COST FA0603
„Plant proteomics in Europe “
WG2 & MC meeting

BOOK OF ABSTRACTS

Institute of Plant Genetics and Biotechnology SAS

Nitra, Slovakia, October 14 - 16, 2009

ISBN 978-80-89088-83-6

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Proteomics of seed development in oilseed crops – current status and future goals

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Oilseeds are important renewable sources of protein and oil, which are produced primarily during the maturation or seed-filling phase of embryo development. To characterize this developmental process at the proteome level, my lab employed high-resolution two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry to quantitatively profile and identify over 500 proteins expressed during seed filling in diverse crop oilseeds including soybean, canola, and castor. As an 'omics approach, proteomics does not require a sequenced genome or the development of analytical tools such as microarrays thus enabling global investigations of nearly any crop. The principal objective of these studies was to quantitatively compare protein expression in developing seed that differ in oil: protein ratio and photosynthetic capacity. I will present some of the findings from these studies and illustrate features of the Oilseed Proteomics web database (www.oilseedproteomics.missouri.edu) that was developed to warehouse these datasets. I will also present results on global, 2-DE based phosphoproteomic screens and propose alternative approaches to 2-DE for in-depth, quantitative proteomics.

Proteome regulation and epigenetic code during *Pinus radiata* needle maturation

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Needle differentiation is a very complex process which leads to the formation of a mature photosynthetic organ. This fact implies important changes in protein accumulation and gene expression which must be regulated, amongst others, by epigenetic mechanisms. We have compared some epigenetic modifications (DNA methylation, Histone H3 trimethylated at Lys 4, Histone H3 trimethylated at Lys 9 and acetylated Histone H4) present in immature (1 month old) and mature (12 month old) *Pinus radiata* needles, determining a tissue specific DNA methylation and the differential expression levels of histone epigenetic marks, being the levels of acetylated Histone H4 and Histone H3 trimethylated at Lys 4, associated to gene expression, higher in immature needles whereas the Histone H3 trimethylated at Lys 9 was only found in mature needles.

To determinate which genes and proteins showed as differential during needle development, we have also characterized and compared proteome and transcriptome of immature and mature needles. Immature needles are characterized by a low tissue differentiation and high morphogenetic capability whereas mature needles are a specialized photosynthetic organ which do not show morphogenetic capabilities. 2-DE profiles determined following a previously described protocol (Valledor L. *et al.,* 2008), showed variations in the relative abundance of 280 spots from a total of 856 that were studied whereas transcriptomic analyses (substractive library building and determination of differential expression by macroarray and real time PCR) resulted in the description of 176 differentially expressed genes in immature and mature needles. The joint analysis of proteomic and transcriptomic data that was performed provided a broad overview of differentially expressed genes and proteins associated with needle maturation. Some spots and genes related to photosynthesis and oxidative phosphorylation were overexpressed in mature needles. On the other hand immature needles were characterized by the overexpression of biosynthesis-, cell division- and differentiation-related proteins.

From these functional groups we have selected five genes related to photosynthesis (*RBCA*), regulation of gene expression (*MSI1*, *CSDP2*), leaf elongation (*CYP78A7*) and stress (*SHM4*) to further investigate its specific DNA methylation. All of these genes showed differential histone modifications, detected by Chromatin Immunoprecipitation, furthermore *CYP78A7* and *CSDP2* showed a sequence rich in CpG in its promoter and first exon. Specific DNA methylation patterns related to tissue differentiation were found for *CSDP2*. This is the first description of a specific gene regulation based on epigenetic mechanisms in conifers.

Acknowledgement

This study was supported by the Spanish Ministry of Science and Innovation (projects AGL2004-00810/FOR and AGL2007-62907/FOR).

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Towards elucidation of temporal and spatial protein patterns during barley grain development by LC-MS^E and MALDI-imaging MS approach

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Recent developments in analytical techniques have enabled high trough-put acquisition of data as a pre-requisite to characterize biological systems in a comprehensive manner. Especially the assessment of proteins has gained substantial impact from the advances in mass spectrometric techniques, which made them better accessible for qualitative and quantitative analysis. We are interested in the proteome analysis of barley tissue. In particular we want to have a closer insight into changes in the proteome during barley grain development, because an improved understanding of cereal seed development is of outmost importance for human nutrition.

