THESIS OF PHD DISSERTATION

EFFECT OF BACTERIUM-INDUCED PLANT BASAL RESISTANCE (BR) ON VIRAL INFECTION AND STUDY OF GENES SHOWING ALTERED EXPRESSION DURING BR

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1. Introduction

Microbes may find ideal conditions in plant intercellular space. Both saps rich in nutrients and the sheltered environment are favorable for bacterial proliferation. Throughout the eons, however, plants have evolved various defense mechanisms against microorganisms.

2. Overview

Király et al. (2007) divide defense mechanisms into two groups in their review article: innate and aquired defense mechanisms.

Innate defense can be either specifix (non-specific or general resistance) and specific (such as the hypersensitive response – HR). In this system, basal resistance (BR) belongs to the non-specific category.

However, nomenclature might differ. Boller and Felix (2009), for instance, use the term PTI (PAMP-triggered immunity) for BR; also implying that the term „basal resistance" is about to cease in literature.

BR can be induced by various microorganisms, pathogenes and saprophytes, as well (Burgyán and Klement, 1979). On the other hand, hypersensitive response is triggered only by living pathogenes with active metabolism. Unlike HR, basal resistance is symptomless, thus observation is possible only through a secondary challenge infection. If there are no HR necroses after the superinfection BR is in effect (Klement et al., 1999).

2.1. *The Hypersensitive Response (HR)*

Nearly all viral, procariotic and eucariotic plant pathogenes were described to have HR inducing capabilities. HR occurs when the interaction between the host and pathogen is incompatible; ie. the pathogen is infecting a non-host plant (eg. bean pathogen is infecting tobacco) or a host plant's resistant cultivar. In this case the infected tissue necrotises. Both the resistance of the given plant an the pathogenicity and hostspecificity of the given bacterium is determined by genes. Therefore the HR is determined by the mutual interaction of genes (gene products) of the pathogen and the host plant (Klement, 1982).

The processes leading to cell death are controlled. Hence, the name programmed cell death (PCD). Unlike necrosis resultet of a passive damage, PCD requires active contribution (eg. protein synthesis) from the cell (Gilchrist, 1998).

2.2. The Basal Resistance (BR)

While the HR is a resistance mechanism solely against pathogens, the BR can be triggered by non-pathogens, as well. This innate form of defense helps plants defeat fight against microorganisms invading the intercellular spaces. This can be achieved by blocking bacterial life processes (eg. proliferation, transcription of pathogenicity related genes, etc.) The BR is not only capable of the inhibition of pathogen proliferation but also the development of HR necroses – at incompatible interactions (Bozsó et al., 1999; Klement et al., 1999; Klement et al. 2003).

The lack of HR necroses after a superinfection with an HR inducing bacterium proves the effectiveness of the BR (Klement et al., 1999, Klement et al., 2003).

2.2.1. Induction of the BR

As the BR can be triggered by both pathogenic and non pathogenic bacteria, BR is induced by bacterial molecular patterns that are common in procaryotes. Eg. lypopolysaccharides (LPS) on Gram negative bacteria. Purified LPS (Newman et al., 1995) and LPS-protein complexes (Mazzuchi és Pupillo, 1976) were successfully used to induce BR. Other molecules, such as the flagellum protein flagellin does also have BR inducing capability (Felix et al., 1999).

Besides these so called elicitor molecules mechanic and osmotic stress can also trigger BR (Novacky and Hanchey, 1976). This cannot be a coincidence, as plants detect microbe associated molecular patterns (MAMPs; eg. flagellin, LPS, EF-Tu) in a similar way as DAMPs (damage associated molecular patterns), molecules that are released upon cell damage (Boller és Felix, 2009).

2.2.2. Cell physiological and ultrastructural changes during BR

During BR pH changes both inside and outside of the cell (Baker et al., 1990). Ca^{2+} ion intake (Grant & Mansfield, 1999), Cl^- ion emission (Nürnberger et al., 1994), and transient increase of active oxygen species (Baker & Orlandi, 1995) can also be observed.

At the site of bacterial attachment cell walls are fortified and papillae form inbetween the cell wall and the cell membrane (Politis & Goodman, 1978; Ott & al., 1997). At these sites callose deposition and enhanced lignification processes can be measured (Brown et al., 1998). Callose deposition can mark not only biotic stress but also mechanic stress (Jaffe et al., 1985). Also, callose deposited in plasmodesmta can block cell-to-cell movement of certain viruses (Radford et al., 1998; Scholthof, 2004; Dong X., 2005).

2.2.3. Signalling and protein degradation genes showing altered activity during BR

Prior to the works included in this dissertation, our research group had identified 160 genes that show altered activity during BR (Figure 1). Over 10 percent of these are related to signal transduction processes and 7 per cent to protein metabolism. (Szatmári et al., 2006).
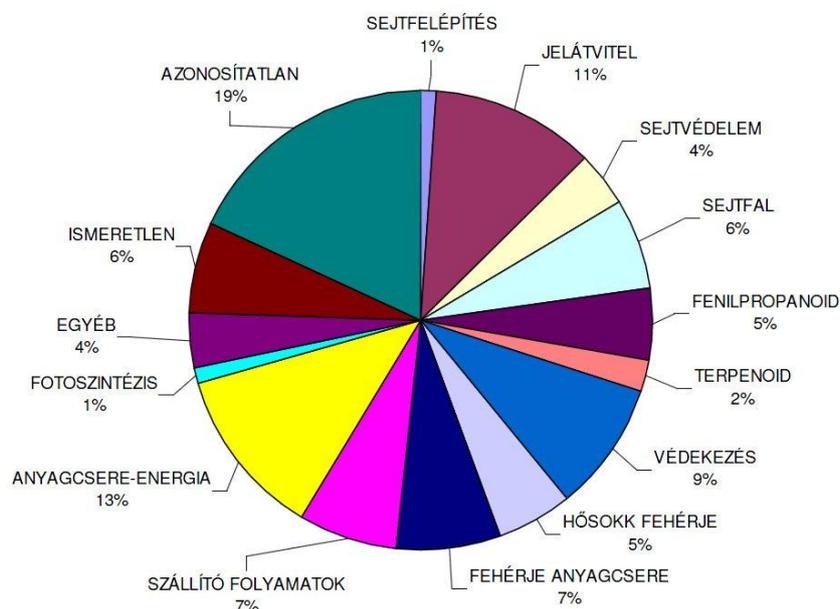
Figure 1. Functional distribution of genes showing altered activity during BR. Jelátvitel = Signaling; Fehérje anyagcsere = Protein metabolism (Source: Szatmári, 2008)

Calcium ion is an important secondary messenger. Calcium can enter the cell not only through calcium ion channels but also through a damaged cell wall or plasmodesmata. Mitogene activated kinase cascades also play an important role in signalling (MAPKKK (vagy MEKK) → MAPKK → MAPK → transcription factor). Generally these cascades start with a GTP-binding protein after being activated by most currently known PAMPs. Phospholipases [phospholipase-A1 (PLA1), A2 (PLA2), C (PLC) and D (PLD)] also take part in these processes (Trewavas, 2000; Pitzschke et al., 2009). Keeping balance of gene products produced during defense is also an important controlling factor (Trewavas, 2000).

Proteins can be degraded either in the ubiquitin pathway, in proteosome complexes or by proteases (eg. Kim & Delaney, 2002; Vierstra, 2003; van der Hoorn & Jones, 2004). Elements of both systems can be found among genes identified by Szatmári et al. (2006). The role of proteases isn't limited to eliminating „leftover" proteins. They may take part in the identification of bacteria, in the signaling processes within the host cells (Xia et al., 2004), or in defense responses against bacteria (Abramovitch et al., 2006; Hao et al., 2006).

