Changes in apoplast protein pattern suggest an early role of cell wall structure remodelling in flagellin-triggered basal immunity

E. SZABÓ[1], Á. SZATMÁRI[1], É. HUNYADI-GULYÁS[2], E. BESENYEI[1], L.R. ZSIROS[1], Z. BOZSÓ[1] and P.G. OTT[1]*

Plant Protection Institute, Hungarian Academy of Sciences, Budapest, H-1022, Hungary[1]
Biological Research Center, Hungarian Academy of Sciences, Szeged, H-6726, Hungary[2]

Abstract

The leaf apoplast is a dynamic compartment in contact with plant pathogenic bacteria after infection. Among the very first interaction events is the receptor-mediated perception of bacterial surface molecules such as flagellin or other conserved microbe-associated molecular patterns (MAMPs). Apoplast proteins likely play a role in basal resistance (BR) or pattern-triggered immunity (PTI). Here, a proteomic approach was carried out on water soluble – potentially the most mobile – apoplast proteins from flagellin-treated tobacco (*Nicotiana tabacum*) leaves. As the quickness of BR/PTI seems crucial for its efficacy, samples were taken as early as 2.5 and 7 h post inoculation. Proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Forty-nine different proteins from 28 protein spots changed in their density compared to the water-inoculated control. Eleven protein spots appeared *de novo* in response to EBR induction. There are glycohydrolases and redox-active proteins besides pathogenesis-related proteins among them, predicting plant cell wall structural modifications and more direct antimicrobial effectors as earliest changes related to BR/PTI.

Additional key words: basal resistance, glycohydrolases, pathogenesis-related proteins, proteomics, tobacco.

Introduction

Plants use their ability to recognise foreign organisms, which is fundamental for basal resistance (BR) and crucial for lowering disease susceptibility. Several bacterial elicitors of BR responses have been determined. Early studies (Lovrekovich and Farkas 1965, Burgyán and Klement 1979, Klement *et al.* 2003) applied whole-cell bacterial preparations or complex elicitors. Later the specific but conserved molecular components, called microbe-associated molecular patterns (MAMPs), such as flagellin (Felix *et al.* 1999, Felix and Boller 2003) have been found, making investigations on downstream processes feasible. Thus, BR comprises elements of pattern-triggered immunity (PTI).

As MAMPs are the first signs reporting potential danger of infection, PTI/BR is a first-line defence response. At increasing temperatures from 10 to 30 °C, the time needed for its development decreases to only 1 - 2 h (Besenyei *et al.* 2005). A period of similar length is required by the bacterium to translocate (a)virulence proteins into the host cytoplasm, a pivotal process for a successful infection. This translocation event requires the expression of bacterial *hrp* genes coding for a syringe-like injector apparatus with a needle piercing through plant cell walls. Importantly, BR is able to inhibit the expression of *hrp* genes (Bozsó *et al.* 1999, Klement *et al.* 2003) and the translocation event itself (Crabill *et al.* 2010).

The leaf apoplast is a dynamic compartment involved in a variety of functions during normal physiological processes and under biotic stress conditions (Patykowski and Urbanek 2003). The plant cell wall facing the intercellular space is the primary stage of plant cell-bacteria interaction. It keeps bacteria outside of plant cells and probably conveys a significant portion of factors

Received 22 July 2010, *accepted* 13 July 2011.
Abbreviations: BR - basal resistance; 2D-PAGE - two-dimensional gel electrophoresis; hpi - hours post inoculation; IWF - intercellular washing fluid; LC-MS/MS - liquid chromatography tandem mass spectrometry; MAMP - microbe-associated molecular pattern; PR - pathogenesis-related; PTI - pattern-triggered immunity; T3SS - type III secretion system.
Acknowledgements: This work was financially supported by the National Scientific Research Fund Nos. AT-049318 and K68386. We are grateful to Georg Felix, Zürich-Basel Plant Science Center, Botanisches Institut der Universität Basel, Switzerland, for providing *Pseudomonas avenae* flagellin.
* Corresponding author; fax: (+36) 1 4877555, e-mail: pott@nki.hu

responsible for the above mentioned pathogen inhibitory effects of BR. Secreted proteins, especially mobile ones, likely have control over these processes. Several research groups characterized plant defence processes by analysing the apoplast (Boller 1995, Bolwell *et al*. 2001, Dahal *et al.* 2010) or the soluble extracellular protein fractions (Jones *et al.* 2004, Chivasa *et al.* 2005, Martinez-Esteso *et al*. 2009). However, very little is known about changes in the leaf apoplast protein pattern accompanying the PTI/BR, especially in the critical early phase.

According to our previous results (Ott *et al.* 2006), changes in some proteins, chitinases, specifically connected to BR could be detected in tobacco apoplast already 2 h following BR-induction. The aim of our study was to describe more comprehensive changes in extracellular water-soluble (*i.e.* mobile) apoplast protein composition during the early events (2.5 and 7 h after induction) of BR, referred to as early basal resistance (EBR). Earliest changes were found in proteins related to modification of cell wall structure, accompanied by a widening array of PR-proteins.

Materials and methods

Plant material and BR induction: Experiments were carried out on 2.5-month-old tobacco (*Nicotiana tabacum* cv. *Samsun*) plants grown in greenhouse. BR was induced by injection of 1 μM suspension of the 22 amino acid long fragment (flg22) of the flagellin protein (a MAMP) isolated from *Pseudomonas avenae* (by courtesy of Prof. Georg Felix) into the leaf intercellular space (Nürnberger *et al*. 2004). The reason for using flagellin suspension for inducing BR instead of using the bacteria suspension itself was to avoid false positive protein spots of bacterial proteins on 2D-PAGE gels. Being symptomless, BR had to be detected by an indirect method: inhibition of hypersensitive response (HR; Klement *et al*. 1999). Water-injected as control and flagellin-injected tobacco plants were incubated at 30 °C because BR develops quickly at this temperature.