Protein extracts from developing barley grains (3, 5, 7, 10, and 16 days after flowering (DAF)) were separated on a nanoUPLC combined with ESI-Q-TOF mass spectrometry. Data acquisition was performed by a data independent strategy, called MS^E. For data processing and protein profiling the Expression^E system solution (Waters) was utilised processing the intensities of molecular ions for quantification and the fragment and molecular ions for identification. Besides, quantification at the peptide level allows also grouping of preliminary unidentified peptides. For an elucidation of statistically significant and objective kinetic patterns and biomarker identification multivariate statistics was applied. Prior to this, data pre-processing and initial visualization was performed to ensure the quality of the data and the appropriateness of the subsequently applied clustering algorithm. A number of computational intelligence based clustering algorithms, such as Self-Organizing Maps (SOM) and Neural Gas (NG), that have proven to be highly suitable in a similar context, were applied for the clustering task. Results, which indicate the validity of our approach for the elucidation and visualisation of changes in protein patterns during developmental processes, are shown.

In addition to the temporal alteration of protein expression during seed development we also want to gain a closer insight into their local distribution. Therefore we started to adapt the fairly new MALDI imaging mass spectrometry (IMS) technique to cryo-dissected grain material. Initial data, showing the applicability of MALDI–imaging MS for analysing barley grains, are presented.
The combination of LC-based separation to analyse temporal changes in protein expression and MALDI IMS to reveal local distribution of proteins provides an entire insight into barley grain development on protein level. The future task will be to transfer the developed analytical methods from the scale of the whole organ down to the level of an individual cell to monitor spatio-temporal patterns in dissected seed tissues.

Arabidopsis protein thiol redox status and changes in response to inoculation with *Pseudomonas syringae*

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Our main goal is to study and characterize the Arabidopsis protein thiol status and its redox changes in response to *Pseudomonas syringae*. On one hand, we are advancing in the characterization of the whole protein disulfide status of non-inoculated *Arabidopsis* plants both, in a qualitative, by thiol affinity chromatography, and in a quantitative way, by 4,4'-dithiodipyridine (DTDP) thiol quantification, redox ICAT and redox DIGE analysis. On the other hand, we are more specifically looking at the S-glutathionylation of the proteins as a response to the inoculation with *Pseudomonas syringae*. Glutathionylated proteins, including its residues, have been detected by ion trap analysis. By using this approach we have identified, after data-base searching, *in vitro* glutathionylated proteins.

Functional analysis of the manganese stabilizing protein of photosystem II in potato

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Manganese stabilizing protein (MSP) is an extrinsic subunit of photosystem II, which is necessary for splitting of water molecules – the reaction generating atmospheric oxygen and electrons for the photosynthetic electron transport chain on the thylakoid membrane.

Analyzing a potato mutant line, we found that the absence of one MSP isoform might be connected with altered photosynthetic activity and spontaneous tuberization. Detailed analysis of potato proteome on 2D gels revealed that MSPs form at least nine different spots differing in isoelectric point and/or size. These nine spots were derived out of only two paralogous genes.

Phylogenetic analysis of MSP sequences from 11 plant species showed that there are just two MSP genes in the majority of species. The respective proteins differ in about 10 aminoacids within a species. Surprisingly, MSPs from different species do not form separated branches of orthologues in the phylogenetic tree, but they are most related in the pairs of paralogues in every species. It suggests that the presence of two different MSP isoforms is advantageous for photosystem II function, but MSP evolution and diversification is somehow restricted, possibly by the interaction with other subunits of photosystem II that, however, displayed only low variability among the species.

Individual MSP isoforms functionally differ as indicated by both literature and our data, but the spectrum of MSP isoforms did not differ under different cultivation and light conditions. Thus, MSPs expression/modification is probably constitutive and the regulatory role of MSP isoforms probably resides in differential binding of individual isoforms on the photosystem II under different conditions that we have analyzed by Blue Native PAGE and MALDI ToF MS/MS.

