LEAF RUST AND CADMIUM-INDUCED CHANGES IN THE PROTEIN PATTERN OF THE APOPLAST OF WHEAT AND BARLEY

VERONIKA PÓS

Supervisor: Noémi Lukács, DSc

Corvinus University of Budapest
Department of Plant Physiology and Plant Biochemistry

Budapest
2010

PhD School
Name: Doctoral School of Horticultural Sciences

Field: Biological Sciences

Head of Ph.D. School: Prof. Dr. Magdolna Tóth, DSc
Department of Fruit Sciences
CORVINUS UNIVERSITY OF BUDAPEST,
Faculty of Horticultural Sciences

Supervisor: Prof. Dr. Noémi Lukács, DSc
Department of Plant Physiology and Plant Biochemistry
CORVINUS UNIVERSITY OF BUDAPEST,
Faculty of Horticultural Sciences

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

.....
Dr. Tóth Magdolna
Head of Ph.D. School

.....
Dr. Lukács Noémi
Supervisor

BACKGROUND

During crop production, agricultural plants have to face a variety of abiotic and biotic stress factors endangering the productivity of high-yielding crop varieties. For successful breeding for stress resistance or tolerance, however, sensitive methods are required to identify early stress responses as well as to follow up the defense processes of the plant.

Proteomics is the branch of systems biology that gives us a high resolution „snapshot" of the total protein composition of an organism, actually expressed in an organ, tissue or cell under a given set of physiological conditions. Based on the differences revealed between certain protein patterns, this method offers a direct way to identify proteins altered in quality or quantity, to reveal their coding genes and to unfold metabolic pathways putatively influenced by the affected proteins.

The intercellular region of plants, the so called apoplast represents an interactive surface and also a barrier between the environment and protoplasts, that actively participates in various normal and pathophysiological processes. Therefore, proteomic analysis of the apoplast can be an efficient tool to monitor the physiological state and health condition of plant populations. Its result can be exploited in molecular breeding.

In this doctoral thesis, proteomic analyses of apoplastic proteins were performed to identify stress-induced changes in leaf rust-infected near-isogenic wheat lines and in cadmium-stressed barley seedlings

AIMS

Our aim was to characterize the protein pattern of the leaf apoplast and stress-induced alterations in its composition.

A. First, we planned to study if near-isogenic resistant and sensitive lines of wheat (*T. aestivum* L.) show any difference at the level of apoplastic proteins after infection with leaf rust (*Puccinia recondita* f.sp. *tritici*), an obligate biotrophic fungus massively threatening cultured populations of the crop. Although there is substantial empirical evidence showing the versatility of wheat genotypes containing Lr1 or Lr9 resistance genes under field conditions, the actual mechanism of action of these resistance genes has not been uncovered. In this work, identification of affected apoplastic proteins was performed by differential proteomics analysis using mass spectrometry and by enzyme activity assays. To confirm

the hypothesized induction of affected proteins at the gene level, transcription analyses were also performed.

B. Cadmium is one of the various polluting heavy metal agents to which crops are frequently exposed to. As an abiotic stress factor of world-wide importance, we examined effects of cadmium on the seedlings of cv. 'Mandolina', a moderately cadmium-sensitive cultivar of barley. The aim of our proteomic analysis was to determine if there were apoplastic proteins specifically expressed in response to heavy metals.

C. Finally, we started reference mapping on the apoplast of healthy seedlings of cv. Chinese Spring', the most examined cultivar in genetic as well as in genomic analysis of wheat. We aim at constructing a reference map that could serve as a tool for the categorization of wheat leaf stress responses in the future.

Our examination of both the biotic and abiotic stress systems was centered mainly on a certain segment of the proteome, the secreted set of the so called "pathogenesis related" (PR) proteins.

MATERIALS AND METHODS

Plant material

To investigate changes in the protein- and gene expression associated with wheat leaf rust infection, susceptible seedlings of *Triticum aestivum* L. cv. 'Thatcher' and two of its near-isogenic, but resistant lines (Lr1 and Lr9), were used. Lr1 and Lr9 show hypersensitivity to leaf rust pathotype 43722 and are known to display differences in their resistance under field conditions. Seeds of Lr1 were a generous gift from J. A Kolmer (U.S. Department of Agriculture, ARS - Cereal Disease Laboratory). Seeds of Tc and Lr9 were provided by Dr. Klára Manninger (Dept. of Pathophysiology, Plant Protection Institute of H.A.S.), who also performed the infection of seven-day-old seedlings - grown at 20 °C under long-day photoperiod (16 h light / 8 h dark regime). Infected and mock-infected first leaves were collected in every 2-2.5 hour for the first 12 h p.i, and daily thereafter for an additional 7 days.

Cadmium stress studies were carried out using barley (*H. vulgare* L.) cv. 'GK Mandolina', a spring cultivar moderately tolerant to heavy metals. Seeds were from Cereal Research Ltd. (Plant Breeding Research Station, Táplánszentkereszt). Ten-day-old seedlings grown in hydroponics at 21 °C under long-day photoperiod (16 h light / 8 h dark regime) were

treated with $CdCl_2$ in the concentration range of 0-(10-30-100-)300 μM. Samples were taken on the 1st, 4th and 7th days post treatment.

For reference mapping of the apoplastic fluid, 'Chinese Spring', a cultivar investigated exhaustively in wheat genetic and genomic researches was selected. Seeds were kindly provided by Dr. Géza Kovács (Agricultural Research Institute of H.A.S., Cereal Gene Bank). The first leaves of wheat seedlings grown at 19 °C under long-day photoperiod (14 h light / 10 h dark regime) were subjected to analysis.

Experiments at the protein level

Isolation of intercellular washing fluid (IWF)

Extraction of IWF was carried out according to Rohringer et al. (1984) with minor modifications: ice-cold 20 mM or 100 mM Tris-HCl (pH: 8.0) buffers (for electrophoretic separation and enzymatic assays, respectively) containing 1 mM phenylmethylsulfonyl fluoride were used for vacuum infiltration (0.56 Pa, 6 min, 4 °C). Apoplastic fluid was obtained and cleared by a two-step centrifugation (2000 and 14 000 rpm, 20-20 min, 4 °C), then,- for gel-electrophoresis experiments , urea was added to a final concentration of 8 M. IWF was stored frozen at -80 °C until use. Determination of the total protein concentration of intercellular proteins was carried out by Lowry (1951) or by the 2-D Quant Kit (*Amersham Biosciences*). Protein samples were concentrated using Icon Concentrator - 9 CO (*Pierce*) molecular filter.