Apoplast protein extraction: BR-induced leaves were harvested 2.5 and 7 h after flagellin injection. The main leaf veins were cut out, the leaf segments were washed with tap water to remove leaky intracellular content on the cut surfaces. Following this, the leaf segments were infiltrated with distilled water with a vacuum pump. This fluid (intercellular washing fluid, IWF) containing water-soluble apoplast proteins was extracted by centrifugation at 600 *g* for 10 min, which was found optimal for sufficient extraction without cell damage insuring that the IWF was not contaminated with cytoplasmic proteins. As an additional precaution, any IWF samples visibly green due to intracellular contamination were discarded. IWF purity was also verified by the glucose-6-phosphate-dehydrogenase assay (Weimar and Rothe 1986). Water soluble apoplast protein samples extracted in three independent experiments were resolved separately on two 2D-PAGE gel pairs (two time points, control and treatment) per experiment.

Protein assay: Intercellular washing fluid of tobacco has low protein content (40 μg cm^{-3}), thus samples were concentrated by ultrafiltration prior to 2D analyses. 3 cm^3 of tobacco IWF was concentrated 30-fold and precipitated with the 2D *Clean-Up* kit (*GE Healthcare*, Wauwatosa, WI, USA). Protein pellets were solubilised in 130 mm^3 rehydration buffer containing 8 M urea, 2 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 50 mM dithiothreitol and *DeStreak* reagent (*GE Healthcare*). Approximately 100 μg IWF proteins were loaded onto 7 cm long immobilized pH gradient strips with 3 - 10 and 4 - 7 pH ranges (*ReadyStrip* IPG strips - *Bio-Rad*, Hercules, USA). Rehydrated strips were isoelectrofocused (*Protean* IEF cell, *Bio-Rad*) at 20 000 Vh at 20 °C. Isoelectrofocused strips were incubated for 20 min in equilibration solution I containing 6 M urea, 0.37 M Tris-HCl, pH 8.8, 2 % sodium-dodecylsulfate (SDS), 20 % glycerol, 2 % (m/v) dithiothreitol, and then for another 20 min in equilibration solution II, which is the same as solution I but containing 2.5 % (m/v) iodoacetamide instead of dithiothreitol. Equilibrated gel strips were loaded on the top of 10 - 20 % gradient denaturating SDS-poly-acrylamide gel (SDS-PAGE). Electrophoresis was performed according to the method of Laemmli (1970) with the *Mini-PROTEAN®3* vertical electrophoresis system (*Bio-Rad*). Gels were stained with Coomassie Brilliant Blue G250. Images of representative gel pairs of three replicates from different sampling periods are shown in Fig. 1. Gel images were scanned and stored in TIF-format and were analysed by *Alpha View* spot densitometer software (*v. 1.3.0.7, Alpha Innotech Corporation*, San Leandro, CA, USA).

Protein identification based on mass spectrometry: Protein spots of interest were excised from the gel. Disulphide bonds were reduced with dithiothreitol, free sulfhydryls were alkylated with iodacetamide prior to tryptic digestion using side-chain protected porcine trypsin from *Promega* (Madison, WI, USA; 4 h at 37 °C). The extracted tryptic peptides were dried and resuspen-ded in 0.1 % formic acid and were analyzed with LC-MS/MS (*LCQ-Fleet*, *Thermo Fisher*, Waltham, MA, USA) instrument in "triple play" acquisition mode where MS scan was followed by ZOOM scans and collision-induced dissociation analyses of the 3 most abundant multiply charged ions. Nano-HPLC separation was carried out on a *Waters Atlantis* (Massachusetts, USA) C18 column, 10 cm × 75 μm, in a gradient mode: 0 - 40 min, 10 - 50 % B in A + B (B: 0.1 % formic acid/ acetonitrile, A: 0.1 % formic acid/water), flow rate was 0.005 $mm^3 s^{-1}$.

Protein identification based on CID spectra: raw data files were converted into searchable peaklist files using *Mascot Distiller* software (*v. 2.2.1.0*; http://www.matrixscience.com). The peak lists were searched on our in-house *Mascot* server (*v. 2.2.04*). Only those peptide matches were considered to be significant that had a *Mascot* score of 50 or higher, and the total number of peptides assigned to a given protein was at least two.

For database search the following parameters were applied. National Center for Biotechnology Information (*NCBI nr 20080718*) database reduced to *Viridiplantae* (372 407 sequences). The type of search was MS/MS Ion Search, digesting enzyme was trypsin. Fixed modifications were carbamidomethyl (C), variable modifications were acetyl (protein N-term), Gln->pyro-Glu (N-term Q), oxidation (M). Mass values were monoisotopic, protein mass was unrestricted, peptide mass tolerance was ± 0.6 Da. Fragment mass tolerance was ± 1 Da, max missed cleavages were 2. Instrument type was *ESI-TRAP*, individual ions scores > 43 indicate identity or extensive homology ($P < 0.05$).

Bioinformatics: For all the identified protein name queries for associated annotations were performed within the *GO* database (http://www.geneontology.org). The annotations (altogether around 800 *GO* terms with variable frequency for the 49 proteins) were classified further manually, following special interests about functions related to cellular location, response to biotic stress and roles in cell wall structure. We note that many different annotations (*e.g.* for intracellular locations) can be assigned to a given protein type.

Database search for cellular localization of the identified proteins was carried out with the *TargetP* software (http://www.cbs.dtu.dk/services/TargetP), according to Emanuelsson *et al*. (2000).