In defense processes mostly cysteine proteases are involved. Many of the some 140 known plant cysteine proteases have been identified as factors in PCD (programmed cell death) triggered by pathogen infection. One example can be caspase-1-like proteins. In animal cells caspases are related to apoptosis, as well. Plant metacaspases (MCA) and VPEs (vacuolar processing enzymes) show similar structures to animal caspases and can be found in PCD processes (van der Hoorn, 2008; Zhang & al, 2010).

2.3. Virus induced gene silencing (VIGS)

As opposed to the use of transgenic plants, VIGS systems have a great advantage: it can be used to silence virtually any kind of genes. This high level of flexibility makes VIGS a handy tool for silencing genes that's phenotypical appearance would require certain environmental conditions. Also, the time and work consuming processes (eg. growing plants, harvesting seeds) can also be eliminated that would be required for producing transgenic plants (Robertson 2004). The bicomponent system developed by Gosselé et al. (2002) is one kind of VIGS. In the so called SVISS (satellite virus induced silencing system) the silencing sequence is carried by a satellite virus that is introduced to the plant together with its helper virus. The latter assists the satellite virus' movement within the host plant. In SVISS components required for silencing and virus replication are separated. This improves silencing performance. Another advantage can be that with the reproduction of the satellite virus, the helper virus' own reproduction is suppressed, thus the symptoms become less severe. However, sometimes enhanced symptoms can be observed on certain hosts (Roossinck et al. 1992).

3. Research tasks

Works discussed in this dissertation can be divided into three main parts:

1. The bacterially induced BR's effect on a viral superinfection (in incompatible tobacco-TMV interactions HR inhibition, in compatible interaction symptom inhibition) was studied. In both cases virus replication was also measured. Viral gene expression assays were supplemented with some of the defense related genes.
2. Transcription changes of genes related to signaling and protein degradation (identified previously by our research group) were studied. Functional genomic assays were also carried out.

3. Methods were elaborated for further functional genomic assays (construction for gene silencing, plant native protease measurement, tissue staining for detection of callose deposition.)

4. Materials and methods

4.1. Plants

Arabidopsis thaliana and 1.5-2 months old greenhouse grown tobacco plants (*Nicotiana tabacum* L.) *N. tabacum* L. 'Samsun' nn and *N. tabacum* L. 'Xanthi' NN were used for studying compatible and incompatible interactions, respectively. Of *Arabidopsis thaliana* both Col-0 ecotype and mutants to the studied genes were used. Seeds for mutant plants were ordered from the Nottingham Arabidopsis Stock Centre (NASC).

4.2. Bacteria

For BR induction *Pseudomonas syringae* pv. *syringae hrcC* (HR negative) mutanat bacteria were used. Bacteria were grown overnight at 27 °C on King's B medium (King & al., 1954) supplemented with 50 μg /ml kanamycin. Bacteria harvested from the medium were suspended in water. Cell count was adjusted to 0,19-0,21-es OD (approx. 10^8/ml). For protease activity studies *Pseudomonas syringae* pv. *tabaci* was used, for its compatible interaction with tobacco. Virus vector for gene silencing was developed using competent *Escherichia coli* DH5α. For certain gene silencing experiments *Agrobacterium tumefaciens* carrying HC-Pro silencing suppressor was used (Wydro et al.; 2006). *Pseudomonas syringae* pv. *tomato* DC3000 expressing luminous lux-gene (Fan et al., 2008) were used with *Arabidopsis* mutants, where bacterium proliferation rate needed to be determined.

4.3. Viruses

For studying the BR's effect on viral superinfection tobacco mosaic virus (TMV) was used. For gene silencing TMV-U2 strain and it's modified satellite, STMV were used.

4.4. Chemicals used for plant treatment

For studying a given protease's role in BR, inhibitor for the given protease was used. Similarly, inhibitors of certain signaling molecules were used in related studies. As a mock treatment plants were infiltrated with water instead of bacterium suspension to cancel out the effect of osmotic and mechanic stress.

4.5. Treatments and sampling

Fully developed leaves were used. In every experiment plant leaves were about the same; with tobacco leaves of the same node range, with *Arabidopsis* similarly developed leaves from the same rosette were used. Samples were collected and mixed from two or more leaves treated the same way. For RNA extraction plant samples were snap-frozen in liquid nitrogen after immediately after harvest and they were stored at -70 °C until further process. For tissue staining samples were bleached in 70-96 % ethanol immediately after harvest.

4.5.1. Infiltration of plant tissues

Bacterium suspension, water and other liquids were injected in leaves using a 26 gauge needle and syringe. (Klement, 1990). This method was enhanced with *Arabidopsis* by puncturing the leafe epidermis with a needle and infiltrating through the tiny hole with a needleless syringe.

4.5.2. Viral infections

Young *Nicotiana tabacum* L. 'Samsun' nn leaves that showed mosaic symptoms were used for viral infection. Leaves were homogenized in water using pestle and mortar. Suspension was rubbed gently onto leaves. Pressure applied to the leaves was enough to break trichomae, thus opening way for virus particles.

4.6. Processing plant samples

4.6.1. Extraction of plant mRNA, cDNA synthesis (for gene expression studies)

100 mg of plant sample wsa homoginezed in liquid nitrogen using pestle and mortar. Commercially available kit was used to extract mRNA following the manufacturer's instructions (Total RNA Extraction Miniprem System – Viogen). Extracted RNA was treated with DNase (RNase-Free DNase Set, Quiagen). 2.5 µg RNA of each sample was used for cDNA synthesis with RevertAid H Minus First Strand cDNS Synthesis Kit (Fermentas). Random hexamer primer or oligo (dT) primers were used depending on the experiments' requirements.

4.6.2. Gene expression studies with real time PCR

After the synthesis of cDNA from plant mRNA real time PCR assays (Opticon MJ Real Time PCR) were carried out. For each reaction 15 µl of reaction micture was used that consisted of 2.5 µl ten-fold diluted cDNA, 1.5 µl primers (3 µM), 2µl water and 7.5 µl reaction mix (iQ SYBR Green 2× Supermix; Biorad). Results were normalized to constitutively expressed tobacco actin gene. Relative gene expression values were compared to untreated plant samples.

4.6.3. Plant sample preparation for native protease activity assay

40 mg of plant sample was hominized in liquid nitrogen and suspended in 1.5 ml of either one of the buffers described in 4.8. Mixtures were shaken on ice for 2 hours and centrifuged at 13,000 rpm for 2 minutes. 25 µl supernatant, 24.75 µl buffer and 0.25 µl protease substrate were mixed and pipeteted in one of the wells of a 96-well PCR reaction plate. 50 µl of blank buffer and substrate mix served as negative control. Commercially available tripsin were used as positive control.

4.6.4. Microarray studies

Microarray studies were carried out based on MIAME (Minimum information about microarray experiment) requirements described by Brazma et al. (2001). Tobacco leaves were inoculated with water, *P. syringae hrcC* suspension, inhibitor (1.5mM $LaCl_3$, 50 µM neomycin-sulphate, 100 µM aristolochic acid, 1.5 µM K252a, 50 µM MG115), and also with a mixture of bacterium suspension and inhibitor. CDNA tagging and hybridization and data normalization were carried out by the TIGR Potato Functional Genomics Project, using TIGR Potato 10K cDNA Array containing some 12,000 potato genes. Genes with significantly (p=5%) altered expression were determined using rank product analysis (Breitling et al., 2004).

4.7. Callose detection

4.7.1. Determination of callose deposition using tissue staining

For callose determination anilin-blue stain was used (Currier, 1957) at 0.1% concentration in K_2HPO_4 (150 mM, pH 9.5) buffer. *A. thaliana* leaves were bleached in boiling ethanol. Leaves were shaken overnight in staining buffer. Excess dye was washed out using blank buffer. Samples were fixed in glycerol and examined using fluorescent microscope (λ_{ex}=393 nm és λ_{em}=479). Callose deposition clusters were counted on microscopic photographs. Number of callose clusters were grouped in either strong or weak decrease, no change, weak or strong increase categories compared to the control samples.