Metabolomics and proteomics data integration for genome annotation and systems biology

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Systems biology is the approach to combine molecular data, genetic evolution, environment and species-interaction with the computer-assisted understanding, modeling and prediction of active biochemical networks. The idea relies strongly on the existence of complete genome sequences and the development of new technologies for the analysis of molecular data. Here, proteogenomic strategies as well as the projection of metabolomics data into genome-wide metabolic networks combined with metabolic modeling emerge as important technologies for improving gene annotation processes (May *et al*., 2008). Using quantitative proteomics and metabolomics we begin to investigate the genome-scale molecular phenotype. However, before data reveal their interrelation, extended statistical and mathematical concepts are required for the integrative analysis of multifactorial phenomena (Weckwerth and Morgenthal, 2005). The detection of significant correlations between the different components based on clustering, dimensionality reduction and other techniques is the basis for biological interpretation (Weckwerth *et al*., 2004; Wienkoop *et al*., 2008). Most of these data mining tools are closely related - based on covariance and/or correlations within a data matrix - and therefore have the potential for comparison of results originating from different procedures. We have developed concepts to systematically connect the genome-wide underlying biochemical regulation with these multivariate data mining procedures (Weckwerth, 2003, 2008; Wienkoop *et al*., 2008). After an introduction into the technological concept of high throughput metabolomic and proteomic analyses I will present a mathematical framework for the application of high throughput profiling data and their relation with genome-scale biochemical networks.

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Integrating multiplexed quantitative proteomic and digital transcriptomics data - possibilities and challenges

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Determining the relative abundances of transcribed genes and their protein products in biological systems is an important aspect to unravel the often subtle molecular differences that regulate biological processes in cells and organisms. Quantitative analytical strategies in Transcriptomics and Proteomics provides the possibility to analyze differences in relative expression in complex samples. In plant proteomics, most often chemical stable isotope labeling strategies instead of metabolic labeling strategies are required (Ong & Mann, 2005; Boersema *et al*., 2009). In such experiments, proteins or peptides from different samples can be labeled with different stable isotopes and their relative amounts are determined from the peptide ion intensities using mass spectrometry. Similar ultra-high-throughput DNA sequencing providing comprehensive tag-based transcriptomic analysis provides an increased sensitivity and cost-effective gene expression profiling (Nielsen, 2008).

We have undertaken a work to optimize and integrate the output data from triplex differential stable isotopic labeling for quantitative mass spectrometry combined with multiplexed digital transcriptomics which will be presented here.

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Systems biology approach to potato - PVY interaction

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In nature plants encounter various factors which influence their growth and development and consequently affect plant product quantity and quality. Potato virus Y (PVY) is a severe plant pathogen responsible for yearly losses in production of *Solanaceae* crops in Europe. Plant responses to viruses and the disease development are different and much less explored in comparison to bacterial or fungal infections. In single component studies the complexity of the plant – pathogen interaction at molecular level can lead to limited conclusions that may fail to notice important changes in physiological processes. Omics approaches, that offer a more holistic view of the processes are therefore a major step forward in understand these interactions.

In our studies, gene expression in the disease response of the susceptible, tolerant and resistant potato (*Solanum tuberosum L.*) cultivars to PVY infection was investigated at different times after infection, using transcriptomics approaches, among them subtractive hybridization, cDNA microarrays and real-time PCR. Transcriptomic studies suggest an important role for genes from various metabolic pathways in the potato – PVY interaction. Functional analysis of a selection of those genes is currently being performed, and their role in the interaction will be confirmed by silencing or over-expression. In parallel with the biological experiments, we have explored and developed several aspects of microarray data analysis and visualization. A potato leaf proteome analysis platform, combining 2D-electrophoresis analysis with identification by LC-MS-MS, was recently established and used for further investigation of the interaction.

A systems biology approach enables us to collect a great amount of data on the interaction and resistance responses between an important crop plant and its significant pathogen. The obtained data will be integrated with pre-existing literature data to construct a structural model of potato - PVY interaction.