Results and discussion

To examine changes in tobacco apoplast, protein composition was investigated during the early period of basal resistance. The number of Coomassie Brilliant Blue G250-stained protein spots in a gel varied around 100 depending on the time of sampling. Coomassie Brilliant Blue has a detection limit of about 10 ng of proteins per spot. The fact that most protein spots were situated mostly in the acidic range of the 2D gels (Fig. 1) was in

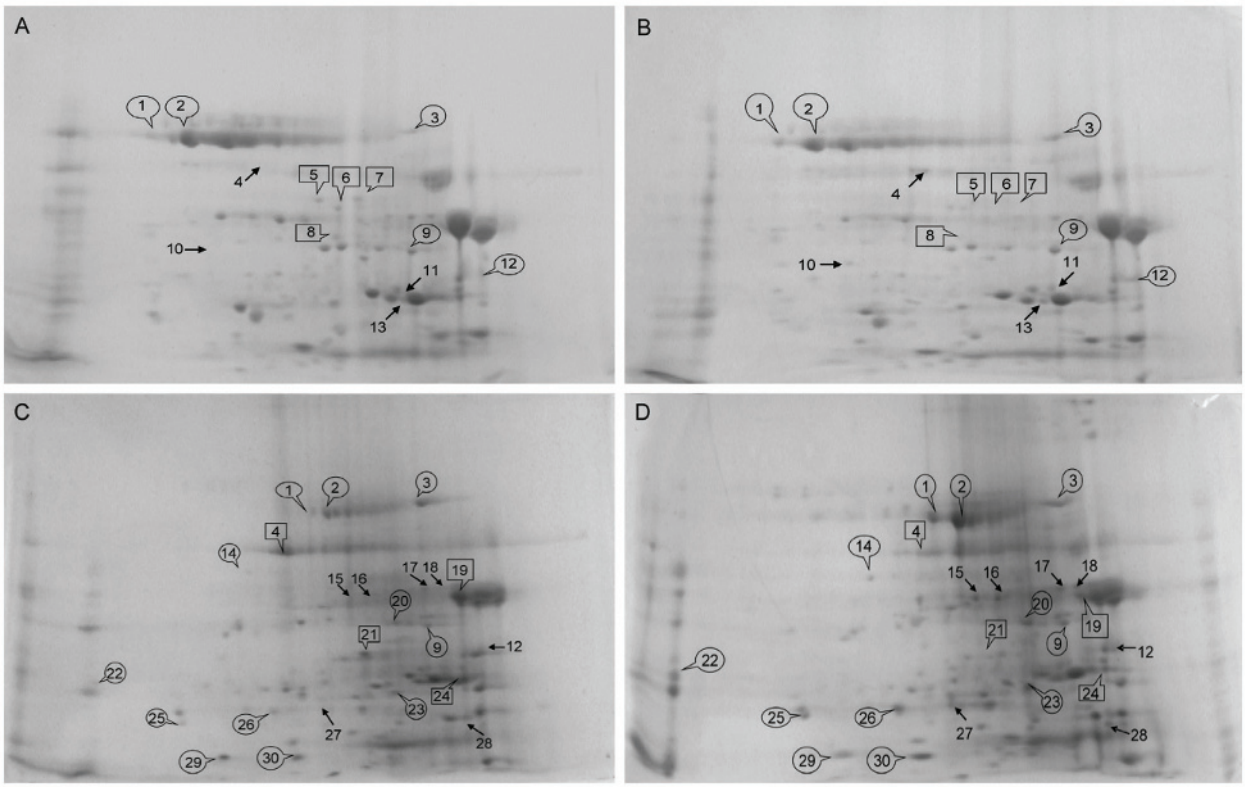

Fig. 1. 2D electrophoretic pattern of water soluble apoplast proteins obtained from control (*A*, *C*) and flagellin-infected (BR-induced, *B*, *D*) tobacco plants 2.5 h (*A*, *B*; pH range 4 - 7) and 7 h (*C*, *D;* pH range 3 - 10) after injection. Representative gels of 3 individual experiments are shown. Protein spots with numbers were identified by mass spectrometry. Numbers in *circles* or *boxes* mark proteins that significantly increased or decreased, respectively, in the gels obtained from BR-induced plants compared to those obtained from control ones. *Arrows* indicate proteins exclusively present in the BR-induced samples.

Table 1. Tobacco leaf apoplast proteins carrying an N-terminal secretory pathway signal peptide, listed in time order and numbered like in Fig 1. Identification data and accession numbers are from the *NCBI* database. Protein spots changing in their density (up = increased, down = decreased, new = *de novo* appeared) are presented. Proteins identified from protein spots, which appeared *de novo* in the BR-induced samples are those which might provide the basis to draw conclusions concerning the early events of BR. Molecular mass (Mr) and isoelectric point (pI) values were calculated from database sequences. Cw - cell wall proteins, PR - pathogenesis related proteins. Detailed information can be found in Results and discussion, where spot numbers are in brackets.

