CORVINUS UNIVERSITY OF BUDAPEST

The inhibition role of basal resistance on the multiplication of saprotrophic and opportunistic pathogen bacteria

Thesis of PhD

Eszter Besenyei

Budapest, 2006

PhD School/Program
Name: PhD School of Horticultural Science
Field: Horticulture, Plant Protection Science

Head: Prof. János Papp
senior scientist, DSc
CORVINUS UNIVERSITY OF BUDAPEST

Supervisor:
Prof. Zoltán Klement†
senior scientist, MHAS
PLANT PROTECTION INSTITUTE, HAS

Prof. Balázs Barna
scientific director, DSc
PLANT PROTECTION INSTITUTE, HAS

Prof. László Palkovics
head of deparment, PhD
CORVINUS UNIVERSITY OF BUDAPEST, faculty of horticultural sciences,
Department of Plant Pathology

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

.....	.....
Head of PhD School	Supervisor
János Papp	Balázs Barna
	.....
	Supervisor
	László Palkovics

1. PRIOR EVENTS AND AIM OF WORK

The development of a new plant resistance mechanisms was studied in low (5 ˚C) and high (20–30 ˚C) temperatures. The bacterial induced hypersensitive reaction (HR) was widely investigated, but the basal resistance (BR) (Lovrekovich and Farkas 1965, Burgyán and Klement 1979), which induced by general bacterial elicitors (flagellin, peptidoglycan, cold-shock protein, EF-TU elongation factor) was only investigated on few plant (Gomez-Gomez 2004): *Nicotiana tabacum*, *Arabidopsis thaliana*, *Medicago truncatula* and *Capsicum annum*. The most data were published connected to tobacco. The BR was activated by the saprotrophic and the heat killed pathogens as well as the molecules of incompatible pathogen bacteria. The HR blocks the incompatible pathogens in plant tissue and the biological significance of BR is the protection of plants against the most of bacteria, which attacked them. Therefore the most plants remain healthy. The BR develops in a few hours and exists 6–7 days in tobacco. It can be divided into one early and one late period. Only indirect methods it could be detected, because the BR is symptomeless. There is a suitable method the inhibition of a second infection or development of HR, for its. The inhibition of HR occurs because of the blocking of type III secretion system and multiplication of incompatible pathogens (Klement et al. 2003, Ott et al. 2006). In the intercellular space, where the cells of plants and bacteria may be close connected, during the BR occurs some physiological alteration. Outher of the plant cell hydrogen-peroxide accumulate and new isoforms of induced peroxidase and chitinase enzymes appear. On the other hand extra– and intracellular processes activated in order to fortificate of plant cell wall and to neutralize of toxic materials (Bozsó 2000, Bozsó et al. 2005a, Ott 2002, Ott et al. 2006, Szatmári et al. 2006). In the compatible interaction the pathogenic bacteria repress the BR in their early phase (early basal resistance, EBR), however it posess general bacterial elicitors, therefore the heat-killed suspension of simmilar pathogen activates the BR (Keshvarzi et al. 2004, Ott et al. 2006).

The BR is the first line defence of the plant resistance system specialised for recognise the strangers. It is activated by the saprotrophic bacteria, it is possible therefore the BR inhibits the multiplication of this bacteria in the plant tissue. I will use saprotroph bacteria which is common in mycology as a synonym of saprotrophic. The “sapro” comes from Greek and it means moulder. The mean of „troph” is lover. So the saprotrophic organisms get some energy from decompose of death organic materials. The short-time incubation of plants at low temperature helps the colonisation of opportunistic pathogen *Pseudomonas syringae* pv. *syringae* (*P. syringae*). They are polyvirulent pathogens which wait the advantageous situation in order to colonisation and cause serious disease on their hosts, but if their hosts are in optimal conditions, they stay healthy. Opportunistic *P. syringae* is a cold-tolerant pathogen and at 5 ˚C it usually causes disease on their hosts, but does not cause at 25-30 ˚C (Hevesi 1986). Are there any reasons of this

phenomenon in the activity of BR or HR? Nowadys we have only little information about the activity of plant defence on different temperature.

Decreasing in temperature influences the plant defences and causes some alteration in physiological processes of plants. It increases the amount of unsaturated fatty acids in cell membrane and alters the circulation of carbon-hydrates and activates the production of poliamine and chaperons which protect the structure of some proteins against denaturation (Mahajan and Tuteja 2005).

