pathogenesis.

- In certain cases of non-host resistance a *SOD* and *BAX-inhibitor1* gene is transiently activated and then repressed. This phenomenon is also linked to the lack of HR-type symptoms.

- If $O_2^{\bullet-}$ generation is inhibited or suppressed by heat shock in mildew-infected, resistant barley, a susceptible reaction is promoted in the originally resistant leaves. This implies that ROS (primarily $O_2^{\bullet-}$ but likely also H_2O_2 and $OH^{\bullet}$) are important, although not the exclusive, determinants of pathogen inhibition or suppression.

2. Characterization of a type of resistance to necrotic symptoms

Nicotiana edwardsonii is an interspecific hybrid derived from the cross of two Nicotiana species (*Nicotiana glutinosa* x *N. clevelandii*). A more recent, similar cross resulted in the hybrid *N. edwardsonii* var. Columbia. The two hybrids are derived from the same parents but they display a different degree of resistance to necrotic symptoms of virus infections. Characterization of the mechanism of this resistance I have obtained the following results:

- *N. edwardsonii* var. Columbia displays a true resistance to *Tobacco necrosis virus* (TNV), i.e. both virus replication and the expression of localized necrotic symptoms are suppressed, when compared to the reaction of the other hybrid, *N. edwardsonii*. However, if 'Columbia' plants are inoculated with *Tobacco mosaic virus* (TMV) enhanced resistance is manifested primarily as a resistance to localized necrotic symptoms but virus replication is not significantly inhibited.

- If var. Columbia plants are crossed with a transgenic tobacco line (*nahG*) unable to accumulate salicylic acid (SA), levels of SA is reduced to a minimum and enhanced virus resistance is lost (in case of both TNV and TMV infections). Therefore, SA indeed plays a pivotal role in this resistance of 'Columbia' plants.

- Based on the experiments described it seems that 'Columbia' plants display a constitutively activated "acquired" resistance because these plants contain an elevated level of not only SA but also superoxide, even in their healthy state, as compared to the other hybrid, *N. edwardsonii*.

- Expression of a resistance marker, a gene encoding pathogenesis-related protein 1 (*NgPR1*) is very high in 'Columbia' plants, even in their healthy state, while in the more susceptible *N. edwardsonii* expression of this marker is hardly detectable even after virus infection. Therefore, activation of the *NgPR1* gene is a reliable marker of the enhanced resistance of 'Columbia' plants.

- The salicylic acid-based enhanced resistance of *N. edwardsonii* var. Columbia is also effective against bacteria that cause necrotic symptoms and against abiotic stresses (exposure to paraquat).

3. Effect of silencing of the TMV resistance gene *N* on infection of an unrelated virus (TNV)

Results of this research answer the question "does the posttranscriptional silencing of the TMV resistance gene *N* influences infection of an unrelated virus, TNV"?

- If we silence the resistance gene *N* in *Nicotiana edwardsonii*, resistance to TMV is impaired, in other words, susceptibility is increased, because both local and systemic virus movement in the silenced plants is visible several days earlier than in the non-silenced controls.

- Silencing of the *N* gene in TNV-infected *N. edwardsonii* does not reduce but, on the contrary, increases resistance. This means that both TNV levels and the extent of HR-type symptoms significantly decrease. Therefore, silencing of the *N* gene has a completely opposite effect on TNV than on TMV.

- In *N. edwardsonii* enhanced resistance to TNV due to silencing of the *N* gene is not caused by an enhanced induction of pathogenesis-related genes. Expression of two such genes (*NgPR-1* and *NtSGT*) after TNV infection is about the same in both wild type and transgenic silenced plants.

Discussing the above mentioned results it seems obvious that one of the most important determinants of non-host resistance is the early accumulation of $O_2^{\bullet-}$. It is very likely that this phenomenon is responsible for the absence of HR-type symptoms (local necrosis) in infected but resistant plant parts during most cases of non-host resistance. $O_2^{\bullet-}$ also accumulates during host resistance and causes similar pathogen inhibition but in most cases HR-type symptoms can develop due to the late inhibition of pathogen growth. In susceptible plants, however, $O_2^{\bullet-}$ does not accumulate at all. I have proved the occurrence of this phenomenon in twenty one host/pathogen combinations. It is known that, as a consequence of $O_2^{\bullet-}$ accumulation other types of ROS are also generated *in planta* which could also play a role in pathogen inhibition or during the development of HR-type local necrotic symptoms. The most typical types of ROS are hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH•) which is generated from H_2O_2. The harmful effects of hydrogen peroxide has been

observed in several plant/pathogen combinations that result in resistance (cf. Király et al., 2007).