Systems analysis of seed filling in *Arabidopsis thaliana*: general linear modeling to determine concordance of transcript and protein expression

Hajduch M.[1], Hearne L.B.[2], Casteel J.E.[1], Joshi T.[3], Agrawal G.[1], Song Z.[3], Zhou M.[4], Wang H.[5], Xu D.[3] & Thelen J.J.[1]

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A fundamental question in systems biology research is 'How well does mRNA and the cognate polypeptide a transcript encodes correlate in expression?' Prior systems biological comparisons of gene and protein expression in plants have largely relied on small datasets or were performed in a static, single time point manner. However, since most biological processes have a temporal component to their regulation it is important to consider how to analyze such data. One such process is plant seed development, for which a cascade of events occurs within a brief time scale when considering the entire life cycle of a plant. Protein and transcript expression data from the model plant *Arabidopsis thaliana* were collected from five sequential stages of seed development spanning the seed-filling (early maturation) phase of development. Two-dimensional gel electrophoresis coupled to tandem mass spectrometry and oligonucleotide microarrays performed in biological triplicate produced high-quality expression data for 523 proteins and 22,746 genes, respectively, across the five developmental stages to establish 319 unique protein/transcript pairs for statistical analysis. To compare temporal expression trends, a technique called general linear modeling was employed to determine concordance among the protein/transcript pairs. Approximately 56% of protein/transcript pairs were concordant in their expression pattern by assessing line curvature, slope and y-intercept. However, concordance varied greatly with protein function. Highest concordance (95%) was observed with transporter proteins and seed storage proteins (63%), and was lowest (25%) for proteins involved in cell structure. The many specific examples of discordant protein /transcript expression suggest this approach could be useful to uncover post-transcriptional regulation.

Construction of soybean proteome database and its application to functional analysis

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Flooding injury is one of the major constraints for cultivation of soybean. Improvement of flooding tolerance in soybean is important for increase in yield. The Soybean Proteome Database aims to be a data repository for functional analyses of soybean responses to flooding injury, recognized as a major constraint for establishment and production of this plant. The current release contains 21 reference maps of soybean (*Glycine max* cv. Enrei) proteins electrophoresed on two-dimensional polyacrylamide gels of which the samples were collected from several organs, tissues and organelles. The Soybean Proteome Database integrates multiple “omes”. An omics table reveals relationships among 106 mRNAs, 51 proteins and 89 metabolites that vary over time under flooding stress. A unified temporal-profile tag attached to the mRNAs, proteins and metabolites facilitates retrieval of the data based on the temporal expression profiles. A graphical user interface based on dynamic HTML facilitates viewing the profiles of multiple omes in a uniform manner. The entire database is available at http://proteome.dc.affrc.go.jp/Soybean/.

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Functional Proteomics: A cornerstone in plant systems biology