Aim of work

The first aim of my work is the investigation of the effect of temperature on basal resistance. Temperature is one of the most important abiotic factors which determinates the physiological condition of plants. On the other hand I would like to study some macroscopic and molecular alterations and the development of defence responses are activated by the saprotrophic *Pseudomonas fluorescens* (*P. fluorescens*) and the opportunistic pathogen *P. syringae* in pepper tissue. It needs to study the next questions:

- The heat-killed and live pathogens activate the local defence responses in tobacco (Hevesi et al. 1981, Klement et al. 1999). The question arrived does the defence system function if plants are kept at low temperatures. I want to detect therefore, the development of BR and appearance of HR in tobacco at this temperature. The results of symptomic experiments will be controlled with bacterial multiplication and the expression of BR marker genes. They will be followed similar experiments in pepper, which are suitable plants for moldelling opportunistic pathogens-plant interaction.
- The effect of durable low temperature on the multiplication of bacteria with different cold-tolerance ability is not resolved yet. I will investigate the *in vitro* and *in planta* multiplication of saprotrophic *P. fluorescens,* opportunistic *Pseudomonas syringae* pv. *syringae* 61, 2214 (wide host range) and *Pseudomonas savastanoi* pv. *phaseolicola* S 21 (limited host range) to control the function of BR and HR.
- I want to detect and to identify some BR marker proteins. The markers will be searched in apoplast of pepper leaves. The specific induction of markers will be proved with effect of temperature and light of their appearance. Increasing in temperature speeds up the development of BR, but the plants, which are kept in dark, inhibit the BR. It needs to analyze the role of some activators and hormone or abiotic steress factors in the induction of BR.
- **Identifing of specific BR markers makes possible the detection of BR in pepper in different conditions. They help to know the role of plant defences and the interaction between plants and saprotrophic (*Pseudomonas fluorescens*) or opportunistic pathogen (*Pseudomonas syringae* pv. *syringae*) bacteria.**

2. MATHERIALS AND METHODS

2.1. Live materials

In the experiments tobacco, bean and pepper were used. The BR was induced by suspension of heat–killed pathogenic *Pseudomonas* or saprotrophic *P. fluorescens*. The HR was induced by live cells of some pathogenic *Pseudomonas* species. The compatible interaction was investigated with *Pseudomonas. savastanoi pv. phaseolicola* pathogenic on bean or *Xanthomonas vesicatoria* pathogenic on pepper. Plants were inoculated with hypodermic syringe or high pressure technique. Bacteria were plated on King B (King 1954) or LB agar (Lennox 1955). The inoculated and controll plants were placed in KLT/04 (Ehret Gmbh., Németország) chambers in different controlled conditions.

2.2. Detection of BR–development and the induction time of HR

The bacteria were killed with antibiotic solution in plant tissue to measure the induction time of HR. The inoculated parts of plant tissue were treated with chloramphenicol in every 30 min. The induction time of HR is the last treating point where the HR did not develop in tobacco (Klement és Goodmann 1967). In similar experimental condition the development time of BR also could be detected. The BR was activated heat–killed pathogens in leaf tissue, followed by subsequent inoculation of HR–causing *Pseudomonas* in every 30 min. The development of BR is the period between two inoculations, where the HR does not appear (Klement et al. 1999).

2.3. *In vitro* and *in planta* bacterial multiplication

Coloniforming units of bacteria were determined from 5 ml liquid King's B media or 5 cm^2 leaf tissue. Inoculated starting material was dilluted. Dilluted solutions were plated on agar. Bacterial cell number was calculated from the number of colonies on plate and the dillution fold (Rudolph 1990).

2.4. Proteomic methods

IWF (intercellular washing fluid) is a water soluted part of plant apoplast. This watery solution is easy of access from vacuum infiltrated and centrifugated leaves. Proteins from IWF were separated 1– or 2D PAGE (Bio-Rad, USA) (Lamelli 1970). The markers were identified with LC-MS/MS (liquid chromatography-mass spectrometry) from silver–stained proteins or determination of chitinase activity direct in gels (Heukeshofen and Dernick 1985, Shevchenko et al. 1996, Kim and Hwang 1994, Zaecho et al. 1995).

2.5. Genomic methods

To detection of expression patterns of BR marker genes we isolated the total RNA from the investigated leaves. The total RNA was translated to DNA. The c(copy)DNA samples served as starting point to

determination and compare the expression level of marker genes with specific primers in a real time PCR reaction.