Spot	Change	Acc. No.	Protein	2.5 hpi	7 hpi	Cw	PR	Species	Mr/pI
7	down	86278368	acid α-galactosidase	*		*		*C. sativus*	45/5.3
10	new	42795462	xyloglucan endotransglucosylase-hydrolase	*		*		*S. lycopersicum*	32/5.2
11		19771	acidic chitinase PR-P	*			*	*N. tabacum*	28/4.9
13		131015	PR protein R major form precursor	*			*		26/5.4
		131017	PR protein R minor form precursor	*			*		25/5.3
7	down	147814943	hypothetical protein	*				*V. vinifera*	49/5.3
9	up	31711507	24K-germin-like protein	*	*	*		*N. tabacum*	22/5.8
		170304	PR-2	*	*		*		38/4.9
21	down	116337	basic endochitinase precursor	*	*		*		33/8.9
4	new	11071974	elicitor inducible β-1,3-glucanase NtEIG-E76	*	*	*	*		50/5.3
		1161573	enolase	*	*			*S. lycopersicum*	35/6.2
14	up	7939623	putative β-galactosidase		*	*			94/6.8
23		31711507	24K-germin-like protein		*	*		*N. tabacum*	22/5.8
22		19771	acidic chitinase PR-P		*		*		28/4.9
		5360263	NtPRp27 protein		*		*		27/9.3
		19783	osmotin		*		*		28/8.1
23		131015	PR protein R major form precursor		*		*		26/5.4
24	down	19771	acidic chitinase PR-P		*		*		28/4.9
		131015	PR protein R major form precursor		*		*		26/5.4
27	new	19962	PR-4A protein		*		*		17/7.6
		100352	PR-4B protein		*		*		16/7.6
15		129837	lignin-forming anionic peroxidase precursor		*	*	*		35/4.7
19	up				*	*	*		
20		19859	glucan endo-1,3-β-glucosidase		*	*	*		38/5.2
24	down	2541876	CND41 chloroplast nucleoid DNA binding protein		*				54/8.9
22	up				*				
23					*				

concord with the data that inducible pathogenesis-related (PR) proteins secreted into the intercellular space are mostly acidic (Van Loon 1997). Their apparent molecular mass varied between 10 and 90 kDa. In total, 28 protein spots were analysed by peptide mass fingerprinting. Most changes between the water-injected and BR-induced samples were quantitative, however, *de novo* appearance of 11 proteins could be detected as well. Several spots contained multiple peptides (Tables 1,2,3). Due to limitations of this MS analysis technique, the relative amount of each identified peptides within one protein spot is not quantifiable, so spot densitometry would neither tell more about the amount of these peptides. Taken into account this fact and the reproducibility and resolution limit of 2D-PAGE technique, it needs to be emphasised, that conclusions concerning the early events of the BR can be drawn mainly on the basis of the 'new' protein spots, which appeared *de novo* in the BR-induced samples. At 2.5 hpi 12 protein spots were found to change in their appearance (Fig. 1*A*,*B*, Tables 1,2,3). Among them four appeared *de novo*, four increased, while four decreased during EBR. At 7 hpi 22 spots changed in their density (Fig. 1*C*,*D*, Tables 1,2,3). Seven of them appeared *de novo*, 13 increased, two decreased in the BR-induced samples relative to the control ones. In some cases the same protein was identified from multiple spots indicating probable posttranslational modification or multigene family origin.

Four proteins with a single-peptide match though were accepted as valid: a putative proline-rich protein [30] and a xyloglucan endotransglucosylase-hydrolase [10], because these proteins were the only match from the given protein spots. The induction of the NtPRp27 protein [22] and glutathione-S-transferase [26] during BR

Table 2. Tobacco leaf apoplast proteins of "other" localization (without chloroplast transit peptide, mitochondrial targeting peptide or secretory pathway signal peptide), listed in time order and numbered like in Fig. 1. For further details see Table 1.

Spot	Change	Acc. No.	Protein	2.5 hpi	7 hpi	cw	PR	Species	Mr/pI
5	down	119354	enolase	*				*S. lycopersicum*	48/5.7
1	up	37359708	beta-D-xylosidase, LEXYL	*	*	*			70/8
12		62719019	basal resistance related chitinase	*	*		*	*N. tabacum*	11/4.8
21	down			*	*		*		
		3023188	14-3-3 like protein A	*	*				29/4.7
		125594744	hypothetical protein	*	*			*O. sativa*	38/4.8
20	up	100325	glucan-endo-1,3-beta-D-glucosidase		*	*		*N. tabacum*	18/5.1
22		62719019	basal resistance related chitinase		*		*		11/4.8
24	down				*		*		
25	up	19948	PR-1b protein		*		*		16/5.3
16	new	10798652	malate dehydrogenase		*				36/5.9
19	up	1063400	glycolate oxidase		*			*S. lycopersicum*	31/9.3
		10798652	malate dehydrogenase		*			*N. tabacum*	36/5.9
20					*				
		76556494	putative cytosolic cysteine synthase 7		*				34/5.3
26		68687853	glutathione S-transferase		*				13/6
27	new	223593	carboxylase/oxygenase,RBP		*				14/5
29	up	134616	superoxide-dismutase		*			*N. plumbaginifolia*	15/5.5
24	down	13310811	ankyrin-repeat protein HBP1		*			*N. tabacum*	38/4.7

Table 3. Tobacco leaf apoplast proteins carrying chloroplast transit peptide, listed in time order and numbered like in Fig. 1. For further details see Table 1.