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We have assembled a proteome map for *Arabidopsis thaliana* from high-density, organ-specific proteome catalogs (Baerenfaller *et al*., 2008). We matched 95,988 unique peptides to 14,179 proteins and provide expression evidence for a number of gene models that are not represented in the TAIR8 protein database. Analysis of the proteome identified organ-specific biomarkers and allowed us to compile an organ-specific set of proteotypic peptides for 4,105 proteins to facilitate targeted quantitative proteomics surveys. On the basis of proteome maps for cell organelles, we compared the quantitative proteome composition of different cell organelles in the different organs, and generated flux-balance models of plastid metabolism in seeds, roots and leaves. Current organellar proteome maps are not exhaustive, and we therefore analyzed the proteome of two plastid protein import mutants, *ppi1* and *ppi2*, lacking components of the plastid protein import machinery. These plastids are depleted of abundant photosynthetic proteins and therefore provide an improved dynamic range for the characterization of low abundance proteins. More than 1500 different proteins were identified and quantified from isolated plastids. Overall, the protein accumulation in the different mutants was surprisingly similar suggesting basic robustness principles and limited plasticity for the assembly of organellar proteomes. In order to further characterize chloroplast protein import in the different mutants, we systematically searched for N-terminal acetylated peptides in genome-scale WT, ppi1 and ppi2 proteomics data. These analyses revealed the accumulation of precursor proteins in the TOC159-deficient mutants (ppi2), probably as a result of the impaired import reaction. The accumulated precursor proteins enter into the cytosolic N-terminal methionine excision (NME) and acetylation pathway. We discuss these observations in the context of protein import specificity and signaling pathways for retrograde communication. In order to expand our grasp on the dynamic regulation of the chloroplast proteome, we analyzed the chloroplast phosphoproteome and its dynamics during a circadian cycle. Motif-X analysis of the phosphorylation sites in chloroplast proteins identified three significantly enriched kinase motifs, which include known casein kinase II and proline-directed kinase motifs. Interestingly, the phosphorylation analyses revealed only minor changes in chloroplast kinase activities and phosphorylation site utilization during a circadian cycle. A notable exception was the beta subunit of ATP synthase (ATPB), which is phosphorylated at CKII phosphorylation sites preferentially in the dark (Reiland *et al.*, 2009). To identify the kinases responsible for the regulation of chloroplast phosphoproteome dynamics, we characterized the phosphoproteome of T-DNA insertion mutants for different chloroplast kinases in comparison to wildtype in order to establish their *in vivo* substrate spectrum. We present here our data obtained with the thylakoid associated kinase STN8.

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Light induced changes in protein expression and uniform regulation of transcription in the thylakoid lumen of *Arabidopsis thaliana*

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In plants oxygenic photosynthesis is performed by large protein complexes found in the thylakoid membranes of chloroplasts. The soluble thylakoid lumen space is a narrow and compressed region within the thylakoid membrane which contains 80–200 proteins. Because the thylakoid lumen proteins are in close proximity to the protein complexes of photosynthesis, it is reasonable to assume that the lumen proteins are highly influenced by the presence of light. To identify light regulated proteins in the thylakoid lumen of *Arabidopsis thaliana* we developed a faster thylakoid preparation and combined this with difference gel electrophoresis (DIGE) of dark-adapted and light-adapted lumen proteomes. The DIGE experiments revealed that 19 lumen proteins exhibit increased relative protein levels after eight hour light exposure. Among the proteins showing increased abundance were the PsbP and PsbQ subunits of Photosystem II, major plastocyanin and several other proteins of known or unknown function. In addition, co-expression analysis of publicly available transcriptomic data showed that the co-regulation of lumen protein expression is not limited to light but rather that lumen protein genes exhibit a high uniformity of expression. The large proportion of thylakoid lumen proteins displaying increased abundance in light-adapted plants, taken together with the observed uniform regulation of transcription, implies that the majority of thylakoid lumen proteins have functions that are related to photosynthetic activity. This is the first time that an analysis of the differences in protein level during a normal day/night cycle has been performed and it shows that even a normal cycle of light significantly influences the thylakoid lumen proteome. In this study we also show for the first time, using co-expression analysis, that the prevalent lumenal chloroplast proteins are very similarly regulated at the level of transcription.

A quantitative method using online RP/RP 2D nanoLC/MS for the analysis of the apoplastic secretome of infected plants

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Nongel-based techniques have been developed for the analysis of complex proteomic samples. In these so-called 'shotgun' experiments a whole proteome is digested with trypsin without prior separation of the proteins via one- or two-dimensional polyacrylamide gel electrophoresis. Subsequently, a single dimension LC-MS approach using reversed phase (RP) chromatography can be applied for the separation and analysis of the resulting tryptic peptides. To obtain increased proteome coverage, we used in the past 2-D LC-MS by separating the peptides first via strong cation exchange chromatography before RP LC-MS analysis. Now, we applied two-dimensional RP/RP chromatography to analyze the apoplastic fluid (AF) of leaves infected by a fungus. Peptides were separated on a nano-RP column at high pH (pH=10) in the first dimension and at low pH (pH=2) in the second dimension using the 2D nanoAcquity UPLC system. Eluting peptides were analyzed using data independent MS^E performed on a Synapt mass spectrometer.