4.8. Measuring native protease activity in tobacco

Proteinase activity was measured using SensoLyte^{TM} Red Protease Assay kit (AnaSpec Co., San Jose, CA, USA). Buffers were made by following the manufacturer's instructions. Opticon MJ Real Time PCR device was used to read samples at every five minutes at $\lambda_{exciation} = 546$ nm and $\lambda_{emission} = 575$ nm..

4.9. Materials and methods related to gene silencing

Satellite virus-induced silencing system (SVISS – Gosselé et al., 2002) with TMV-U2 strain

and its satellite virus (STMV) was used. Silencing sequences were inserted into plasmid pVE349 containing the STMV using PstI and NotI restriction enzymes. Restriction digestion was carried out by following the instruction of the endonuclease provider company (Fermentas). T4 DNA ligation was done at 4°C overnight.

4.9.1. Plasmid purification
Desired plasmid was purified from *E. coli* grown overnight at 37 °C in liquid LB medium using Miniprep Express[TM] kit (Bio101, Vista, CA, USA).

4.10. Searching BR related tobacco gene orthologues in *Arabidopsis*
Ortologues of tobacco genes responsible for protein degradation and signaling were searched in *Arabidopsis*. For this SeqMan (LaserGene) software, Genevestigator (Zimmermann et al., 2004) database and gene mapping application available online at the SALK Institute homepage (http://signal.salk.edu/cgi-bin/tdnaexpress) were used.

4.11. Determining sequence of plasmid pVE349
Sequence of plasmid pVE349 was reconstructed *in silico* based on its patent documents (Metzlaff et al., 2003) and the article of Gosselé et al. (2002) using SeqBuilder 7.0.0 software (DNASTAR, Madison, USA).

4.12. Statistical methods
Mean results from repeated experiments were compared using Student's t-test. Confidence interval was p>90%.

5. Results

5.1. Effect of bacterium induced basal resistance on viral superinfection

5.1.1. Symptom inhibition (compatible tobacco-TMV interaction)
Only mild mosaic symptoms were observed on plants that were superinfected with tobacco mosaic virus (TMV) 6 or 24 hours after bacterial infection compared to the water treated or the non-treated plants (data not shown). Results also suggest that even water infiltration has effect on symptoms, although, not as strong as the bacterial infection.

5.1.2. Inhibition of virus replication (kompatibilis dohány-TMV kapcsolat)
Two days after the viral infection the TMV mRNA expression level was very high, then from the dropped significantly in the bacterium pre-infected samples (Figure 2).
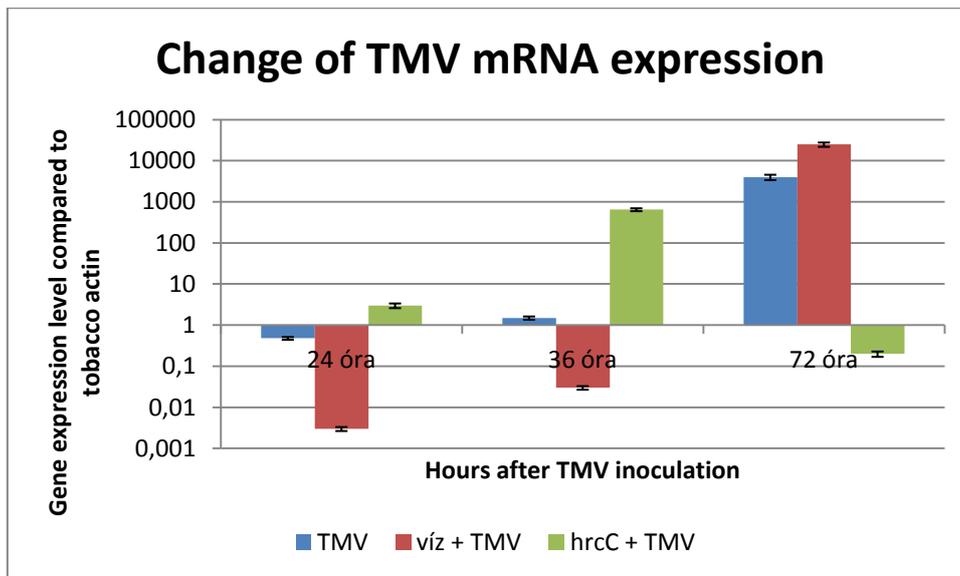
Figure 2. Change of TMV mRNA expression levels over time in inoculated leaves of Samsun nn plants on logarithmic scale. Control (only TMV), water mock treatment [Víz + TMV] and *P. syringae hrcC* HR-negative bacterium infection [hrcC + TMV]. Viral infection followed bacterial infection in six hours.

5.1.3. Inhibition of viral HR (incompatible tobacco-TMV interaction)

Strong HR inhibition was observed with plants where viral superinfection followed bacterial infection in six hours. Not only the number of necrotic lesions but also their sizes changed. Similar HR inhibition was observed where superinfection followed pretreatments in 24 hours. However, in this case even water mock treatment inhibited HR necroses effectively (Figure 3).

Figure 3. Bacterium induced BR inhibits HR necroses on *N. tabacum* L. 'Xanthi' NN leaves. X = untreated leaf. V = water mock treatment, C = *P. syringae* pv. *syringae hrcC* infection. TMV superinfection followed pretreatments in 6 hours. Figure shows representative results of repeated experiments.

5.1.4. Inhibition of virus replication (incompatible tobacco-TMV interaction)

Figure 4 shows virus replication inhibition at plants receiving superinfection six hours after pretreatment. TMV mRNA expression levels are increasing after all three treatments, however, the rate is different. Water and bacterium suspension pretreatment suppresses virus replication.
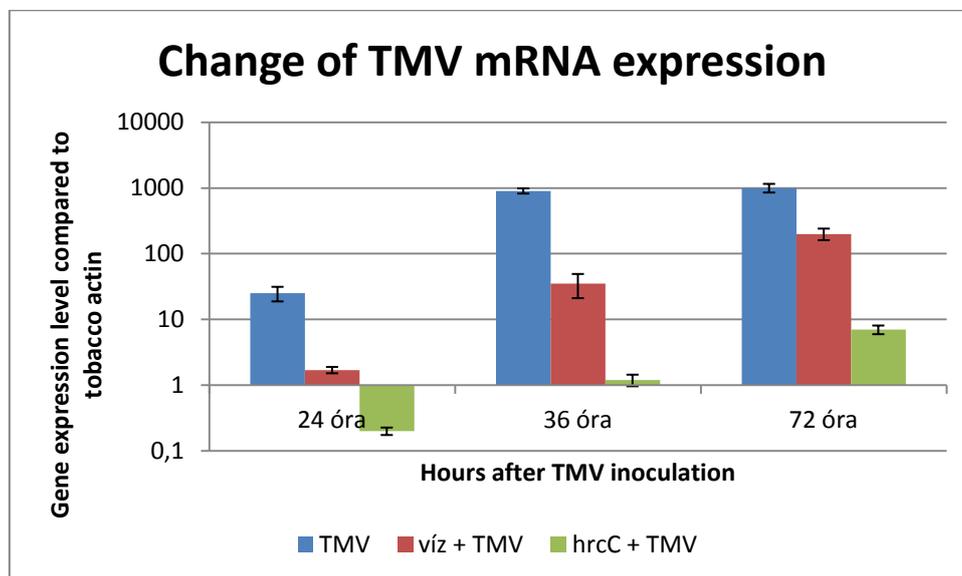
Figure 4. TMV mRNA expression level changes on logarithmic scale over time in Xanthi NN tobacco plants over time. TMV = plants receiving only TMV infection; víz + TMV = water mock inoculation prior to TMV infection; hrcC + TMV = *P. syringae hrcC.*

5.1.5. Activity changes of plant defense genes (incompatible tobacco-TMV interaction)

Two genes with antioxidant role (catalase and dehydroascorbate reducatase) showed increased expression 48 hours after TMV infection in samples pre-treated with *hrcC* bacterium suspension. However, this high level of expression does not persist: by 72 hours after inoculation it drops to nearly half of this value (Figure 5.)