Spot	Change	Acc. No.	Protein	2.5 hpi	7 hpi	cw	PR	Species	Mr/pI
8	down	4827251	plastidic aldolase NPALDP1	*				*N. paniculata*	43/6.9
		4827253	plastidic aldolase	*					43/6.3
		2492782	alpha-galactosidase precursor	*				*C. arabica*	42/5.6
3	up	399942	stromal 70 kDa heat shock-related protein, chloroplast precursor	*	*			*P. sativum*	76/5.2
4	new	132000	RuBisCO large subunit	*	*			*N. acuminata*	53/6.4
21	down	115473	carbonic anhydrase, chloroplast precursor	*	*			*N. tabacum*	35/6.4
30	up	48375046	putative proline-rich protein		*				21/9.1
15	new	4827253	plastidic aldolase		*			*N. paniculata*	43/6.4
16		4827251	plastidic aldolase NPALDP1		*				43/6.9
17		125579	phosphoribulokinase		*			*S. oleracea*	45/6
		4827253	plastidic aldolase		*			*N. paniculata*	43/6.4
18		125579	phosphoribulokinase		*			*S. oleracea*	45/6
		4827253	plastidic aldolase		*			*N. paniculata*	43/6.4
19	up	32746733	mRNA-binding protein precursor		*			*N. tabacum*	45/6.5
		4827251	plastidic aldolase NPALDP1		*			*N. paniculata*	43/6.9
		125578	phosphoribulokinase, chloroplast precursor		*			*M. crystallum*	44/6
20		4827253	plastidic aldolase		*			*N. paniculata*	43/6.4
23		131393	oxygen-evolving enhancer protein 2-2		*			*N. tabacum*	29/7.7
		52000782	oxygen-evolving enhancer protein 2-3		*				
		52000814	oxygen-evolving enhancer protein 2-1		*				
27	new	132080	ribulose bisphosphate carboxylase		*			*N. sylvestris*	21/6.6
28		1350820	30 kDa ribonucleoprotein		*			*N. plumbaginifolia*	31/4.8
		1762130	chaperonin-60 beta subunit		*			*S. tuberosum*	63/5.7

was confirmed also on mRNA level. The expression of other multi-peptide matches as superoxide dismutase, basal resistance related chitinase, lignin-forming anionic peroxidase and PR-1 protein was also confirmed

(Szatmári *et al.* 2005, Szatmári *et al.* unpublished). As the protein database of *Nicotiana tabacum* is still not complete, in some cases homologous proteins were identified from other plant species.

Changes in the expression of different glycoside hydrolase enzymes were detected at both 2.5 and 7 hpi. At 7 hpi (Fig. 1*C*,*D*) the increasing amount of PR proteins is most notable. Six protein spots [1, 3, 4, 9, 12, 21] were detected with a relatively long persistence in the IWF. They showed early increased density in the BR-induced samples at 2.5 hpi and their concentration was still high at 7 hpi.

Although great attention was paid on IWF extraction, still several proteins with a reported cytoplasmic origin – and a probable high affinity to the cell wall matrix, making it hard to eliminate during the washing step – were detected on the 2D gels. From the 49 different proteins identified from 28 analysed protein spots 16 (33 %) were found to possess a chloroplast transit peptide. These are not further analysed as we do not know whether they are artefacts of the sampling. Intriguingly, several recent studies (*e.g.* Dahal *et al.* 2010) have reported components of primary/energy metabolism in a plant secretome and discussed their possible roles in defence.

We explored further the *GO* annotations focussing on sugar metabolism and binding as this property may have influenced protein redistribution during sampling. We found that from the non-chloroplast targeted proteins (33 acc., Tables 1,2), 22 (67 %) had annotations related to carbohydrate metabolism and/or binding, 17 (51 %) to nucleotide (molecules with a sugar moiety) binding. Their overlap was 13 proteins. The remaining 7 (21 %) consisted of five, biochemically less known PR-proteins and two redox-active proteins (24K-germin, lignin-forming peroxidase). The lack of sugar binding could confer a higher mobility that is probably advantageous for resistance-related functions like reaching microbial targets or quick restructuring of the extracellular matrix. Chloroplast-targeted proteins (Table 3) had similar distribution (not shown), however, with a swap of preference for nucleotides over carbohydrates. Interestingly all the carbohydrate-active proteins here have been annotated to the extracellular milieu as well. The only protein not annotated to bind these molecule types was oxygen-evolving enhancer protein (3 isoforms), which is also redox-active.

From the 33 non-chloroplast targeted proteins 15 (45 %) were known PR-proteins and further five (15 %) have been *GO*-annotated as responsive to biotic stress, while only 7 to other stresses. Even from the chloroplast transit sequence-containing proteins 10 (63 %) were found to be responsive to stress. Here phosphoribulokinases are also known (by the *GO* database) to respond to bacterial pathogens. A portion (4 accessions, 27 %) of PR-proteins has reported plant cell wall-modifying properties, all four having a secretion signal sequence (Table 1). Thus, proteins tend to be close to the location of the stress they respond to. Cell wall-modifying enzymes (9 accessions, 27 % of non chloroplast-targeted proteins) are marked in Tables 1,2.

We follow with the discussion of those proteins, which were isolated from *de novo* appeared protein spots belonging to two main functional groups related to cell wall structure and response to biotic stress.

The plant cell wall polysaccharides are usually grouped into three classes: cellulose, hemicelluloses and pectic polysaccharides. Usually, polysaccharides make 80 - 90 % of the wall, structural proteins, enzymes and phenolic compounds like lignin constitute the remaining 10 - 20 % (Carpita *et al*. 1993). Plant enzymes that belong to the superfamilies of glycoside hydrolases and transglycosidases are involved in the structural reorganization of cell wall polysaccharides (Minic *et al*. 2006). Glycoside hydrolase enzymes, which possibly take part in plant cell wall polysaccharide modification during EBR, changed in their amount at both 2.5 and 7 hpi.

In the primary cell wall, xyloglucans tethering cellulose microfibrils are cleaved and rebounded again by xyloglucan endotransglucosylase-hydrolase [10]. It loosens up the cell wall structure, through which cell wall extension can occur (Fry *et al*. 1992, Cosgrove 2001). Xyloglucan endotransglucosylase-hydrolase may function also to integrate newly secreted xyloglucans into the existing wall network (Nishitani *et al*. 1992). It appeared *de novo* 2.5 hpi during EBR.

The β-D-xylosidase LEXYL2 [1] is a xylan-remodelling enzyme hydrolyzing xylo-oligosaccharides and xylobiose from their non-reducing ends to release D-xylose (Minic *et al*.2004). It appeared at 2.5 hpi in the course of EBR.