3. RESULTS

3.1. The influence of low temperature on BR and HR.

We made an experimental system to compare two species of bacteria (*Pseudomonas syringae* pv. *syringae* 61, *P. syringae* 61; *Pseudomonas savastanoi* pv. *phaseolicola* S21, *P. phaseolicola* S21) with different cold-tolerance abbility in tobacco which were incubated at 5-30 ˚C (Table. 1.).

It was a very exciting result that after the inoculation of phytopathogen *Pseudomonads* the HR appeared 10-12 hpi at 20 ˚C, but within only some days at 5 ˚C. *P. syringae* 61 caused HR 3-4 dpi and *P. phaseolicola* S21 5-7 dpi at this temperature. Delay in appearance of the necrosis was caused by the longer induction and latent priod of HR, as well. At low temperature the induction period of HR was influenced by the cold-tolerance abbility of used bacteria. The HR-induction time of *P. phaseolicola* S21 increased much stronger than of the *P. syringae* 61. It is determining what type of defence response develops at the finishing of a plant-pathogen interaction. Last years the development time of BR was the period between the first and the subsequent second inoculation, when it prevented the appearance of HR.

Klement et al. (2003) and Bozsó et al. (1999) published that the BR inhibited the activity of *hrp* genes in pathogenic bacteria, therefore it is able to prevent the HR during its induction time. Low temperatures increased this measured time period between the first and second inoculation, and the HR-induction time of challenge bacteria used in the second inoculation as well. Therefore there is more time for the development of BR. The time between the two inoculations was influenced by the properties of challenge bacteria. The knowing these results the "total" development time of BR is the sum of the measured time period between first and second inoculation and the HR-induction time of second used bacteria (Table. 1.). $BR_{det} = BR_{total} - IT$, where BR_{det} came from the period between the first and second inoculation and IT was the HR-induction time of the bacteria used for detection. Low temperatures strongly increased the development of BR induced by heat-killed *P. syringae* 61 or *P. phaseolicola* S21 in tobacco. So the total development time of BR was similar in both combinations (Table. 1.).

The *tpoxN1* gene was isolated from TMV-infected tobacco leaves at first, then it was reported as a BR marker gene (Bozsó et al. 2002). The *EBR-43* codes an orthometil-transferase enzyme (Szatmári et al. 2006). This BR-marker has role in synthesis of lignin in tobacco. The transcription of these BR-marker genes were also proved that low temperatures inhibited the BR and HR. These tested genes were activated stronger than BR, if the HR was induced in tobacco. These resultes were explained if these defence responses use similar processes in plant to function (Bozsó et al. 2002).

Table. 1. The "total" development time of BR corrected by the HR-induction time in tobacco between 5-30 ˚C temperature.

	Ps+Ps	Pp+Pp
Temperature (˚C)	**"total" development time of BR** (hours)	
30	**4,28**	no data
20	**6,39**	**6,33**
15	**8,83**	**10,5**
10	**21,83**	**22,72**
5	no data	**71,83**

The "total" development time of BR is the sum of the measured time period between first and second inoculation and the HR-induction time of second used bacteria. Ps+Ps: first inoculation with heat-killed *P. syringae* 61 (4×10^8 sejt/ml) and second inoculation with live *P. syringae* 61 (10^8 sejt/ml) Pp+Pp: first inoculation with heat-killed *P. phaseolicola* S21 (4×10^8 sejt/ml) and second inoculation with live *P. phaseolicola* S21 61 (10^8 sejt/ml)

The activity of BR or HR also could be investigated by the change of bacterial cell number came from second inoculation of a HR-causing pathogen. At the and point of HR the cell number of investigated bacteria decreased and the necrosis occurred (Bozsó et al. 1999, Klement et al. 1999). It usually appears 10-12 hpi at 20 ˚C temperature. In the plants incubated at 5 ˚C and inoculated with *P. syringae* 61 or *P. phaseolicola* S 21 no response could be detected within two days after inoculation. The cell number of bacteria also did not change in these conditions. The HR did not appeare and did not decrease the concentration of bacteria. The necrosis of plant tissue appeared 3-4 or 4-7 days after inoculation. If the BR induced by heat-killed bacteria, it can be investigated independently from processes of HR. The BR also had decreasing effect on bacterial cell number in plant tissue, but its effect was much weaker than the effect of HR on this area. *P. syringae* 61 was more sensitive the influence of BR. The BR decreased the concentration of *P. syringae* 61 10-folds than *P. phaseolicola* S 21. At 5 ˚C we also could not any response in cell number (BR and HR) detect during 2-2,5 days after inoculation. At low temperatures the plants are without active defence system (BR and HR) against colonisation of pathogenic or non-pathogenic bacteria.