Catalase gene

Dehydroascorbate reductase gene

■ TMV ■ víz + TMV ■ hrcC + TMV
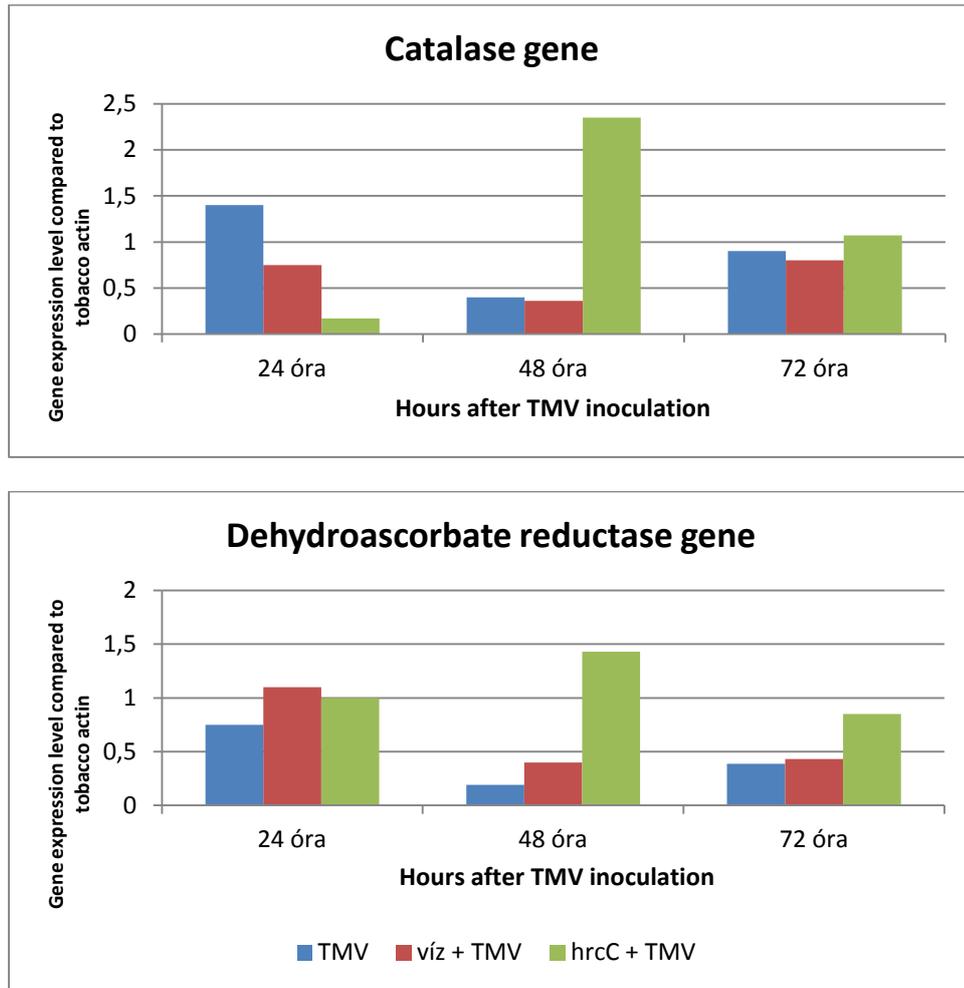
5. Figure Catalase (NtCAT) and dehydroasscorbate reducates (NtDHAR) gene expression changes in Xanthi NN tobacco over time after infection with TMV. TMV = only TMV infection; víz+TMV = water mock treatment and TMV infection; hrcC + TMV = *P. syringae hrcC* pre-treatment and TMV infection.

5.2. Study of signaling and protein degradation genes that show altered activity during BR

5.2.1. Signaling genes that show altered activity during BR

Figure 6 shows some typical changes in activities of genes that play role in signaling and were previously identified by our research group.

The MAP3K kinase gene shows increased activity usually at an early stage of BR compared to the untreated samples. It might play a role in the induction of the defense mechanism. Other genes (such as map-kinases or receptor-like protein kinases) show activity at later times. There were also some genes that decreased their activity during BR compared to mock treated or untreated samples (eg. annexin-like protein gene 24 hours after inoculation.)
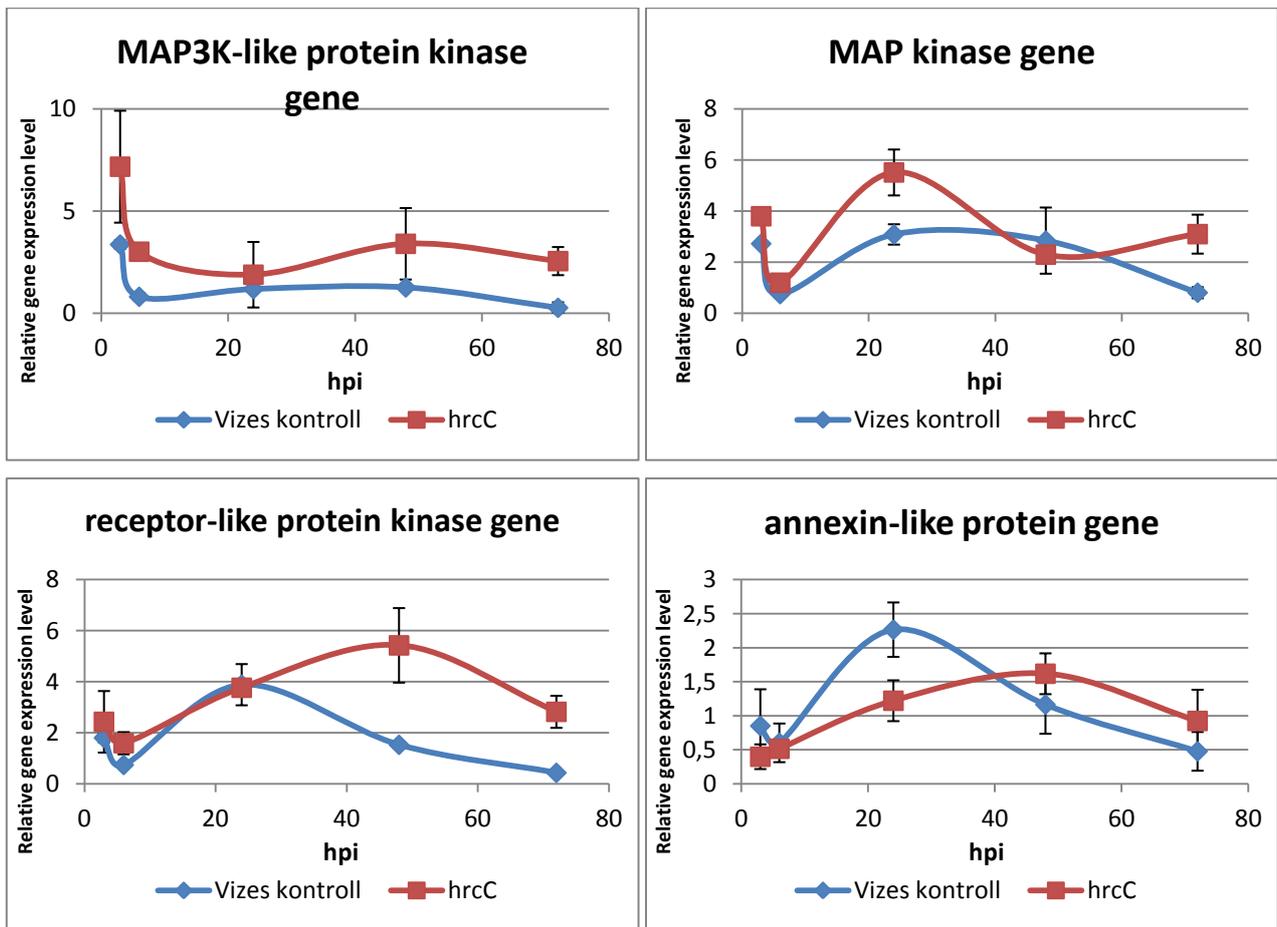
Figure 6. Gene expression changes over time of different BR related genes. Blue line: water mock treatment, Red line: *P. syringae* pv. *syringae hrcC* infection. Expression levels were compared to tobacco actin and to untreated samples.