Proteomic analysis

Isoelectric focusing (Ettan IPGPhorII, Amersham and / or Multiphor™ II (*AP*)) was carried out on IPG strips (13 cm, pH 3-10 NL) after solubilization (Rabilloud 1998) and passive rehydration of proteins (60-120 μg for analytical and 300 μg for semi-preparative analysis).

For one- and two-dimensional SDS-PAGE, 12.5 % (w/v) slab gels were prepared in a Protean II xi chamber (*Bio-Rad*) according to Laemmli (1970). Before loading into the second dimension proteins in IPG-strips were reduced with 1% dithiothreitol and then alkylated by treating with 4 % iodoacetamide. After electrophoretic separation, proteins were stained either with CBB G-250 according to Neuhoff et al. (1985) or with silver (Shevchenko et al., 1996).

Gels were scanned using the EPSON Expression 1680 Pro (EPSON) gel documentation system and evaluated visually or with the help of ImageMaster™ 2D Platinum v4.9 (Amersham Biosciences) image-analysing program.

After selection, excised protein bands and spots were subjected to MALDI-TOF and their composition confirmed with PSD-spectrum (Bruker Reflex III) and LC-MS/MS (Agilent 1100LC XCT Plus IonTrap; Thermo LCQ Fleet LC-MS/MS ill. Waters LC-ESI-qTOF) mass spectrometry in the Laboratory of Proteomics Research (Biological Research Center of H.A.S) by Dr. Éva Hunyadi-Gulyás and Dr. E. Szájli. Raw data were pre-processed with Mascot Distiller software (ver:2.2.1.0). The resulting peak lists were used to identify the corresponding proteins in the NCBInr and/or SwissProt 52.5 protein databases and also - if needed - at the EST database (*T. aestivum)* of TIGR and NCBI, via the Mascot (2.2.04. Matrix Science) and UCSF ProteinProspector search engines.

Enzyme activity measurements

Activity assays were performed colorimetrically. Endo-1,3-beta-D-glucosidase activity was measured using reduced laminarin as a substrate (Deanult et al. 1978). The assay described by Dygert et al. (1965) and Zheng and Wozniak (1997) for microtiter plates was modified and extended to the range of 10–600 μM of glucose equivalents (Kabai 2008). Extracellular chitinase-assay was carried out by Wirth and Wolf (1990).

Experiments at the RNA level

Isolation and purification of nucleic acids

Total RNA was isolated with TRIzol® reagent (*Invitrogen*) and was further purified by digestion with RNase-free DNase I.

RT-PCR

Primers (approx. 30) of different specificity to given isoforms or wider categories of chitinases and glucanases as well as to ubiquitin were designed using Primer3, IDT and OligoCalc programs after collecting sequence homologues from the NCBI database.

Reverse transcription with either oligo$(dT)_{18}$ or gene-specific primers was performed by SuperScript^{TM} II and III (*Invitrogen*) enzymes according to the manufacturer's instructions. cDNAs or isolated plasmids derived from *E. coli* clones were used as templates for PCR using Double-Taq (*Zenon-Bio*) polymerase. The PCR protocol was as follows: 95 °C, 5 min; T_m-(0,5-2)°C, (30 sec), 72 °C (30 sec)] x 30 cycles; 72 °C, 5 min.

Cloning of PCR products

Amplified PCR products were separated on 1 % or 2,5 % agarose gels in 1x TBE or - in preparation for cloning - in 1x TAE buffer (Sambrook *et al*. 1989). Excised and purified PCR products were ligated into pTZ57R/T cloning vector, transformed into *E. coli* XL1 Blue or JM109 competent cells (Inoue *et al.* 1990) and cloned according to Sambrook *et al.* (1989).

Sequence analysis

Nucleotide sequences were determined in the BRC of H.A.S. using an automated DNA sequencer (ABI PRISM® 3100 Genetic Analyzer). Sequences were compiled, analyzed, and aligned by Chromas Lite v2.01 and Vector NTI Advance 10 or the ClustalW2 program of the Jalview 2.4.0.b2 software. Database searches of the cDNA clones as well as amino acid sequences derived either from conceptual translation of the inserts or identified by mass spectrometry were performed with the NCBI Blast program and the TIGR Plant Transcript Assembly BLAST Server.

Extracellular localization of the proteins was predicted using the SignalP 3.0 server, whereas probable N- and O-glycosylation sites were identified using the prediction servers of CBS (NetNGlyc 1.0 and YinOYang 1.2).

Phylogenetic analyses

Evolutionary relationships were revealed using MEGA4 software (Tamura *et al.* 2007, Kumar *et al.* 2008). The trees were generated by applying the neighbour-joining method, using a distance matrix calculated from the Kimura 2-parameter model of nucleotide substitution and a pairwise deletion option to remove gaps with missing data. The data sets were subjected to 1,000 bootstrap replicates to check the reliability of the findings. Outgroups were not defined.

RESULTS AND DISCUSSION

A.) Apoplastic proteins induced during the stress response against leaf rust infection

The biotic stress response was investigated in wheat – leaf rust interaction, using near-isogenic lines of the 'Thatcher' cultivar (the susceptible Tc, and two of its resistant genotypes, bearing Lr1 and Lr9 resistance genes). Based on the proteomic results, we identified 24 proteins secreted in the apoplast of the near-isogenic lines of cv. 'Thatcher' in association with the infection. These proteins were found to be the members of seven, functionally different, protein groups, among which six belong to different pathogenesis-related (PR-type) protein families. Several of these families have been characterized as glycohydrolases (GH) capable of cleaving various cell wall polysaccharides of fungal origin. That is, members of the GH 17 family (five isoforms of the PR2-type endo-1,3-glucanases (Subfamily A), and an endo-1,3-1,4-glucanase (Subfamily B)) and also isoforms of PR3-type (class I, II and IV) chitinases and PR4-type chitin-bonding proteins (3 members of the Barwin family) could be distinguished. Among them, induction of a class II chitinase of PR3 origin (BAB82471) and an endo-1,3-glucanase (AAY88778/AAY96422) was detected in all three genotypes, but both temporal and intensity differences were observed in the resistant lines (Lr1 and Lr9). On the other hand, induction of endo-1,3-1,4-glucanase (ABB96917) was only found in the susceptible line, as part of the late phase of its defense process. It is worth mentioning however, that – although proteins with the latter activity are not considered to be classical PRs – their putative efficacy against pathogen fungi has just recently been investigated (Nishizawa et al. 2003, Akiyama et al. 2009).