Plants have evolved sophisticated active defense mechanisms against potential pathogens. One of the responses to pathogen attack commonly observed is the production of so-called pathogenesis-related (PR) proteins. PR proteins accumulate in large amounts in vacuoles and the apoplast, where they inhibit growth of penetrating fungal pathogens. To obtain a more comprehensive overview of the response of a plant to fungal attack, we initiated LC- MS^E analysis of the changes in apoplastic fluid (AF) protein content of tomato upon infection with *Cladosporium fulvum*. AF was collected from susceptible and resistant plants at several time points after inoculation. The results from this comparative LC- MS^E study using the above mentioned RP/RP 2D set up for separation of tryptic peptides will be presented at the meeting.

Thioredoxin targets of the plant chloroplast lumen and their implications for plastid function

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The light-dependent regulation of stromal enzymes by means of thioredoxin-catalyzed disulphide/dithiol exchange is a classical mechanism for control of chloroplast metabolism. Recent proteome studies show that thioredoxin targets are not only present in the stroma but in all chloroplast compartments ranging from the envelope to the thylakoid lumen. Thioredoxin-mediated redox control appears to be a common feature of important pathways, such as the Calvin cycle, starch synthesis and the tetrapyrrole biosynthesis. Hitherto, the extent of thiol-dependent redox regulation in the thylakoid lumen has not been systematically explored. In this study, we addressed thioredoxin-linked redox control in the chloroplast lumen of *Arabidopsis thaliana.* Using complementary proteomics approaches, we identified 19 thioredoxin target proteins, thus covering more than 40 percent of the currently known lumenal chloroplast proteome. We show that the redox state of thiols is decisive for degradation of the extrinsic PsbO1 and PsbO2 subunits of photosystem II. Moreover, disulphide reduction inhibits the activity of the xanthophyll cycle enzyme violaxanthin de-epoxidase that participates in thermal dissipation of excess light absorbed. Our results indicate that redox-controlled reactions in the chloroplast lumen are critical for the function of photosystem II and the regulation of adaptation to light intensity.

The study of the *Musa* spp. plasma membrane proteome using a gel-based and gel-free approach

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The plasma membrane proteome is of interest to many plant physiologists since plasma membrane proteins play a crucial role in response to several types of biotic and abiotic stress. Its study is challenged by the fact that (i) the plasma membrane is a complex membrane system, (ii) its proteome changes with the developmental and physiological state of the plant and (iii) integral membrane proteins can not readily be studied by classical proteomics techniques. Although the plasma membrane proteome of *Arabidopsis thaliana* has been studied quite intensively, the studies on non-model plants are still limited.

We present a study of plasma membrane proteins of the non-model plant banana (*Musa* spp.) which implies protein identification through MS/MS combined with a cross-species and EST database search.

First, an aqueous two phase partitioning technique was optimized for banana tissue. This method is based on the different physicochemical properties of plasma- and endomembranes, and their solubility in the polymers PEG 3350 and dextran T500. Plasma membranes preferentially reside in the (PEG rich) upper phase, while endomembranes will be concentrated in the (dextran rich) lower phase (Widell *et al*., 1982)

Subsequently a gel-based and gel-free approach was compared for the ability to identify proteins present in the plasma membrane rich phase. In the gel-based approach proteins were extracted first in a mixture of chloroform and methanol (C/M). Both the C/M soluble, which is expected to contain the more hydrophobic proteins, and the C/M insoluble fraction were analysed using one dimensional SDS-PAGE and MALDI-TOF/TOF. In the gel-free approach the organic solvent based extraction method published by Mitra and coworkers was applied (Mitra *et al.*, 2009). It involves a methanol assisted tryptic digestion followed by a chloroform-based extraction to remove remaining chlorophylls and undigested proteins which can affect downstream analysis. Since the gel-free approach is not obvious for application in non-model studies (Carpentier *et al*., 2008), two strategies were followed to ensure reliable protein identification, namely 2-D LC-ESI and LC-MALDI TOF/TOF. The complementarity of both ionization methods as well as the differences between the gel-based and gel-free approach were evaluated.