β-Galactosidase is usually associated with pectin and galactan degradation during fruit ripening processes. Esteban *et al*. (2003) have reported β-galactosidase gene induction in relation to vegetative organ elongation. The degradation of pectin side chains could increase cell wall pore sizes and allow access to cell wall substrates of other wall-modifying proteins such as expansins, xyloglucan endotransglycosylase-hydrolase or other hydrolases. The amount of β-galactosidase [14] increased 7 hpi during EBR.

PR-2 proteins are β-1,3-glucanases believed to take part in plant protection against pathogen attack (Bucher *et al*. 2001). It has been suggested that the callose accumulated in plants in response to biotic and abiotic stresses may serve as a reservoir of plant β-glucan elicitors (Esquerré-Tugayé *et al*. 2000), which could be a hypothetical role of callose depositions, although experimental support of that is still lacking. *De novo* appearance of the elicitor inducible β-1,3-glucanase [4] was detected at 2.5 hpi, and the enzyme was still persisting at 7 hpi in the IWF. It is thought to be an important defence-related protein against fungal pathogens and able to take part in callose and hemicellulose degradation (Takemoto *et al*. 2003).

De novo appearances of lignin-forming anionic peroxidase (PR-9) [15, 19] isoforms were identified at

7 hpi supporting the idea of papilla formation and cell wall enforcement through cross-linking between lignin precursors. Lignin-forming anionic peroxidase responds to wounding, pathogen attack (Lagrimini *et al*. 1987b, Egea *et al*. 2001) and oxidative stress. The lignified cell wall is water resistant and thus less accessible to microbial cell wall degrading enzymes (Von Röpenack *et al*. 1998). Lignin-forming anionic peroxidase also contributes to the removal of H_2O_2, oxidation of toxic reductants, suberization and auxin catabolism. Its function is well understood in tobacco (Lagrimini *et al*. 1987a). Samples gained from the BR-induced tobacco leaves both at 2.5 and 7 hpi showed in every case a brownish colour compared to the ones gained from the control leaves, which may be an indicator of oxidised phenolics resulting from phenylpropanoid pathway activation where lignin precursors are also formed.

Content of proline-rich protein [30] increased at 7 hpi. It is a main component of cell wall (Showalter 1993) and with its basic character it may interact ionically with the acidic pectin network. Proline-rich protein may become rapidly insolubilized in response to stress, which may be mediated by the release of hydrogen peroxide and catalyzed by a wall peroxidase (Bradley *et al*. 1992). They are possibly involved in lignification processes (Ye *et al*. 1991). The protein found here contains a chloroplast transit peptide, so on the basis of that it rather seems to be contaminant. Consistent with the increased pro-oxidant activity, enzymes with antioxidant activity accumulated in BR-induced tobacco IWF, possibly controlling an excess of reactive oxygen species like glutathione S-transferase [26] and superoxide dismutase [29].

PR proteins may contribute to EBR development by improving its efficacy and inhibit bacterial metabolism in a direct or indirect way. Eight PR proteins were identified from protein spots which appeared *de novo.* All of them carry N-terminal signal peptides supporting their probable extracellular role. Their appearance mainly took place in the later stage of BR at 7 hpi. Our earlier studies have already revealed an acidic extracellular chitinase [12] whose appearance is in strong correlation with EBR (Ott *et al*. 2006). Its increasing density in BR-induced samples was confirmed also by the present 2D-PAGE results. Their role during EBR is still not well understood, though they might release elicitors from bacterial cell wall further amplifying the defence mechanism. Many plant endochitinases, especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme-like activity, hydrolyzing peptidoglucan in the bacterial cell wall (Boller *et al*. 1983, Brunner *et al*. 1998). Chitinases can also contribute to the release of intracellular bacterial elicitors, which can amplify the resistance mechanism. Such elicitors might be the cold shock proteins (Felix and Boller 2003). Two other chitinases showed quantitative changes during EBR: acidic chitinase PR-P [11] and basic endochitinase precursor [21].

PR-lb [25], is a protein of less known function whose level under standard conditions is very low but shows a substantial increase in infected plants (Kim *et al*. 2001). It is strongly induced during the onset of systemic acquired resistance (SAR) in tobacco (Uknes *et al*. 1993). The PR-1 family is also associated with tobacco mosaic virus resistance (Cutt *et al*. 1988), and antifungal properties (Alexander *et al*. 1993). *PR-1* gene silencing reduced callose deposition after cryptogein treatment in tobacco (Riviére *et al*. 2008), indicating that PR-1 protein can positively influence this process during defence responses.

PR-4A, 4B proteins [27] with endochitinase activity were identified from a protein spot appearing *de novo* in the BR-induced samples 7 hpi; these proteins may confer antifungal properties (Linthorst *et al*. 1991).

PR protein R major and minor forms [13] are considered to be thaumatin-like proteins. They are members of the PR-5 family and their abundance increased during EBR 7 hpi. Transgenic rice overexpressing thaumatin-like protein showed enhanced resistance against bacterial blight and led to changes in proteins related to oxidative stress and energy metabolism in addition to changes in proteins related to defence (Mahmood *et al.* 2009). Transgenic tobacco over-expressing thaumatin-like protein showed enhanced resistance against different abiotic stresses and fungal pathogens (Munis *et al*. 2010).

The present proteomical analysis of tobacco intercellular washing fluid leads to the conclusion, that basal resistance is accompanied by the change of the protein pattern of the tobacco apoplast. Eleven protein spots appeared *de novo* as an answer to basal resistance induction by bacterial flagellin. The results were supported by bioinformatic analysis concerning the probable cellular location and putative function of these proteins. Appearance of glycoside hydrolases, which are potentially involved in the modification of the cell wall polysaccharide matrix in the apoplast, suggests, that the reorganization of the cell wall structure, maybe through the loosening of its hemicellulose component, might be an important early step during EBR development.