Besides chitinases and glucanases, in the resistant lines (Lr1 and Lr9) we also confirmed at least two members of the PR1 family. The mode of action of PR1s is still not completely understood, in spite of the fact that their antifungal activity has long been known and it is also presumed that they may act as endopeptidases with cell wall and cell membrane permeabilizing activity due to their extracellular SCP domain (Park *et al*. 2010).

A more detailed analysis of the apoplastic protein pattern in the resistant Lr9 line revealed the induction of more protein families in association with leaf rust infection. That is, at first, four isoforms of the thaumatin-like (PR5) proteins were identified indicating activation of a wider range of PR5 proteins. Some members of the PR5 family have been described that cause cell membrane leakage (Abad *et al*. 1996) as well as intensive binding or even hydrolytic activity on the 1,3-beta-D-glucans of the fungal cell wall (Osmond *et al*. 2001, Grenier *et al*. 1999).

These features might explain why PR5 proteins can inhibit growth of both fungal spores and hyphae.

In addition five different isoforms of secretory peroxidases belonging to cluster I (Liu *et al*. 2005) of the PR9 family with hydrogen-peroxide producing activity were also identified. Moreover, the presence of a plant-type, extracellular GDSL-like lipase was also proved, the transcript of which has just recently been sequenced among the clones of an SSH-cDNA library of Lr9/Tc subjected to leaf rust infection (Lasota *et al*. 2009). The putative plant protecting role of lipases has just started to be investigated both in the nectrotrophic interaction between *Arabidopsis* and the fungus *Alternaria* (Oh *et al*. 2005) and in the biotrophic connection in rust-infected *Phaseolus* (Lee *et al*. 2009). Their induction is considered to be a manifestation of the strict connection between basal and R gene-mediated resistance mechanisms, whereas a direct inhibiting effect on spore germination and other indirect action mechanisms are also hypothesized (e.g. creating signal molecules of lipid origin by excising them from the oxidatively stressed cell membrane).

Among the proteomically identified PR families, we performed activity measurements for extracellular endo-1,3-glucanases (PR2) and chitinases (PR3 and PR4) to find out whether their increased protein expression occurs in parallel with increased activities in the resistant Lr1 and Lr9 lines and as such may contribute to resistance. We demonstrated that although the first increase in activity appeared 1-2 hours earlier in the susceptible Tc line than in the resistant ones, it remained relatively low and started to regress to the basal level after 3-4 days. In contrast, in the two resistant lines on the 3rd day post inoculation the activities exceed the activity levels of the susceptible Tc and remain at a permanently higher level thereafter.

Our results do not prove unambiguously that the studied enzyme groups contribute to resistance, since endo-1,3-glucanases and chitinases can be induced by other stressors as well. Nevertheless, since these enzymes are able to hydrolyze fungal cell walls and their activities increase remarkably in the apoplast of Lr1 and Lr9, their presence may have a direct antifungal potential.

As the result of differences in genetic background for any given resistance gene, expression of secreted glucanases and chitinases may show differences between the near isogenic lines bearing one of the nearly 60 known leaf rust resistance genes. While in some cases, similar to Lr1 and Lr9 intensive induction of these enzymes has been reported, in other cases (e.g. in Lr35/Tc) constitutive expression has been described. Moreover, in the resistant genotypes Lr29/ and Lr34/Palmiet the induction dynamics was very similar to that in the suscepti-

ble Palmiet cultivar (Anguelova *et al*. 1999, Anguelova-Merhar *et al*. 2002, Kemp *et al*. 1999), indicating that the studied glucohydrolases do not always play a substantial role in leaf rust resistance, although their high expression level nonetheless suggests some form of contribution.

In order to confirm changes in expression identified at the protein level and also to distinguish between differentially expressed isoforms, we performed gene expression analyses for the GH17 and GH19 families in both the susceptible Tc and the resistant Lr9 genotypes. Using primers with stricter or wider specificity, deduced from sequences of mass spectrometrically identified chitinases and glucanases, we succeeded in amplifying the targeted transcripts or their close and also annotated homologues in the cases of some isoforms (e.g. AB029934, AF112963; DQ078255/DQ090946 and Y18212, AB244638.2, AB244642.1). In addition, however, by sequencing more clones, we also revealed transcripts of new wheat chitinase and glucanase isoforms (four and three, respectively) not yet represented in the NCBI database, but found in the TIGR EST database.

Our transcription analyses (RT-PCR) confirmed and also complemented the results of the proteomics, proving that in the course of leaf rust-induced stress response various isoforms of a given protein family can be expressed in parallel. Furthermore, the diversity of proteins revealed by MS has been extended by transcript analysis to gene variants not yet described at the level of proteins or mature mRNAs.

By combining results of proteomic and transcriptional studies, we proved that the expression of genes *Chi1* and also *TaGlb2b, 2f* and *2a* contribute to the prominently enhanced activity of the apoplastic chitinase and endo-1,3-glucanases in Lr9 line.

For future studies we suggest RT-qPCR analyses to quantify genotype-dependent expression differences, and promoter analysis to identify modified regulation processes causing alterations in gene expression patterns. Such experiments are however, only feasible if we can provide reliable distinction between the many isoforms present in the wheat genome. Because of the incomplete sequence data in wheat databases, we see no opportunity for reaching this final goal at present.