One basis of resistance to virus-induced (TMV and TNV) necrotic symptoms is the effect of salicylic acid (SA) that enhances this type of resistance. In *Nicotiana edwardsonii* var. Columbia plants that I have crossed with a transgenic tobacco line (*nahG*) unable to accumulate SA, resistance to virus-induced local necrosis has significantly declined. 'Columbia' plants display a constitutively activated resistance because these plants contain an elevated level of not only free and bound SA but also superoxide, even in their healthy state, as compared to the control hybrid, *N. edwardsonii*. The resistance conferred by SA is also effective against bacterial infections and abiotic stresses that cause necrotic symptoms. In 'Columbia' plants expression of a gene encoding pathogenesis-related protein 1 (*NgPR1*) is very high, even in a healthy state, while in the more susceptible *N. edwardsonii* expression of this gene is hardly detectable even after virus infection. Therefore, activation of the *NgPR1* gene proved to be a reliable marker of the enhanced resistance of 'Columbia' plants.

During my experiments I could show that posttranscriptional silencing of a virus resistance gene influences infection by two unrelated viruses (TMV and TNV) in a distinctly different manner. If the TMV resistance gene *N* in *Nicotiana edwardsonii* is silenced, resistance to TMV is impaired. Unexpectedly, however, silencing of the *N* gene in TNV-infected *N. edwardsonii* does not reduce but, on the contrary, increases resistance to this virus. Such a phenomenon indicates that the same plant gene could act as either a resistance gene or a susceptibility factor, depending on the invading virus pathogen.

NEW SCIENTIFIC RESULTS OF DOCTORAL RESEARCH

1. Based on characterization of twenty one plant/pathogen combinations I found that in case of susceptible host/pathogen interactions (with typical disease symptoms) *no superoxide ($O_2^{\bullet -}$) accumulation* can be detected. In host resistant plants, where HR-type symptoms develop, *$O_2^{\bullet -}$ accumulates* 48 hours after inoculation. However, in non-host resistant plants, *$O_2^{\bullet -}$ accumulates very early* (i.e. 24 hours after inoculation). This early ROS accumulation is linked to the symptomless (HR^-) phenotype of non-host resistance. Early $O_2^{\bullet -}$ accumulation is also related to the enhanced activation of NADPH oxidase, the enzyme that plays a pivotal role in superoxide generation during plant pathogenesis.

2. Inhibition or suppression of $O_2^{\bullet -}$ generation in resistant plants partially converts resistance to susceptibility. This implies that ROS (primarily $O_2^{\bullet -}$ but likely also H_2O_2 and $OH^{\bullet}$) are important, although not exclusive, determinants of pathogen inhibition or suppression. In certain cases of non-host resistance, transiently activation of a *SOD* and a *BAX-inhibitor1* gene is linked to the lack of HR-type symptoms.

3. Previous research has shown that the interspecific hybrid *N. edwardsonii* var. Columbia displays enhanced resistance to local necrotic symptoms of virus infections caused by *Tobacco mosaic virus* (TMV) and *Tobacco necrosis virus* (TNV) and contains high levels of salicylic acid (SA) both in healthy as well as in diseased states (Cole et al. (2004). This means that 'Columbia' plants are in a genetically activated “resistant” state as compared to the control hybrid, *N. edwardsonii*. We have shown that artificial reduction of SA contents results in the inhibition or significant suppression of this resistance.

4. Enhanced resistance in *N. edwardsonii* var. Columbia plants infected with TNV is effective both against virus replication and local necrotic symptoms. However, when 'Columbia' plants are infected with TMV, virus replication is only slightly inhibited, while expression of localized necrosis is significantly suppressed. The SA-based enhanced resistance of *N. edwardsonii* var. Columbia causes a profound reduction in necrotic symptoms and pathogen multiplication during bacterial infections and also suppresses tissue necrosis associated with abiotic stresses (exposure to paraquat).

5. Silencing of the TMV resistance gene *N* in *Nicotiana edwardsonii* impairs resistance to TMV by increasing virus spread. Unexpectedly, silencing of the *N* gene in *N. edwardsonii* does not reduce but, on the contrary, increases resistance to an unrelated virus, TNV by dramatically reducing virus titers. In *N* gene-silenced *N. edwardsonii*, enhanced resistance to TNV is not caused by an enhanced induction of pathogenesis-related genes, because expression of two such genes (*NgPR-1* and *NtSGT*) during TNV infection is about the same in both wild type and silenced plants.

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