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The role of protein oxidation in cellular metabolism

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The production of reactive oxygen- and reactive nitrogen-species in plant cells can lead to a variety of modifications of proteins through oxidation of amino acid side groups. The widespread occurrence of such modifications is becoming appreciated as new proteomic approaches allow their systematic identification. Oxidized amino acid residues can be identified directly by mass spectrometry if the modification is stable, but it is more common to covalently tag the oxidised group by reaction with a marker molecule. The marker molecule generally allows visualisation through immuno-detection and isolation of modified proteins by affinity purification. Although there are several technical caveats with such approaches, they have been useful in documenting the extent of oxidative modification of proteins and have highlighted a number of proteins where oxidative modification is critical for protein function. A view that such modifications could have signalling ramifications is emerging. However, in many cases there is a lack of information as to the effect of oxidation on protein activity or function. Severe protein oxidation is costly to the cell since oxidatively damaged proteins need to be degraded by specific proteases or damaged cellular components recycled via the autophagy pathway. Avoiding this cost is clearly advantageous, and it has been proposed that proteins may have an over-representation of easily oxidisable amino acids on their surface to act as decoy or sacrificial residues, thus preventing or postponing oxidation of residues more important for the function of the protein.

Oxidation of an adjacent methionine residue inhibits regulatory phosphorylation of pyruvate dehydrogenase

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A Met residue is located adjacent to phosphorylation site 1 in the sequences of mitochondrial pyruvate dehydrogenase E1alpha subunits. When synthetic peptides including site 1 were treated with H2O2, the Met residue was oxidized to methionine sulfoxide (MetSO), and the peptides were no longer phosphorylated by E1alpha-kinase. Isolated mitochondria were incubated under state III or IV conditions, lysed, the pyruvate dehydrogenase complex (PDC) immunoprecipitated, and tryptic peptides analyzed by MALDI-TOF mass spectrometry. In all instances both Met and MetSO site 1 tryptic-peptides were detected. Similar results were obtained when suspension-cultured cells were incubated with chemical agents known to stimulate production of reactive oxygen species within the mitochondria. Treatment with these agents had no effect upon the amount of total PDC, but decreased the proportion of P-PDC. We propose that the redox-state of the Met residue adjacent to phosphorylation site 1 of pyruvate dehydrogenase contributes to overall regulation of PDC activity *in vivo*. Similar results were obtained in studies of cytoplasmic nitrate reductase.

Associating wound-related changes in the apoplast proteome of *Medicago* with early steps in the ROS signal-transduction pathway

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Early wound-related changes in the leaf apoplast proteome of Medicago truncatula has been characterised by 2-DE and MALDI-TOF/TOF and the differential expression of 28/110 extracellular proteins could be reproducibly observed 6 h after wounding. Wounding induced an initial (0-15 min) burst of O_2^-, followed by a later (3-6 h) production of O_2^- and H_2O_2. Suppression of the initial O_2^- production with the application of DPI $\leq$ 3 min after wounding also suppressed wound-regulated changes in 9/28 extracellular proteins, whereas DPI application after 15 min only partially inhibited early O_2^- production and was ineffective in suppressing wound-related changes in these proteins. Further studies with wounded plants treated with DPI concomitant with exogenous sources of ROS confirmed the regulation of these proteins with early ROS-signalling events. This indicates that rapid O_2^- signalling is required for mobilising the downstream (3-6h), differential expression of several wound-regulated proteins. Apoplastic proteins mobilised within the ROS-dependent pathway included SODs, peroxidases and germin-like proteins, suggesting their involvement within wound-activated, ROS regulatory loops.

Ongoing work is now seeking further wound-responsive and ROS-regulated proteins within cytosolic and integral plasmalemma proteins in order to form an integrated view of ROS-targeted proteins in this system and the identification of early, wound-signalling intermediates.

Ozone-triggered rapid stomatal response involves production of reactive oxygen species and is governed by SLAC1 and OST1

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The air pollutant ozone spontaneously generates reactive oxygen species (ROS) in the leaf apoplast. ROS as signaling molecules have important functions in plant stress responses and development. Consequently, ozone has been used as a tool to unravel in planta ROS-induced processes. ROS are also signaling components in plant guard cells. Here we have utilized ozone to study ROS-dependent stomatal signaling. We show that ozone triggered rapid transient decrease in stomatal conductance (RTD) coincided with the elevated burst of ROS in guard cells suggesting that RTD is induced by the ROS triggered by the application of ozone.