B.) Cadmium stress associated changes in protein pattern of barley apoplast

In association with the cadmium treatment (0-300 μM Cd^{2+}) of cv. 'Mandolina', a moderately sensitive cultivar of barley, dramatic changes were observed in both the one- and two dimensional protein pattern of the intercellular fluid in the molecular weight range of 10-40 kDa. Mass spectrometric analysis revealed induction of different members of six pathogenesis-related (PR) protein families, indicating that a general stress response in the apoplast is part of the plant defense against cadmium. Accordingly, we detected an increase in intensity of certain PR1 proteins, of endo-1,3-glucanases (PR2), chitinases (PR3, PR4) and especially, of several members of the thaumatin-like (PR5) proteins. In addition, a PR17 protein and one of its homologues, a hypothetical protein referred to as a member of the basic secretory proteins (BSP superfamily) were also identified. Several proteins showing differential expression after cadmium treatment have not yet been analyzed in our experiments. According to the literature we expect induced expression of at least one more PR-family, namely that of secreted forms of inducible peroxidases (PR9).

Investigations during the last two decades on the signaling pathways of various stress factors, and especially the deeper studies of the last few years on their interaction and fine tuning by cross talk, have shown that a stereotypical induction of many PR protein families may be typical of heavy metal stresses, (Jonak *et al*., 2004, Maksymiec *et al*., 2007). According to recent results, they probably contribute to plant defense by a complex mode of action, including protection against some primary or secondary effects of heavy metals.

It had been shown some years ago in transgenic tobacco that overexpression of a basic PR1 protein of pepper origin and a fungal PR3-type chitinase (Sarowar *et al.* 2005, Dana *et al*. 2006) can confer resistance to a broad range of fungal and bacterial pathogens as well as to some biotic stressors. Among other effects, these plants were found to be more tolerant to heavy metals (Cd^{2+}, Hg^{2+} and Cu^{2+}). Sarowar et al (2005) suggested that overexpression of *CABPR1* may result in disturbance of the cellular redox-status leading to the accumulation of hydrogen-peroxide, which is a protecting agent in many stress conditions.

A secondary effect of the cadmium treatment is the initiation of dehydration stress. Induction of the MS-identified beta-1,3-glucosidase isoforms after cadmium stress in the leaf apoplast raises the possibility that some members of the PR2 group (members of subfamily A of the GH17 family) could also help the plant to overcome dehydration, e.g., by cleavage of ABA glycoconjugates, delivering the active form of the stress hormone (Perfus-Barbeoch et al. 2002, Leubner-Metzger and Meins 1999).

Our investigations were restricted to intercellular proteins which can be extracted at low ionic strength. However, several publications report that heavy metals affect the rigidity of the cell wall and the cell membrane as well as the adsorption of certain secreted proteins to the cell wall (Hirano *et al.* 1994, Lagrimini *et al*. 1997 ill. Kataoka *et al.* 2003, Scheel *et al*. 2008). Therefore, the identification of the complete set of heavy metal- or cadmium-induced proteins in the apoplast by screening protein fractions strongly bound to the cell wall is an inevitable future task.

C.) Reference proteome mapping of the wheat apoplast

One of the basic requirements of any appropriately performed proteomic analysis of stress induced agents is reference mapping. To this end, we initiated a sub-project aiming at the proteomic mapping of apoplast proteins present in healthy seedlings of the wheat cultivar 'Chinese Spring' which is the cultivar most frequently studied by both domestic and international research groups. In my experiments up to now MS analysis of 21 spots derived from 2D protein separation was carried out. This resulted in the identification of 9 proteins.

By functional classification of these proteins we first identified a subgroup involved in cell wall formation, including arabinoxylane-arabinofuranohydrolase (AXAH II, ~AAK21880), a beta-D-xylosidase (~ABA92796) and finally an isoenzyme sharing enzymatic activities with both of them (ARA I, ~AAK38481). These enzymes are particularly characteristic for various grasses, as they play a pivotal role in the synthesis of the so-called Type II. cell wall structure, typically low in pectin and xyloglycan and characteristic for the Poaceae (Dornez et al. 2009). In this type of cell wall (arabino)xylans constitute the overwhelming majority of hemicellulose, followed by pectins and mixed-bound (1→3),(1→4)-β-D-glucans (Carpita 1996, Fincher 2009). The identified (1,3;1,4)-beta-glucanase (most probably CAA80493) is involved in both sprouting and growth regulation of developing vegetative tissue (Meikle *et al*. 1994). In addition, the identified ARA I and a secreted beta-galactosidase (BG904072*) represent enzymes that, by performing specific partial cleavage of sugar residues on the essential cell wall protein arabinogalactans, might even serve to deliver endogenous elicitors (Etzler 1998), and hence to stimulate intracellular signaling under both physiological and pathologic conditions (Hirano *et al*. 1994, Showalter 2001, Hawes *et al*. 2007).
A second subgroup of the identified proteins can be distinguished as a set of enzymes involved in the preformed defense with the capability of acting directly against microbial path-

ogens or herbivores. In this set we identified a protein homologous to a bifunctional alpha-amylase inhibitor / endochitinase (P15326) isolated originally in a wild relative of cultivated cereals and also an endo-1,3-glucanase (CAI64809), already identified in the stress response of leaf rust infected Lr9 wheat line. In addition, we also detected a PR17 homologue similar to barley hypothetical protein (CAA74594) found to be induced under cadmium stress.

Finally, the most dominant triple spots in the 2D protein pattern of seedling stage leaves were shown to correspond to protein CAC85479 which is supposed to participate in a number of normal and pathophysiological processes. First, due to its ADP-glucose-pyrophosphatase/phosphodiesterase activity, one of its possible roles could be to assist in the glycosylation of the phenoloid-types of antimicrobial phytoanticipins in the apoplast (Vermerris and Nicholson, 2007). Furthermore, on the basis of two, MS-identified conservative boxes (B, C) and a KGD motif participating in cell adhesion processes, CAC85479 belongs to the "do all" germin-like plant proteins (GLPs), and is a putative member of the PR16 family (Bernier and Berna 2001).

OUTLOOK

Our proteomic investigations aimed at revealing apoplastic proteins that participate in biotic or abiotic stress responses. The findings described in my thesis indicate that not only infections by pathogens, but also other stress factors may induce signal transduction pathways leading to massive and stereotypic expression of PR proteins. Moreover, it has been shown that healthy plants grown under normal physiological conditions can also secrete proteins that may act as protective agents when the plant is confronted with stress situations.