Further examination of two PR proteins bearing cell wall modifying properties, such as elicitor inducible β-1,3-glucanase and lignin-forming anionic peroxidase, might be interesting. The appearance of the latter enzyme might indicate that during EBR the rearranged plant cell wall structure can be reinforced by enzymes cross-linking between lignin precursors. Chitinases and glucanases increased in their protein density during EBR could liberate elicitor-type saccharides from the bacterial cell wall further stimulating the defence response. All these mechanisms together may contribute to the complexity and efficiency of EBR which enable the plant to inhibit bacterial colonization in its intercellular space in an early stage of plant-bacteria interaction and thus alleviate disease.

References

Alexander, D., Goodman, R.M., Gut–Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E.: Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. - Proc. nat. Acad. Sci. USA **90**: 7327-7331, 1993.

Besenyei, E., Ott, P.G., Bozsó, Z., Czelleng, A., Szatmári, Á., Varga, G.J., Klement, Z.: Low temperature delay and inhibition of a plant defense mechanism: early basal resistance in tobacco. - Acta phytopathol. entomol. Acad. Sci. hung. **40**: 323-332, 2005.

Boller, T.: Chemoperception of microbial signals in plant cells. - Annu. Rev. Plant Physiol. Plant mol. Biol. **46**: 189-214, 1995.

Boller, T., Gehri, A., Mauch, F., Vögeli, U.: Chitinase in bean leaves: induction by ethylene, purification, properties and possible function. - Planta **157**: 22-31, 1983.

Bolwell, P.P., Page, A., Pislewska, M., Wojtaszek, P.: Pathogenic infection and the oxidative defense in plant apoplast. - Protoplasma **217**: 20-32, 2001.

Bozsó, Z., Ott, P.G., Kecskés, M.L., Klement, Z.: Effect of heat and cycloheximide treatment of tobacco on the ability of *Pseudomonas syringae* pv. *syringae* 61 *hrp/hrmA* mutants to cause HR. - Physiol. mol. Plant Pathol. **55**: 215-223, 1999.

Bradley, D.J., Kjellbom, P., Lamb, C.J.: Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. - Cell **70**: 21-30, 1992.

Brunner, F., Stintzi, A., Fritig, B., Legrand, M.: Substrate specificities of tobacco chitinases. - Plant J. **14**: 225-234, 1998.

Bucher, G.L., Tarina, C., Heinlein, M., Di Serio, F., Meins, F. Jr., Iglesias, V.A.: Local expression of enzymatically active class I beta-1,3-glucanase enhances symptoms of TMV infection in tobacco. - Plant J. **28**: 361-369, 2001.

Burgyán, J., Klement, Z.: Early induced selective inhibition of incompatible bacteria in tobacco plants. - Phytopathol. Med. **18**: 153-161, 1979.

Carpita, N.C., Gibeaut, D.M.: Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. - Plant J. **3**: 1-30, 1993.

Chivasa, S., Simon, W.J., Yu, X.L., Yalpani, N., Slabas, A.R.: Pathogen elicitor-induced changes in the maize extracellular matrix proteome. - Proteomics **5**: 4894-4904, 2005.

Cosgrove, D.J.: Wall structure and wall loosening. A look backwards and forwards. - Plant Physiol. **125**: 131-134, 2001.

Crabill, E., Joe, A., Block, A., Van Rooyen, J.M., Alfano, J.R.: Plant immunity directly or indirectly restricts the injection of type III effectors by the *Pseudomonas syringae* type III secretion system. - Plant Physiol. **154**: 233-244, 2010.

Cutt, J.R., Dixon, D.C., Carr, J.P., Klessig, D.F.: Isolation and nucleotide sequence of cDNA clones for the pathogenesis-related proteins PR1a, PR1b and PR1c of *Nicotiana tabacum* cv. Xanthi nc induced by TMV infection. - Nucl. Acids Res. **16**: 9861, 1988.

Dahal, D., Pich, A., Braun, H.P., Wydra, K.: Analysis of cell wall proteins regulated in stem of susceptible and resistant tomato species after inoculation with *Ralstonia solanacearum*: a proteomic approach. - Plant mol. Biol. **73**: 643-658, 2010.

Egea, C., Ahmed, A.S., Candela, M., Candela, M.E.: Elicitation of peroxidase activity and lignin biosynthesis in pepper suspension cells by *Phytophthora capsici*. - J. Plant Physiol. **158**: 151-158, 2001.

Emanuelsson, O., Nielsen, H., Brunak, S., Von Heijne, G.: Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. - J. mol. Biol. **300**: 1005-1016, 2000.

Esquerré-Tugayé, M.T., Boudart, G., Dumas, B.: Cell-wall-degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. - Plant Physiol. Biochem. **38**: 157-163, 2000.

Esteban, R., Dopico, B., Munoz, F.J., Romo, S., Martin, I., Labrador, E.: Cloning of a *Cicer arietinum* beta-galactosidase with pectin-degrading function. - Plant Cell Physiol. **44**: 718-725, 2003.

Felix, G., Boller, T.: Molecular sensing of bacteria in plants. The highly conserved RNA binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. - J. biol. Chem. **278**: 6201-6208, 2003.

Felix, G., Duran, J.D., Volko, S., Boller, T.: Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. - Plant J. **18**: 265-276, 1999.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., Matthews, K.J.: Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. - Biochem. J. **282**: 821-828, 1992.

Jones, A.M.E., Thomas, V., Truman, B., Lilley, K., Mansfield, J., Grant, M.: Specific changes in the *Arabidopsis* proteome in response to bacterial challenge: differentiating basal and *R*-gene mediated resistance. - Phytochemistry **65**: 1805-1816, 2004.