3.2. BR markers (CaPOX, CaCHI) in pepper. The role of the BR and the HR in the colonisation strategy of a cold-tolerant opportunistic pathogen and saprotrophic bacterium

The BR also was active in pepper, but it developed slowlier than we detected in tobacco. Similar to tobacco only the EBR could be detected in pepper which were kept on dark and at low temperatures no BR could be detected. The development of HR was as similar as in tobacco.

Table 2. Extracellular BR marker proteins from pepper, which are similar to some identified or hypothetical proteins from database TIGR.

Code	Size (kDa)	Enzyme activity	Amino acid identity %	Hypothetical protein[a] (TIGR)	Similar, identified protein (NCBI/Swiss-Prot)	Plant of similar protein	References
CaPOX EC 1.11.1.7.	32	peroxidase	41	TC3709	AF442386.1 (total)	pepper	Do et al. (2003)
			36		AF442386.1 (total)	pepper	Do et al. (2003)
			39		AJ810540.1 (total)	pepper	Do et al. (2003)
CaCHI EC 3.2.1.14.	22-24	chitinase	48	TC3666	Q7XB39 (part, 64 %)	grapevine	
			48	TC3667	Q43151 (part, 61 %)	elder	
			22	TC4206	O82552 (part, 81 %)	pepper	Hong et al. (2000)
			16	TC3587	Z15139.1/Q05540 (part, 79 %)	tomato	Danash et al. (1993)

[a]hypotetical protein: protein comes from *in silico* identified open reading frame. Its appearance and role in biological system have not been verified yet.

Table 3. Hypotetical proteins from database TIGR and their references.

		Calculated		References				
Code	**Hypothetical protein (TIGR)**	**Size (kDa)**	**pI**	**Size (kDa)**	**pI**	**Inducing bacteria**	**Inducing fungi**	**Abiotic factors**
CaPOX	**TC3709** Do et al. (2003)	36,129	7,54	36,07	8,8	*Xc*	*Pc, Cg*	SA, H_2O_2
CaCHIs	**TC3666**	15,072	4,65	-	-	-	-	-
	TC3667	15,095	4,8	-	-	-	-	-
	TC4206 Hong et al. (2000)	23,106	6,1	25,161	9,39	*Xc*	*Pc*	E
	TC3587 Danash et al. (1993)	26,439	4,7	27	4,3	-	*Cf*	-

Xc: Xanthomonas vesicatoria
Pc: Phytophtora capsici
Cg: Colletotrichum gloeosporoides
Cf: Cladosporium fulvum
SA: salicylic acid
E: ethylene

Table 4. The production of CaPOX and CaCHI marker proteins from pepper and the development of BR under different induction treatments.

	activation		
Treatment	**BR**	**CaPOX**	**CaCHI**
water	-	+	+
heat-killed *P. syringae* 61	+	+++	+++
P. fluorescens	+	+++	+++
P. tabaci	**0**	+++	+++
P. pisi	**0**	+++	+++
X. vesicatoria	-	-	-
mannithol	-	+	-
sodium-chlorid	-	+	-
paraquat	-	+	-
hydrogen-peroxide	-	+	-
salicylic acid	-	-	+
jasmonic acid	-	-	-
ethylene	-	-	+
BR activation treatment+incubation temperature 30 ˚C	+	+++	+++
BR activation treatment + incubation temperature 5 ˚C	-	-	-
BR activation treatment+ incubation in constant light (72 hours)	+	+++	+++
BR activation treatment+ incubation in constant dark (72 hours)	-	++	-

-: no produce/develop, +: weakly, ++: közepesen, +++: strongly produce/develop
0: The appearance of HR caused: the BR was not detectable.

We have investigated the *in planta* multiplication of *P. fluorescens*, *P. syringae* 61 and *P. phaseolicola* S21 in their host (pepper or bean) at high and low temperatures with the development of BR and HR paralell. The saprotrophic bacteria (*P. fluorescens*) activated the BR in pepper but did not able to cause HR or disease. Its multiplication rate *in vitro* and in pepper had as similar as opportunistic *P. syringae* 61 at both 5 and 30 ˚C temperatures. It could be caused the similar cold-tolerance ability. On the other hand it proved the idea, that the defence of test plants developed rapidly at 30 ˚C and inhibited the multiplication of opportunistic and saprotrophic bacteria. At 5 ˚C no BR and no HR was detected, therefore pepper plants did not defence against colonizing bacteria. The opportunistic pathogens take advantages of these conditions without response, than the saprotrophic bacteria. In contrast of *P. fluorescens* and *P. syringae* 61 the multiplication and disease causing activity of *P. phaseolicola* S21 was inhibited by low temperature.