Because of the intensive cross-talk between overlapping stress signal transduction pathways it might seem profitable to permanently enhance the basic level of the plant defense to achieve cross-resistance (Jonak *et al*. 2004, Maksymiec 2007, Poschenrieder *et al*. 2006). In my opinion, however, we should say no to these agrochemical- or biotechnology-based techniques because of the significant anti-nutritive and allergenic potential of a broad range of PR families, and the long-term, putatively hazardous by-effects on the plant itself (e.g. reduced rates of growth and productivity, metabolism shifted to earlier senescence, etc. - Heil *et al.* 2000, Iakimova *et al*. 2006, Maksymiec 2007, Van Hulten *et al*. 2006).

Expression differences between PR isoforms can substantially influence the outcome of the defense response. Since PR proteins have several isoforms and allele–variants, identification

of a given isoform was not always possible at our present level of sequence knowledge. Therefore, our first aim for the future is to find experimental approaches to reliably identify isoforms taking part in a given stress reaction. Secondly, we would like to determine the degree and time course of their individual contribution to the total response, and finally, to find individual and/or common promoter motifs in their regulatory regions.

NEW SCIENTIFIC RESULTS

A.) Stress response of wheat to leaf rust infection

1. In association with leaf rust infection we detected altered expression of at least 24 proteins in the near-isogenic lines of the 'Thatcher' cultivar showing differences in the resistance to the fungus. These proteins are secreted into the apoplast and belong to seven, functionally different protein families: one protein is a GDSL-like lipase and all others are members of the so called "pathogenesis-related" (PR) proteins: **GLIP** - ABL11233; PR1 - AA07473, AAK60565/AAP14676; **PR2** - AAY88778/AAY96422, CAA77085, CAI64809, AAD28732, BAE96089, továbbá ABB96917; **PR3** - BAB82471, AAG53609, AAD28733; **PR4** - 2209398A/O64393, AAS78780, O64392; **PR5** - AAK55326, AAK55325/AAB71680, CAA66278, AAK60568; **PR9** - CAA59486, AAW52716, AAW52720, CAA59485, Q05855.
2. Among these proteins, accumulation of a PR3-type chitinase (BAB82471) was detected in all three wheat lines, whereas that of at least one PR2-type endo-1,3-glucanase (AAY88778/AAY96422) and two PR1 proteins (CAA07473, AAK60565/AAP14676) occurred only in the resistant genotypes. Furthermore, after infection these proteins were found to be expressed earlier and / or in higher amounts in the resistant lines than in the susceptible Tc.
3. Using primers deduced from tryptic peptide sequences of the MS-analysis we confirmed the expression of transcripts largely corresponding to the proteomically identified chitinases and endo-1,3-glucanases: identical to the sought isoforms were *Chi1* - AB029934 and *Chi IV* - AF112966, and different in some position (instead of *TaGlb2a* - AB244637 we found *TaGlb2b* - AB244638.2 - Δ: 0 nt and *TaGlb2f* - AB244642 - Δ: 1 nt, as well as *beta-glucanase* Y18212 - Δ: 2-2 nt and DQ090946/DQ078255 - Δ: 3-4 nt).

4. We proved that the beta-1,3-endoglucosidase and chitinase activities of non-treated seedlings were low in all three genotypes, but were enhanced after pathogen infection and showed genotype-dependence. We detected that these enzymes were induced after leaf rust infection with a significantly higher activity in the apoplast of the resistant lines Lr1 and Lr9, and remained at a permanently higher level than those of the susceptible Tc line. Our proteomic and transcriptional analysis confirmed that the expression of the genes *Chi1* and *TaGlb2b, 2f* and *2a* definitely contribute to the increased activity of the resistant Lr9 line.

B.) Apoplastic protein response in barley to cadmium stress

1. We showed that in cadmium–stressed barley (cv. 'Mandolina') expression of several apoplastic proteins has a tendency to change, it usually increases during a one-week $CdCl_2$ treatment in the concentration range of 0-300 μM. After one- and two dimensional separation we succeeded in identifying two 1,3-glucanases (putative PR2 family- 1607157A and P15737/AAM75342), two chitinases (PR3 family - CAA55344 and CAA55345) and two chitin-binding proteins (PR4 family - CAA71774, P28814), as well as two PR1s (CAA52893, P35793) and a rich set of PR5 proteins (e.g. AAB71680, AAK55325, AAK55326). In addition, the presence of a PR17 protein (ABV22582) and a member (CAA74594) of the antimicrobial basic secretory proteins (BSP), closely related to the PR17 protein were also confirmed.
2. This is the first time that the apoplastic stress response to cadmium has been analysed proteomically in barley leaves (Pós *et al*. 2011, *in press*). Analyzing the protein fraction extractable at low ionic strength we found that a wide set of PR proteins is (also) induced as part of the non-cadmium specific, generalized stress response of the plant.

C.) Reference proteome mapping of wheat apoplast:

1. Two-dimensional mapping of the apoplast proteome of healthy seedlings of wheat cv. 'Chinese Spring' revealed 11 spots corresponding to 9 relevant, secreted wheat proteins or to homologues originating from other Poaceae. Part of these proteins are involved in remodeling the plant cell wall (arabinoxylan arabinofuranohydrolase isoenzyme AXAH-II - AAK21880, alpha-L-arabinofuranosidase / beta-D-xylosidase isoenzyme ARA-I - AAK38481, beta-D-xylosidase - ABA92796, (1,3;1,4)-beta-glucanase - CAA80493, beta-D-galactosidase - BG904072*), take part in normal metabolic processes and / or are capable of generating endogenous elicitors. Another group of them

includes protecting agent against microbial pathogens and herbivores as a part of the preformed defense (alpha-amylase inhibitor / endochitinase - P15326, putative glucan endo-1,3-beta-D-glucosidase - CAI64809). In addition, we identified a multifunctional protein promoting glycosylation of phenoloid-type phytoanticipins (adenosine diphosphate glucose pyrophosphatase - CAC85479) and a hypothetical protein (CAA74594) with still unknown function, but sequentially related to the PR17 family.

2. Based on a detailed MS-analysis proving the presence of two conservative boxes (B and C) and a KGD motif characteristic in cell adhesion process, we presume that the ADP-glucose pyrophosphorylase (CAC85479), is a dominant protein in the developmental stage in wheat apoplast we examined and that it can be classified into the germin-like proteins (GLPs) and is therefore a putative member of the PR16 family.