5.2.2. Effect of signal inhibitors on BR related genes

Studies of 16 BR related genes were carried out after inhibiting the following signaling molecules and/or processes: calcium influx (lantane chloride), phoshoplipase-A2 (aristolochic acid), phospholipase-C (neomycin sulphate), phospholipase-D (n-butanol), protein synthesis (cycloheximide), proteosome degradation (MG115). Times after infection where gene activity differed from samples that were not treated with inhibitors are shown in Table 1. Some processes affected gene activity particularly at early stages of BR (eg. PLA2 on MAP-kinase, MAP3K, annexin-like protein and PTS protein), while others did so at rather later times (eg. inhibition of PLC on MAP kinase and receptor-like kinase).

Table 1. Left column: genes that showed altered expression levels. First row: signaling processes that were inhibited. Numbers in table show time after infection where change in expression levels occurred. N.a. = data not available

	Calcium influx	Phospholipase-A2	Phospholipase-C	Phospholipase-D	Protein synthesis	Proteosome
MAP kinase		3	24-48	6	6	12
MAP3K	12-48	3	12	6	6	6
Receptor-like kinase	6		24		6-24	
PTS-protein		3-6				
Annexin-like protein	6	3	6-12	½-6	6	3-12
SPL						12
Heat shock protein	n.a.	½-48			n.a.	
Cinnamic acid hydroxilase	n.a.	3-6		6	n.a.	
Epoxide hydrolase	n.a.	3-6	48	12	n.a.	3-12; 48
Orthometil transferase	n.a.	3-6		3-6	n.a.	
Glycine rich protein	n.a.	6-48	24		n.a.	12-48
Gluthation-S-transferase	n.a.	6	6	3	n.a.	3-6
Peroxidase	n.a.	3-6	48	12	n.a.	3-6; 48
Cysteine protease	n.a.	n.a.	n.a.	12	n.a.	n.a.
Ubiquitin-binding protein	n.a.	n.a.	n.a.	3	n.a.	n.a.
Proteosome δ-subunit	n.a.	n.a.	n.a.	12	n.a.	n.a.

PLA2 and PLD affected studied genes in the early stages of BR (until 12 hours post infection). The glycine-rich protein gene was an exception as it showed altered activity at 48 hours after infection, as well. PLC seemed to affect different genes at different times.

5.2.3. CDNA microarray studies of gene expression changes due to signal inhibitor treatments

Samples were taken 6 hours after inoculation. Signal inhibitors (lantana chloride – calcium influx, aristolochic acid – PLA2, neomycin sulphate – PLC, K252a – kinases, MG115 – proteosome) were used.

Inhibitors had effect on the expression of the 18% (99) of the 547 BR related genes six hours after infection. The directions of expression changes of any two treatments are compared in Table 2. The direction of changes showed the most similarity when comparing kinases and PLA2 (28 cases). PLC and proteosome inhibition changed 17 genes' activity the same way.

Table 2. Comparison of the direction of gene activity changes after signal inhibitor treatments. Results based on microarray studies. Same direction is indicated with red, opposite with green.

	Calcium influx	Phospholipase-C	Phospholipase-A2	Protein kinase	Proteosome	
Calcium influx		9	3	5	7	
Phospholipase-C	0		8	10	17	Same direction
Phospholipase-A2	0	0		28	6	
Protein kinase	2	2	0		6	
Proteosome	0	0	1	5		
	Opposite direction					
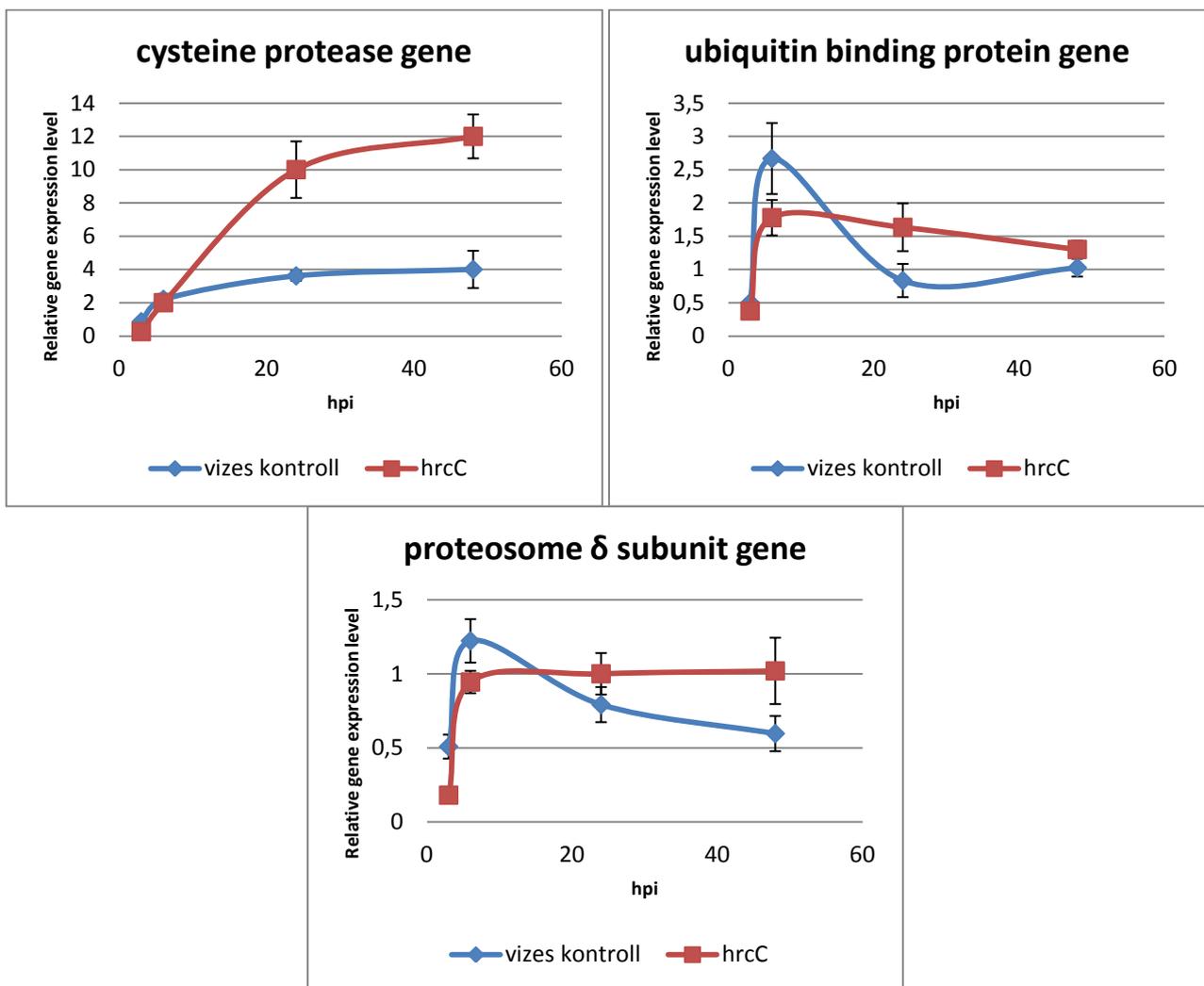
Figure 7. Gene expression changes over time of different BR related genes. Blue line: water mock treatment, Red line: *P. syringae* pv. *syringae hrcC* infection. Expression levels were compared to tobacco actin and to untreated samples.