Kim, S., Ahn, I.P., Lee, Y.H.: Analysis of genes expressed during rice - *Magnaporthe grisea* interactions. - Mol. Plant Microbe Interact. **14**: 1340-1346, 2001.

Klement, Z., Bozsó, Z., Ott, P.G., Kecskés, M.L., Rudolph, K.: Symptomless resistant response instead of the hypersensitive reaction in tobacco after infiltration of heterologous pathovars of *Pseudomonas syringae*. - J. Phytopathol. **12**: 479-489, 1999.

Klement, Z., Bozsó, Z., Kecskés, M.L., Besenyei, E., Czelleng, A., Ott, P.G.: Local early induced resistance of plants as the first line of defense against bacteria. - Pest Manage. Sci. **59**: 465-474, 2003.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Lagrimini, L.M., Burkhart, W., Moyer, M., Rothstein, S.: Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. - Proc. nat. Acad. Sci. USA **84**: 7542-7546, 1987a.

Lagrimini, L.M., Rothstein, S.: Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. - Plant Physiol. **84**: 438-442, 1987b.

Linthorst, H.J., Danhash, N., Brederode, F.T, Van Kan, J.A., De Wit, P.J., Bol, J.F.: Tobacco and tomato PR proteins homologous to win and pro-hevein lack the 'hevein' domain. - Mol. Plant Microbe Interact. **4**: 586-592, 1991.

Lovrekovich, L., Farkas, G.L.: Induced protection against wildfire disease in tobacco leaves treated with heat-killed

bacteria. - Nature **205**: 823-824, 1965.

Mahmood, T., Jan, A., Komatsu, S.: Proteomic analysis of bacterial blight defence signalling pathway using transgenic rice overexpressing thaumatin-like protein. - Biol. Plant. **53**: 285-293, 2009.

Martinez-Esteso, M.J., Sellés-Marchart, S., Vera-Urbina, J.C., Pedreńo, M.A., Bru-Martinez,R.: Changes of defense proteins in the extracellular proteome of grapevine (*Vitis vinifera* cv. Gamay) cell cultures in response to elicitors - J. Proteomics **73**: 331-341, 2009.

Minic, Z., Jouanin, L.: Plant glycoside hydrolases involved in cell wall polysaccharide degradation. - Plant Physiol. Biochem. **44**: 435-449, 2006.

Minic, Z, Rihouey, C., Do, C.T., Lerouge, P., Jouanin, L.: Purification and characterization of enzymes exhibiting beta-D-xylosidase activities in stem tissues of *Arabidopsis*. - Plant Physiol. **135**: 867-878, 2004.

Munis, M.F.H., Tu, L., Deng, F., Tan, J., Xu, L., Xu, S., Long, L., Zhang, X.: A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against *Verticillium dahliae* and other stresses in transgenic tobacco. - Biochem. Biophys. Res. Commun. **393**: 38-44, 2010.

Nishitani, K., Tominaga, R.: Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. - J. biol. Chem. **267**: 21058-21064, 1992.

Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L.: Innate immunity in plants and animals: striking similarities and obvious differences. - Immunol. Rev. **198**: 249-266, 2004.

Ott, P.G., Varga, G.J., Szatmári, Á., Bozsó, Z., Klement, É., Medzihradszky, K.F., Besenyei, E., Czelleng, A., Klement, Z.: Novel extracellular chitinases rapidly and specifically induced by general bacterial elicitors and suppressed by virulent bacteria as a marker of early basal resistance in tobacco. - Mol. Plant Microbe Interact. **19**: 161-172, 2006.

Patykowski, J., Urbanek, H.: Activity of enzymes related to H_2O_2 generation and metabolism in leaf apoplastic fraction of tomato leaves infected with *Botrytis cinerea*. - J. Phytopathol. **151**: 153-161, 2003.

Riviere, M.-P., Marais, A., Ponchet, M., Willats, W., Galiana, E.: Silencing of acidic pathogenesis-related PR-1 genes increases extracellular beta-(1/3)-glucanase activity at the onset of tobacco defence reactions. - J. exp. Bot. **59**: 1225-1239, 2008.

Showalter, A.M.: Structure and function of plant cell wall proteins. - Plant Cell **5**: 9-23, 1993.

Szatmári, Á., Ott, P.G., Varga, G.J., Besenyei, E., Czelleng, A., Klement, Z., Bozsó, Z.: Characterisation of basal resistance (BR) by expression patterns of newly isolated representative genes in tobacco. - Plant Cell Rep. **25**: 728-740, 2005.

Takemoto, D., Yoshioka, H., Doke, N., Kawakita, K.: Disease stress-inducible genes of tobacco: expression profile of elicitor-responsive genes isolated by subtractive hybridization. - Physiol. Plant. **118**: 545-553, 2003.

Uknes, S., Dincher, S., Friedrich, L., Negrotto, D., Williams, S., Hope, T.T., Potter, S., Ward, E., Ryals, J.: Regulation of pathogenesis-related protein-1a gene expression in tobacco. - Plant Cell **5**: 159-169, 1993.

Van Loon, L.C.: Induced resistance in plants and the role of pathogenesis–related proteins. - Eur. J. Plant Pathol. **103**: 753-765, 1997.

Von Röpenack, E., Parr, A., Schulze-Lefert, P.: Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. - J. biol. Chem. **273**: 9013-9022, 1998.

Weimar, M., Rothe, G.: Preparation of extracts from mature spruce needles for enzymatic analysis. - Physiol. Plant. **69**: 692-698, 1986.

Ye, Z.-H., Song, Y.-R., Marcus, A., Varner, J.E.: Comparative localization of three classes of cell wall proteins. - Plant J. **1**: 175-183, 1991.