LITERATURE CITED

1. ABAD L.R., D'URZO M.P., LIN D., NARASIMHAN M.L., RENVENI M., ZHU J.K., NIU X., SINGH N.K., HASEGAWA P.M., BRESSAN R.A. *Plant Sci* (1996) 118: 11-23.
2. AKIYAMA T., JIN S., YOSHIDA M., HOSHINO T., OPASSIRI R., KETUDAT CAIRNS J.R. *J Plant Physiol* (2009) 166: 1814—1825.
3. ANGUELOVA V.S., VAN DER WESTHUIZEN A.J., PRETORIUS Z.A. *Physiol Plantarum* (1999) 106: 393-401.
4. ANGUELOVA-MERHAR V.S., VAN DER WESTHUIZEN A.J., PRETORIUS Z.A. *Journal of Plant Physiology* (2002). *159(11): 1259-1261*
5. BERNIER F., BERNA A. *Plant physiology and Biochemistry* (2001) 39: 545-554.
6. CARPITA N.C. *Annu Rev Plant Physiol Plant Mol Biol* (1996) 47: 445–76.
7. DANA M.M., PINTOR-TORO J.A., CUBERO B. *Plant Physiol* (2006) 142: 722–730.
8. DENAULT L.J., ALLEN W.G., BOYER E.W. et al. *American Society of Brewing Chemists Journal* (1978) 36: 18-23
9. DORNEZ E., GEBRUERS K., DELCOUR J.A., COURTIN C.M. *Trends in Food Science & Technology* (2009) 20 (11-12): 495-510.
10. DYGERT, S., LI L. H., FLORIDA D. *et al. Analytical Biochemistry* (1965) 13: 367-374.
11. ETZLER M.E. *J. Cell. Biochem.* (1998) Suppl. 30-31: 123–128.
12. FINCHER G.B. *Plant Physiology* (2009) 149: 27-37.
13. GRENIER J., POTVIN C., TRUDEL J., ASSELIN A. *The Plant Journal* (1999) 19(4): 473-80.
14. HAWES M., CELOY R., PRICE I., WEN F., EBOLO J. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* (2007) 146 (4), Suppl. 1, S276.
15. HEIL M., HILPERT A., KAISER W., LINSENMAIR K.E. *The Journal of Ecology* (2000) 88(4): 645-654.
16. HIRANO Y., TSUMURAYA Y., HASHIMOTO Y. *Physiologia Plantarum* (1994) 92(2): 286-296.
17. IAKIMOVA E., KAPCHINA-TOTEVA V., DE JONG A., ATANASSOV A., WOLTERING E. In: Blume Y et al. (Szerk.): *Cell Biology and Instrumentation: UV Radiation, Nitric Oxid and Cell Death in Plants*. (2006) Proceedings of the NATO Advanced Research Workshop, Yalta, Ukraine 8-11. 09. 2004. IOS Press, Amsterdam, 2006.
18. INOUE H., NOJIMA H., OKAYAMA H. *Gene* (1990) 96(3): 23-28.
19. JONAK C., NAKAGAMI H., HIRT H. *Plant Physiol* (2004) 36: 3276–3283.
20. KABAI M. (*Szakdolgozat) Budapesti Corvinus Egyetem, Kertészettudományi Kar* (2008)

21. KATAOKA T., FURUKAWA J., NAKANISHI T.M. *Biol Plant* (2003) 36, 445-449.
22. KEMP G., BOTHA A-M., KLOPPERS F.J., PRETORIUS Z.A. *Physiological and Molecular Plant Pathology* (1999) 55(1): 45-52.
23. LAEMMLI U.K. *Nature* (1970), 227(5259): 680-685.
24. LAGRIMINI L.M., GINGAS V., FINGER F., ROTHSTEIN S., LIU T.Y. *Plant Physiol* (1997) 114: 1187–1196.
25. LASOTA E., DMOCHOWSKA M., KAWALEK A., NADOLSKA-ORCZYK A., ORCZYK W. *19th ITMI / 3rd COST Tritigen Joint Workshop 2009. (Aug.31-Sept.4. 2009 Clermont-Ferrand, France) Abstracts p. 166.*
26. LEE J., FENG J., CAMPBELL K.B, SCHEFFLER B.E., GARRETT W.M., THIBIVILLIERS S., STACEY G., NAIMAN D.Q., TUCKER M.L., PASTOR-CORRALES M.A., COOPER B. *Mol Cell Proteomics* (2009) 8(1): 19-31.
27. LEUBNER-METZGER G., MEINS F.J.R. In: Datta SK, Muthukrishnan S (Szerk.): Pathogenesis-related proteins in plants. pp 49-76, CRC Press LLC, Boca Raton, Florida, 1999.
28. LIU G., SHENG X., GREENSHIELDS D.L., OGIEGLO A., KAMINSKYJ S., SELVARAJ G., WEI Y. *Mol Plant Microbe Interact* (2005) 18: 730–741.
29. LOWRY O.H., ROSEBROUGH N.J., FARR A.L., RANDALL R.J. *J. Biol. Chem*. (1951) 193(1): 265–75.
30. MAKSYMIEC W. *Acta Physiologiae Plantarum* (2007) 29(3): 177-187.
31. MEIKLE PJ, BONIG I, HOOGENRAAD NJ, CLARKE AE, STONE BA. *Planta*(1991) 185: 1-8.
32. MINIC Z. *Planta*(2008) 227(4): 723-740.
33. NEUHOFF V., STAMM R., ELBL H. *Electrophoresis* (1985) 6: 427-448.
34. NISHIZAWA Y., SARUTA M., NAKAZONO K., NISHIO Z., SOMA M., YOSHIDA T., NAKAJIMA E., HIBI T. *Plant Mol Biol.* (2003) 51(1): 143-52.
35. OH I.S., PARK A.R., BAE M.S., KWON S.J., KIM Y.S., LEE J.E., KANG N.Y., LEE S., CHEONG H., PARK O.K. *Plant Cell* (2005) 17(10): 2832–2847.
36. OSMOND R.I.W., HRMOVA M., FONTAINE F., IMBERTY A., FINCHER G.B. *Eur J Biochem* (2001) 268: 4190-4199.
37. PARK S-C., LEE J.R., KIM J-Y., HWANG I., NAH J-W., CHEONG H., PARK Y., HAHM K-S. *Biotechnology Letters* (2010) 32(1): 125-130.
38. PERFUS-BARBEOCH L., LEONHARDT N., VAVADDEUR A., FORESTIER C. *Plant J* (2002) 32: 539-548.
39. POS V., HUNYADI-GULYÁS É., KABAI M., CAIAZZO R., JÓCSÁK I., MEDZIHRADSZKY K., LUKÁCS N. (2011): *Acta Alimentaria - An International Journal of Food Science* 40, (Suppl 1) (*in press*)
40. POSCHENRIEDER CH., TOLRA R., BARCELO J. *TRENDS in Plant Science* (2006) 11 (6): 288-295.
41. RABILLOUD T. *Electrophoresis* (1998) 19: 758-760.
42. ROHRINGER R., EBRAHIM-NESBAT F., WOLF G. *Journal of Experimental Botany* (1983) 34(149): 589-605.
43. SAMBROOK J., FRITSCH E.F., MANIATIS T. 2nd Ed.; *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.* 1989.
44. SAROWAR S., KIM Y.J., KIM E.N., KIM K.D., HWANG B.K., ISLAM R., SHIN J.S. *Plant Cell Rep* (2005) 24: 216–224.
45. SCHEEL T., PRITSCHK., SCHLOTER M., KALBITZ K. *Journal of Plant Nutrition and Soil Science* (2008) 171(6): 900-907.
46. SHEVCHENKO A., WILM M., VORM O., MANN M. *Anal Chem* (1996) 68 (5): 850-858.
47. SHOWALTER A.M. *Cell Mol Life Sci* (2001) 58: 1399–1417.
48. VAN HULTEN M., PELSER M., VAN LOON L.C., PIETERSE C.M.J., TON J. *Proc Natl Acad Sci USA* (2006) 103(14): 5602–5607.
49. VERMERRIS W., NICHOLSON R. In: *Phenolic Compound Biochemistry*, pp. 211-234. Springer Netherlands, 2006.
50. WIRTH S.J., WOLF G.A. *J. Microbiol. Meth.* (1990) 12: 197–205.
51. ZHENG Y., WOZNIAK C.A. *BioTechniques* (1997) 5 (22): 922-926.