5.2.4. Changes of protease gene activities during BR

Three genes are shown here as examples. A cysteine protease and a proteosome δ subunit gene showed altered expression activity 12 hours after the infection, while a ubiquitin bindig protein gene was affected by the infection in 3 hours after infection.

Most significant difference between water mock treated and bacterium infected samples were observed in case of a cysteine protease gene. Other types of activity changes were also observed (Fiugre 7).

5.2.5. Effect of protease inhibitors on gene expressions during BR

Most studied genes showed higher level of expression during the early stages of BR after plants were treated with protease inhibitors compared to mock treatment and untreated plants. This was especially true for cysteine and serine protease inhibitors.

Expression changes of 14 genes were studied with real time PCR. Different patterns of changes in expression were observed. For example the orthomethyl transferase didn't change its expression level after phenantroline (metallo-protease) treatment, while the expression level dropped significantly when the bacterium suspension was supplemented with the same inhibitor. The same inhibitor alone increased the expression of an annexin-like protein gene, while together with the bacterium suspension, it did not affect the gene activity. The cysteine protease inhibitor zinc chloride affected OMT activity both alone and together with the bacterial infection, while it hardly affected the annexin-like protein gene's expression (Figure 8).
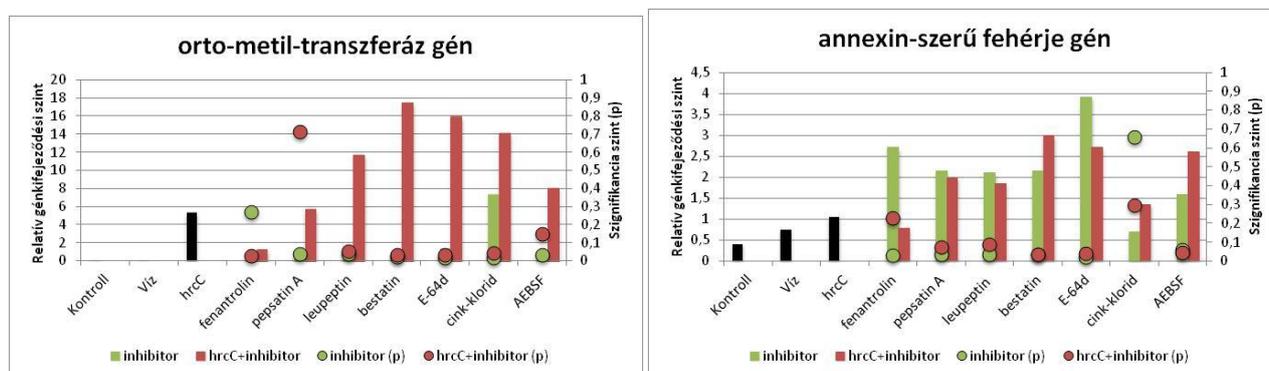
Figure 8. Expression levels of two BR related genes (orthomethyl transferase and an annexin-like protein) with various protease inhibitors 6 hours after infection. Expression levels were compared to tobacco actin. Black: control BR samples (Kontroll – untreated, Víz – water mock treatment and hrcC – *Pseudomonas syringae* pv. *syringae hrcC*); Green: inhibitor only; Red: inhibitor + bacterium suspension. Inhibitors used: phenantroline: metalloprotease; pepstatin: asparagic acid protease; leupeptine: cysteine and serine protease; bestatin: aminopeptidase; E-64d and zinc chloride: cysteine protease; AEBSF: serine protease.

5.2.6. Changes in callose deposition in *Arabidopsis* after protease inhibitor treatment

BR was induced in *Arabidopsis* plants using *hrcC* bacterium suspension. Some samples were also treated with various protease inhibitors. 24 and 48 hours after infection leaves were stained with aniline blue to detect callose with a fluorescent microscope. Wherever there was a

change in the rate of callose deposition, inhibitors on their own enhanced, while inhibitor and bacterium suspension together suppressed the process (Table 3.)

Table 3. Callose deposition after protease inhibitor treatments in *Arabidopsis*. Water mock treatment served as control for inhibitor (only) treatments, while blank bacterium suspension was used as control for bacterium + inhibitor mixture treatments. Green: less callose, yellow: more callose.

	Sampling 24 hours after treatment		Sampling 48 hours after treatment	
	Inhibitor	Bacterium + Inhibitor	Inhibitor	Bacterium + Inhibitor
Pepstatin				
Leupeptin		Slightly decreased	slightly increased	
Bestatin				
E-64d	slightly increased			
AEBSF	slightly increased	Slightly decreased	slightly increased	slightly decreased
Cink-klorid		Slightly decreased		slightly decreased
TPCK	slightly increased		slightly increased	
E-64	slightly increased			
Jodo-acet-amid		significantly decreased	slightly increased	slightly decreased
NEM		significantly decreased		slightly decreased
Aprotinin		Slightly decreased		
PMSF				

5.3. Introduction of new methods for functional genomic studies

5.3.1. Native protease activity measurement in tobacco

For these studies a kit developed for animal and human research[1] was adapted to our conditions. *N. tabacum* 'Samsun' plants were incubated with water, a compatible pathogen (*P. syringae* pv. *tabaci*), suspension of heat killed pathogens, and with an incompatible bacterium (*P. syringae* pv. *syringae hrcC*). A buffer suggested by the manufacturer for papain measurement produced the first results.

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[1] SensoLyte[TM] Red Protease Assay Kitet (AnaSpec Co., San Jose, CA, USA)

The dynamics of the protease activity changes were similar in all treatments. However, we observed significant differences htree and 48 hours after inoculation between the treatments. The compatible bacterium affected native protease activity the most: it remained lower than the other treatments. Also, 12 hours after the infection protease level in these samples dropped almost to zero. (Figure 9).

Putative papain like protease
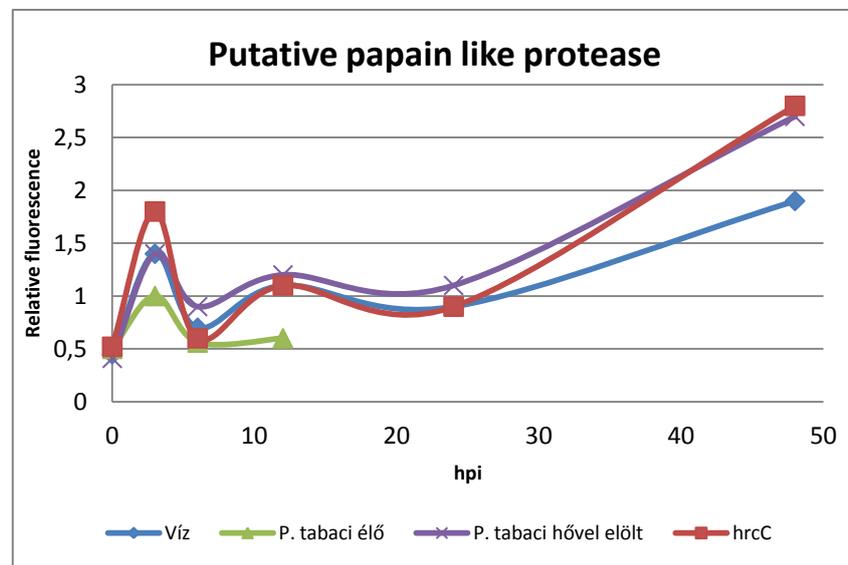
9. Figure Activity changes of a protease with the same pH optimum as papain in tobacco. Values were compared to untreated samples based on fresh leaf mass. Fluorescence of samples show direct correlation to protease activity.