The inhibition of HR as a type of symptome and the decrease in bacterial cell number *in planta* there was a third pillar to report the BR. Some BR marker proteins were isolated and identified from apoplast of pepper leaves. At high temperature we have identified two BR proteins. One of them was a peroxidase the other was a chitinase enzyme (Table 2., 3.).At low temperature these proteins were not induced. We have donne some treatment similar to kwovn results in tobacco to prove the specificity of marker proteins (Table. 4.).

3.3. New scientific results

- **The development time of BR induced with heat-killed bacteria were modified. The development time of BR is the sum of the period between the pre-treatment and the challenge inoculation and the HR-induction time of challenge bacteria.**
- **Decreasing in temperature injured the active defence system of tobacco (BR, HR).** The plants which were kept in 5 ˚C stood without active defence against colonising bacteria.
- **The BR developed more slowly in pepper than in tobacco, but the effect of temperature and light on the BR was similar in both plants.**
- **BR marker proteins induced by saprotrophic bacteria were detected and isolated from apoplast of pepper leaves. The induced CaPOX and CaCHI marker proteins showed peroxidase or chitinase activity.**
- **At low temperature (5 ˚C) the absence of active defence system made without defence the plants against cold-tolerant pathogen bacteria, one of the parameters of their disease causing ability.**

4. DISCUSSION AND CONCLUSIONS

In previous studies Klement et al. (1999, 2003) were investigated the development of BR and HR on different temperatures (20 és 30 ˚C). It is a very intresting question if we have focused on the thesis, that the BR protects the plants against epiphytic bacteria.

Decreasing in temperature delayed the development of BR in tobacco. It was demonstrated the delayed appearance of the HR-inhibition and increasing the induction and the latent period of HR, as well. The BR developes and the HR appeares within several hours at 20-30 ˚C but they can not detectable at 5 ˚C. These results come from symptom-experiments the detecting of bacterial cell number (CFU) and expression of two marker genes also proved under similar conditions. In contrast of control experiments at 20 ˚C, we did not detect the reducing effect of plant defense in the bacterial cell number and increased expression of BR marker genes (*tpoxN1*, peroxidease, *EBR-43*, orthomethil-transferase) at 5 ˚C. So some type of experiments proved that tobacco plants are without defence system against bacteria at 5 ˚C.

The BR and HR developed much slower at the investigated temperatures in pepper than in tobacco. It was characteristic of both plants that no defence response was detected during days of experiments at 5 ˚C.

The proteins secreted from the plant cells into the apoplast are in direct connection to the bacteria, which live there. Two extracellular, induced enzyme of pepper marked the induction of BR. They were separated on SDS-PAGE, stained peroxidase-specific and idetified with mass spectrometry. CaPOX is a peroxidase and it is similar to AF442386.1 or AJ810540.1 proteins (NCBI). CaCHI protein is simmilar to four chitinase isoenzymes. Because of investigation of some environmental and abiotic stress factors the induction of marker proteins specified to BR. Their induction did not occure if the plants were incubated in dark or at low temperature.

The BR blocked the multiplication of saprotrophic bacteria and the both, BR and HR, together control the colonisation of opportunistic pathogen at 30 ˚C. But at 5 ˚C the bacteria, they are pathogen or not, did not need fight to effects of plant defences. At this low temperature the extracellular level of carbohydrates increased in the apoplast. These are important factors in colonisation of *P. syringae* and *P. fluorescens* which are able to multiplicate at 5 ˚C.

BR marker proteins in pepper plants made possible the detection of BR, when HR-inhibition is not suitable method for it, because the HR not appears during the experimental conditions. It can occure at the too low or high temperatures for test plants. For instance a strain of *Xanthomonas vesicatoria* did not cause HR on pepper at 32 ˚C and other Pseudomonas species also not cause HR at 37 ˚C on tobacco.

Among the apoplast proteins separated on 2D-PAGE, further possibly BR markers appeared. The identification of their and getting to know their function will give more new information about BR and new possibilities in plant beerding.

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