MOST IMPORTANT PUBLICATIONS RELATED TO THE SUBJECT OF THESIS

Articles

Journal with impact factor

Pós V., Hunyadi-Gulyás É., Kabai M., Caiazzo R., Jócsák I., Medzihradszky K., Lukács N. (2011): Induction of pathogenesis-related proteins in intercellular fluid by cadmium stress in barley (*Hordeum vulgare* L.) – a proteomic analysis. *Acta Alimentaria - An International Journal of Food Science* 40, (Suppl 1) (*in press*)

Journal without impact factor

Pós V., Halász K., Mesterházy Á., Csősz L.né, Manninger K., Hunyadi-Gulyás É., Medzihradszky K., Juhász T., Lukács N. (2005): Proteomic investigation of wheat intercellular washing fluid. *Acta Biologica Szegediensis* 4 (1-2): 31-32.

Conference papers

Congress full papers in English

-

Abstract in English

Pós V., Manninger K., Halász K., Hunyadi-Gulyás É., Szájli E., Cserháti M., Duan H., Medzihradszky K., Györgyey J., Lukács N. (2007): Proteomic changes of the wheat apoplast associated with resistance against leaf rust. 15th International Congress of the Hungarian Society for Microbiology (18-20 July 2007, Budapest, Hungary) *Acta Microbiologica Hungarica* (2007) Vol 54, Suppl. pp. 104-105.

Pós V., Manninger K., Halász K., Hunyadi-Gulyás É., Szájli E., Cserháti M., Medzihradszky K., Györgyey J., Lukács N. (2007): Proteomic analysis of leaf rust-infected wheat. 2nd World Conference of Stress – "Hans Selye 1907-2007". (23-26 August 2007, Budapest, Hungary) Abstracts pp. 199-200.

Pós V., Manninger K., Halász K., Hunyadi-Gulyás É., Szájli E., Cserháti M., Duan H., K. Medzihradszky, Györgyey J., Lukács N. (2007): Protein pattern of the wheat apoplast. 1st Central and Eastern European Proteomic Conference – "Proteomics Driven Discovery and Applications" (29-31 October 2007, Prague, Czech Republic). Abstracts pp. 141-142.

Pós V., Hunyadi-Gulyás É., Cserháti M., Manninger K., Medzihradszky K., Györgyey J. and Lukács N. (2008): From apoplast proteomics to gene identification in hexaploid wheat by analyzing the host response in leafrust-resistant Lr1 and Lr9 lines. 9th International Congress of Plant Pathology - ICPP 2008 (24-29 August 2008, Torino, Italy) *Journal of Plant Pathology* Vol. 90 (2, Suppl.), p. 202.

Pós V., Szikriszt B., Hunyadi-Gulyás É., Manninger K., Medzihradszky K., Lukács N. (2008): Chitinase Isoenzymes from Proteomic and Transcriptomic Aspects in Wheat Leaf Rust Infection Response in Near-Isogenic Lines of the 'Thatcher' Cultivar. 2nd Central and Eastern European Proteomic Conference (Jena, 12-15. 10. 2008.) Book of Abstracts p. 56.

Pós V., Hunyadi-Gulyás É., Manninger K., Szikriszt B., Rab E., Kabai M., Medzihradszky K., Lukács N. (2009): Wheat leaf-rust infection response – from apoplast proteomics to transcriptional aspects in near-isogenic lines of the 'Thatcher' cultivar. 3rd International Symposium of 'Plant Protection and Plant Health in Europe' (14-16 May, Berlin, Germany). In: Feldmann F, Alford D V, Furk C: Crop Plant Resistance to Biotic and Abiotic Factors (2009) 192-193, ISBN 978-3-941261-05-1 © Deutsche Phytomedizinische Gesellschaft, Braunschweig, Germany

Pós V., Hunyadi-Gulyás É., Kabai M., Caiazzo R., Jócsák I., Medzihradszky K., Lukács N. (2009): proteomic analysis of the apoplast of cadmium stressed barley leaf (*Hordeum vulgare* L.). 3rd Central and Eastern European Proteomic Conference (06-09. 10. 2009., Budapest, Hungary) Book of Abstracts p. 67.