5.3.2. Use of *Arabidopsis* model for functional genomic studies

78 T-DNA protease mutant *Arabidopsis* plants were ordered from NASC. They were tested with a luminescent lux gene carrier *Pseudomonas syringae* pv. *tomato* DC3000 strain. The change of luminescence correlated with the change of bacterium cell count. Those plants that had the biggest difference in bacterium cell count compared to control plants were used for further studies.

Three days after infection there were significant differences in bacterium cell count compared to wild type plants in a carboxypeptidase and a subtilase mutant plants (Figure 10).
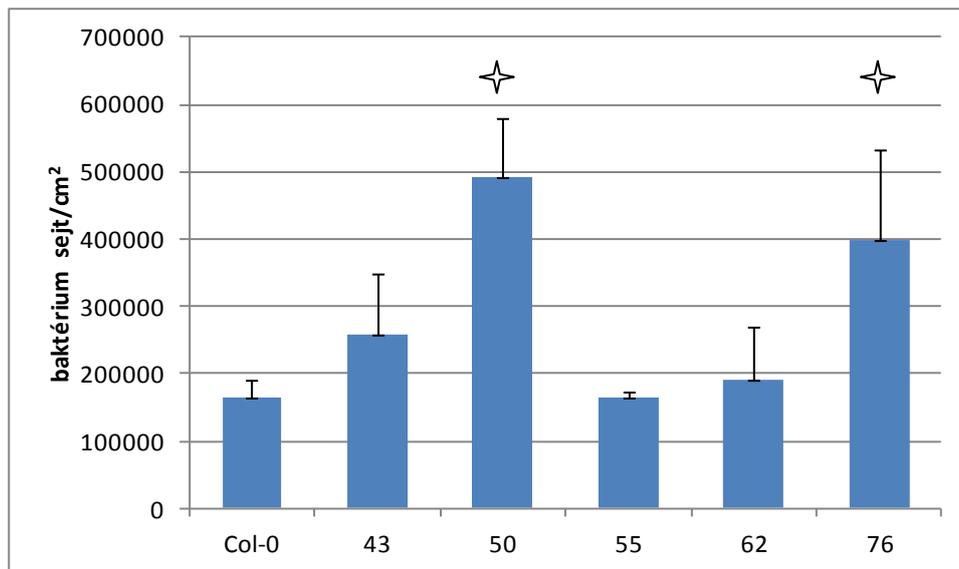
Figure 10. Cell count of a compatible *Pseudomonas syringae* pv. *tomato DC3000* bacterium in wild type (Col-0) and protease mutant *Arabidopsis* plants (indicated with code numbers). 50: carbodypeptidase; 76: subtilase. Samples were taken 3 days after inoculation.

5.3.3. Constructing *Agrobacterium* vector for transient expression of STMV satellite virus in tobacco

Virus induced gene silencing (VIGS), namely satellite virus induced silencing system (SVISS – Gosselé et al., 2002) method was used with tobacco plants.

Satellite virus RNA was first synthetized using *in vitro* transcription. As this method is cost intensive and difficult to handle, satellite virus was built in an *Agrobacterium* binary vector together with the silencing sequence (Figure 11).
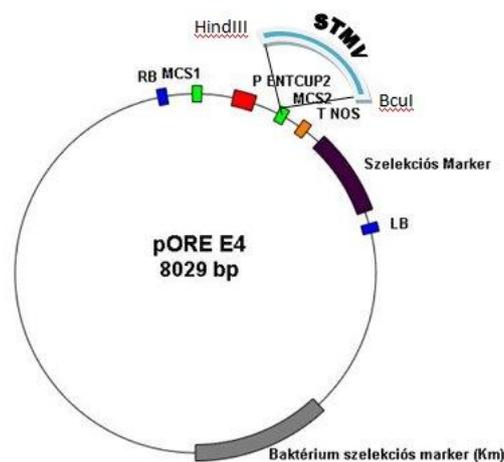
Figure 11. *Agrobacterium* binary plasmid pORE E4 encoding STMV satellite virus. RB: right boarder of the T-DNA; LB: left boarder of the T-DNA; MCS: multiple clonging site; pENTCUP2: plant promoter region; T NOS: terminator; szelekciós marker: selection marker was kanamycine.

Three weeks after infection young Samsun tobacco plants were bleached around major leaf veins when PDS (phytoen desaturase) gene was silenced using the method described above (Figure 12).

Figure 12. *N. tabacum* cv. „Samsun" nn plant in greenhouse under strong light conditions when phytoen desaturase (PDS) gene was silenced. Bleached leaves occurred three weeks after inoculation.

5.4. Theses

1. Basal resistance (BR) induced by *P. syringae* pv. *syringae* HR negative mutant may suppress a later tobacco mosaic virus infection. In compatible TMV-tobacco interactions the symptoms are less severe, in incompatible interactions the number of HR necroses are reduced. BR also keeps TMV replication low.

2. Antioxidant genes counterweighing hydrogen peroxide show higher activity at later stages of BR (48 hpi) when there is a TMV superinfection. They might take part in suppressing necrotic lesions.

3. Activity changes of many signaling and protein metabolism genes (defined by our research group prior to these studies) were followed over time. Many genes were identified to be active at certain stages of BR.

4. Inhibition of PLA2 and PLD results in change of 16 BR marker genes shortly after infection. On the other hand, PLC inhibition affects gene expression at later times.

5. Protein degradation factors are typically negative regulators of transcription changes at early stages of BR – based on our studies of seven different protease inhibitors. Similar studies with 12 different protease inhibitors showed that proteases may enhance callose deposition during BR in *Arabidopsis* leaves.

6. Using potato cDNA chip the effect of five different signal inhibitors was studied on tobacco genes six hours after inoculation. The correlation with these signal pathways of 99 BR related genes was determined.

7. A putative papain-like (cysteine) protease was proved to be accumulated in tobacco leaf tissue during BR.

8. Studies with *Arabidopsis* protease mutants suggested that two serine proteases (carboxypeptidase and subtilase) may play a positive regulatory role during BR.

9. Based on satellite virus-induced silencing system (SVISS) new TMV satellite virus (STMV) expression and silencing vector was constructed for further functional genomic studies.

6. Conclusions

6.1. Effect of bacterium induced basal resistance on viral superinfection

There are similarities in defense responses given to different types of pathoges (bacteria, viruses, fungi). Responses to biotic and abiotic stresses may also overlap. For example, wounding as a mechanic stress induces similar mechanisms in plants as pathogens (Cheong, 2002). It is possible, that a bacterium can induce responses developed for viruses as well. This can be perhaps because in the plant kingdom the ability to give very specific responses has not (yet) evolved. It can also be possible that this strategy is very effective for plants, as one stress may forecast another stress factor's appearance (eg. a wounding may open way to bacteria). However, this type of general response takes too much energy to be maintained for longer time.

The way a bacterium induced BR can suppress a viral infection is still unclear. It can be due to the inhibition of virus replication or viral movement. Viral RNA expression can be suppressed by activating gene silencing mechanisms (Waterhouse et al., 1999). Virus movement can be blocked by callose deposition in plasmodesmata (Roberts and Oparka, 2003; Beffa et al., 1996; Wenlong et al., 2012).

Our findings of the changes in antioxidant gene expression suggest that H_2O_2 degradation may be more intense at certain phases of a viral infection. At physiological conterntrations hydrogen peroxide is not enough to trigger cell death but together with other molecules (eg. NO) it might be possible (Delledonne et al., 1998). Thus a lower hydrogen peroxide level may inhibit cell death (tissue necrosis). But it can also act as signal molecule of other types of defense mechanisms (Hafez, 2005).