Szikriszt B., **Pós V.**, Manninger K., Lukács N. (2010): Comparison of the expression of beta-1,3-glucanase and chitinase isoforms in leaf rust resistant and sensitive near-isogenic wheat lines. 2nd International Conference on Horticulture Post-graduate Study (30-31 August 2010, Lednice, Czech Republic). Book of Abstracts p. 6. In: Conference proceedings, ISBN:978-80-7375-419-8)

Pós V., Hunyadi-Gulyás É., Manninger K., Szikriszt B., Cserháti M., Medzihradszky K., Györgyey J., Lukács N. (2010): Proteomic and transcriptional Aspects of the Stress Response of Leaf Rust infection in Wheat. 12th International EMBL PhD Symposium -"From Science Fiction to Science Fact: What's next?" (21-23 October 2010, Heidelberg, Germany). Abstract Book p. 81.

Congress full papers in Hungarian

Pós V., Manninger S.né, Hunyadi-Gulyás É., Cserháti M., Miklódy D., Györgyey J., Medzihradszky K., Lukács N. (2007): Levélrozsda fertőzéssel asszociált változások a búza apoplaszt fehérjemintázatában. 4. Erdei Ferenc Tudományos Konferencia "A TUDOMÁNY MINDENKIÉ" (Kecskemét, 2007. augusztus 27-28.) Programfüzet (II. kötet) 963-966. o.

Abstract in Hungarian

Pós V, Halász K, Mesterházi Á, Csősz Lné, Manninger Sné, Hunyadi-Gulyás É, Medzihradszky K, Juhász T, Lukács N (2005): Rozsdarezisztenciával asszociáltan megjelenő fehérjék azonosítása a proteomika módszereivel. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak (Budapest, 2005. 10. 19-21.) Összefoglalók - Kertészettudomány pp. 126-127.

Pós V., Halász K., Rab E., Manninger S.né, Hunyadi-Gulyás É., Szájli E., Kovács G., Csősz Lné, Medzihradszky K., Lukács N. (2006): Búza apoplasztállományának proteomikai analízise. Magyar Biokémiai Egyesület 2006. évi Vándorgyűlése (Pécs, 2006. 08. 30. - 09. 02.) Biokémia XXX. évf. 3. szám p. 77.

Pós V., Halász K., Rab E., Kabai M., Manninger S.né, Hunyadi-Gulyás É., Szájli E., Kovács G., Csősz L.né, Medzihradszky K., Juhász T., Lukács N. (2007): Búza apoplaszt – A proteomika szemszögéből. 53. Növényvédelmi Tudományos Napok (Budapest, 2007. 02. 20-21.) p. 29.

Pós V., Manninger S.né, Halász K., Kabai M., Hunyadi-Gulyás É., Szájli E., Kovács G., Juhász T., Medzihradszky K., Lukács N. (2007): Búza apoplaszt proteomikai vizsgálata. XIII. Növénynemesítési Tudományos Napok (Budapest, 2007.03. 12.) Összefoglalók p. 47.

Pós V., Manninger S.né, Halász K., Kabai M., Hunyadi-Gulyás É., Szájli E., Kovács G., Juhász T., Medzihradszky K., Lukács N. (2007): Levélrozsda-rezisztencia proteomikai elemzése a búza apoplaszton. VII. Magyar Genetikai Kongresszus (Balatonfüred, 2007. 04. 15-17.) Program Összefogalók pp. 158-159.

Pós V., Szájli E., Kovács G., Medzihradszky K., Lukács N. (2007): Búza referencia apoplaszt proteomikai térképezése. A Magyar Proteomikai Társaság Vándorgyűlése (Debrecen, 2007. 08. 25-27.) p. 24.

Pós V., Cserháti M., Hunyadi-Gulyás É., Manninger S.né, Györgyey J., Medzihradszky K., Lukács N. (2007): Közös cisz-reguláló elemek levélrozsda-fertőzéssel asszociált búza-apoplasztfehérjék génexpressziójában. A Magyar Biokémiai Egyesület 2007 évi Vándorgyűlése (Debrecen, 2007. 08. 26-29.) Biokémia XXXI. évf. 3. szám p.66.

Pós V., Szájli E., Kovács G., Medzihradszky K., Lukács N. (2007): Búza referencia apoplaszt fehérjemintázatának analízise. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak (Budapest, 2007. 11. 7-8.) Összefoglalók - Kertészettudomány pp. 128-129.

Kabai M., **Pós V.**, Szájli E., Hunyadi-Gulyás É, Caiazzo R., Jócsák I., Kovács G., Medzihradszky K., Lukács N. (2009): Búza (Triticum aestivum L.) és árpa (Hordeum vulgare L.) apopalszt proteomikai vizsgálata. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak (Budapest, 2009. 10. 28-30.) Összefoglalók - Kertészettudomány pp. 110-111.

Szikriszt B., **Pós V**., Manninger K., Lukács N. (2010): Levélrozsdafertőzés kapcsán indukálódó glükanáz és kitináz izoformák azonosítása és génexpressziós vizsgálata közel izogén, rezisztens és fogékony búzavonalakban. Magyar Biokémiai Egyesület 2010. évi Vándorgyűlése (Budapest, 2010. augusztus 25-28.) Biokémia, XXXIV. évf. 3. szám p.39.

Publication reference

References in international journals

Chen P., Li Y.-H., Cheng Z-H., Chen T. (2007): Intercellular 27 kD Protein is a Chitinase Induced by water stress or Pseudoperonospora cubensis in Cucumber Leaves. *Journal of Plant Physiology and Molecular Biology* 33 (6): 581-588.

Li Y.-H., Chen Z-H., Zhan G.J., Dong J (2009): Purification and Identification of a Pathogenesis-related Protein in Intercellular Fluids of Cucumber Leaves induced by BTH. *Acta Horticulturae Sinica* 36 (4): 507-512.