6.2. Study of signaling and protein degradation genes that show altered activity during BR

6.2.1. Signaling genes that show altered activity during BR

Activity changes of MAP kinases may be because osmotic stress trigger similar pathways as pathogen infections. At later stages of the infections, however, other – more specific – response processes may be stronger. Genes with higher expression level in mock treated plants than in bacterium infected plants may play a negative regulatory role in defense. For example, in our studies an annexin-like protein acted this way when we blocked PLA2 and PLC. In animal cell research it was proven that annexins inhibit PLA2 (Parente és Solito, 2004).

6.2.2. Effect of signal inhibitors on gene expressions during BR

Based on our research we can conlude that PLA2 and PLD play role at early stages of BR. Also, many kinase genes and the gene of an annexin-like protein play role in many signaling pathways. Annexin-like proteins may serve as a linking molecule between these pathways. Especially that it was proven that they can bind to phosphatidil inositol – a molecule that takes part in a PLC mediated pathway (Hoshino et al., 1995). PLA2 may play in important role in cell defense through cell wall fortification through affecting glycine rich proteins that are key elements of cell wall fortification (Ringli et al., 2001). Our researches also suggest that the ubiquitination of proteins to be degraded in the PLD pathway happen shortly after infection.

6.2.3. Changes of protease gene activities during BR

It seems that certain proteases play role at early, while other at later stages of BR. For instance cysteine proteases in our studies showed stronger expression levels in 24 and 48 hours after infection, while had no activity change (compared to mock treatment) at 3 or 6 hours after infection.

6.2.4. Effect of protease inhibitors on gene expression during BR

Several protease inhibitors were studies. Many of them has already be proven to play important role in plant defense. Such as cysteine and serine proteases (van der Hoorn, 2008., Baek and Choi, 2008). Our results seem to prove these observations found in references: more significant changes were observed in these two protease families. Other proteases may also be important parts of defense mechanisms. For instance, our studies on certain metalloproteases suggest that they may have negative regulatory roles in these processes.

6.2.5. Changes in callose deposition after protease inhibitor treatment

Our results suggested that only cysteine and serine proteases have an effect on callose deposition. The fact that there were changes in both 24 and 48 hours after inoculation suggest that callose deposition is a longer process within BR, and its defensive effect reaches longer as well.

6.3. Introduction of new methods for functional genomic studies

6.3.1. Native plant protease measurement

Our primary experiments suggest that a certain cysteine protease (a putative papain-like protease) activity is present not only on gene expression level but also on enzymatic levels.

6.3.2. Use of an *Arabidopsis* model

Both subtilase and carboxypeptidase (serin proteases) may play a positive regulatory role during BR, based on our studies on protease mutant *Arabidopsis* plants using lux gene expressing *P. syringae* pv. *tomato* DC3000 bacteria.

6.3.3. Gene silencing in tobacco

Using the satellite virus induced silencing system (SVISS) designed by Gosselé et al. (2002) was difficult. Most problems originated from the laborintensive handling of the satellite virus synthetized *in vitro*. Direct infection with this RNA was generally not too effective. Overpassing with inocula from bleached leaves of PDS (phytoene desaturase) silenced plants increased effectivity. The answer is 42. *Agrobacterium* pORE4 plasmid was used to increase effectivity.

7. Summary

In natural environments plants are always subject to stress factors of various microorganisms. One of the effective defense system against them is the so-called basal resistance (BR). Unlike hypersensitive response (HR), BR does not produce visible symptoms. While HR is a result of pathogen infection in non-host plants, BR can be induced by pathogens, saprophytes and heat killed bacteria as well.

Our results suggest that BR induced in tobacco plants may inhibit a tobacco mosaic virus superinfection – in terms of both symptoms (HR lesions) and virus replication.

We also concluded that signal molecules, such as phospholipase-A2 and phospholipase-D play role in the early phases (6-12 hours) of BR, while phospholipase-C is active in later times (24 and 48 hours after inoculation).

There is a strong relationship among signaling pathways. MAP kinase cascades and phospholipase-A2, and also phospholipase-C and protein degradation can be found in closely related pathways. This observation is also supported by the expression changes of an annexin-like protein after various inhibitor treatments. Up- and downregulation were found both among signaling and protease enzyme genes. The direction of expression changes could also change in time.

Results of active plant protease level measurement showed that a – putative papain-like – cystein protease increased in concentration in tobacco leaves during BR.

Bacterium proliferation in protease mutant *Arabidopsis* plants suggest that two serin-proteases (subtilase and carboxypeptidase) may play an important role in keeping bacteria at bay during BR-

An *Agrobacterium* vector constructed by our research group may prove to be an effective transient expression tool in further functional genomic research if used with tobacco mosaic virus satellite (STMV).

List of publications

Articles with IF
Szabó E, Szatmári Á, Hunyadi-Gulyás É, Besenyei E, <u>Zsiros LR</u>, Bozsó Z, Ott PG. 2012. Changes in apoplast protein pattern suggest an early role of cell wall structure remodelling in flagellin-triggered basal immunity *Biologia Plantarum* 56:551-559 [IF: 1,974]
Bozsó Z, Maunoury N, Szatmári Á, Mergaert P, Ott PG, <u>Zsiros LR</u>, Szabó E, Kondorosi É, Klement Z. 2009. Transcriptome analysis of a bacterially induced basal and hypersensitive response of Medicago truncatula *Plant Molecular Biology* 70:627-646 [IF: 3,978]
<u>Zsiros L</u>, Szatmári Á, Palkovics L, Klement Z, Bozsó Z. 2007. Bacterium induced basal resistance inhibits viral infection in tobacco plants *Cereal Research Communications* 35(2):1345-1348 [IF: 1,19]

Articles with no IF
Czelleng A, Bozsó Z, Ott PG, Besenyei E, Varga GJ, Szatmári Á, Szabó E, <u>Zsiros LR</u>, Klement Z. 2009. Efficient transposon mutagenesis in a wide range of phytopathogenic Gram-negative bacteria *Acta Phytopathologica et Entomologica Hungarica* 44(1):19-24

Conference abstracts
<u>Zsiros LR</u>, Szatmári Á, Szabó Erika, Ott PG, Bozsó Z. 2008. A baktériumok által kiváltott általános védekezés és hatása más fertőzési mechanizmusú kórokozókra *Pro Scientia Aranyérmesek VIII. Konferenciája* 87-92
<u>Zsiros LR</u>, Szatmári Á, Szabó E, Ott PG, Bozsó Z. 2008. Baktériumok indukálta általános védekezés (BR) jelátviteli folyamatai dohány levélben *54. Növényvédelmi tudományos napok. Összefoglaló* 33
Szabó E, <u>Zsiros LR</u>, Szatmári Á, Szamos J, Bozsó Z, Ott PG. 2008. Isolation and characterization of EBR specific induced chitinases from tobacco (*Nicotiana tabacum*) *Acta Biologica Szegediensis* 52(1):251-252
<u>Zsiros LR</u>, Szatmári Á, Szabó E, Ott PG, Bozsó Z. 2008. Plant protein degradation affects transcription of genes associated with bacterium-induced basal resistance *Acta Biologica Szegediensis* 52(1)253-255
Szatmári Á, Szabó E, Ott PG, Mergaert P, Kondorosi E, <u>Zsiros L</u>, Bozsó Z. 2007. Characterisation of proteins and novel types of peptides with putative antimicrobial activities in plan-microbe interactions *XIII. International Congress on Molecular Plant-Microbe Interactions Abstracts* 168

Other scientific publications
Ködöböcz L, <u>Zsiros LR</u>, Murányi A. 2011. A szójaoltás hatása csernozjom talajon *Agrokémia és talajtan* 60(1):233-244
Ködöböcz L, <u>Zsiros LR</u>, Murányi A. 2008. Symbiotic effectiveness of inoculation on soybean plant (*Glycine max* L.) *Magyar Mikrobiológiai Társaság 2008. évi Nagygyűlése és XI. Fermentációs Kollokvium. Összefoglaló* 44-45

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