By analyzing RTD in 51 mutants carrying mutations in proteins shown to be involved in stomatal regulation, we show that OST1 and SLAC1 are required for the ROS-induced fast stomatal closure. We have also identified in the N-terminus of SLAC1 two putative phosphorylation sites which are required for the ozone-triggered RTD. Furthermore, a split-ubiquitin yeast two-hybrid assay demonstrated interaction between SLAC1 and OST1, providing indirect evidence that SLAC1 might be activated by OST1 kinase. Complete absence of the RTD in dominant negative ABA-insensitive mutants *abi1-1* and *abi2-2* also suggest a regulatory involvement of the protein phosphatases ABI1 and ABI2 in the processes required for the activation of the S-type anion channel by ROS.

Proteome-wide characterization of seed vigor and identification of markers for the seed industry

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During this presentation, I will review proteomic work performed in my laboratory (published and not yet published) on seed vigor by using model plants (e.g. *Arabidopsis*) and plants of agronomic interest (e.g. sugar beet). Seed vigor can be evaluated from germination assays and seedlots naturally exhibiting variations in seed vigor, or by using a reference seedlot submitted to seed treatments altering its vigor (priming or controlled deterioration assay). Some salient features could be evidenced as the importance of the stored proteins and mRNAs, protein translation, metabolic activity, and the extent of proteome oxidation in the mature seeds.

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2D DIGE,PCA/PLS and de novo identification, a powerful combination to explore crop biodiversity and to explain variety specific phenotypes?

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Water stress and desiccation tolerance can be studied using different systems: (i) tolerant systems, species such as resurrection plants or structures such as seeds, (ii) genetic model systems and (iii) crop plants. Through breeding and selection, crop varieties are available with varying degrees of tolerance. *Musa* (banana) is an interesting species to study. The genus originated in Southeast Asia, where the wild types *Musa acuminata* (AA) and *Musa balbisiana* (BB) still occur. The A (acuminata) genome sometimes in combination with the B (balbisiana) genome lays at the origin of the cultivated banana varieties which are diploid (AA, AB and BB), triploid (AAA, AAB, ABB and BBB) or tetraploid (AAAA, AAAB, AABB and ABBB). The B genome is often associated with drought tolerance and hardiness to environmental factors. We designed an experimental set-up to study osmotic stress acclimation in banana (Carpentier *et al*., 2007). This *in vitro* model is based on the regeneration capacity of meristems following acclimation and severe abiotic stress. Since banana is a non model crop, we decided to use proteomics instead of other, sometimes more performing, techniques involving transcriptomics (Carpentier *et al*., 2008a, 2008b).

We compared a tolerant ABB variety with a sensitive AAA variety subjected or not to osmotic stress acclimation. After acclimation, the regeneration rates of the ABB and AAA variety were 78 and 16%, respectively. Without osmotic acclimation, regeneration rates of the ABB and AAA variety were 68 and 2% respectively. PCA analysis clearly demonstrates that the variety specific variance is much bigger than the treatment specific variability. We observe many variety specific isoforms of which we presume they originate from the B and the A genome. However, the questions remains “Which variety specific differences are correlated to the observed phenotype?” For this a supervised PLS/DA analysis coupled with VIP was applied. This analysis combined with previous observations on the evolution of the proteome over time led us to an important insight. We demonstrate that the control of sucrose metabolism and a switch from oxidative phosphorylation towards fermentation for ATP production is an important mechanism during acclimation (Carpentier *et al*., in preparation).

Using automated *de novo* sequence analysis of derivatized peptides (Samyn e*t al*., 2007) on proteins extracted from a AA and BB variety, we were able to identify B- and A-genome specific isoforms.

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Proteomic insights into *Nicotiana tabacum* trichome metabolism

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Nicotiana tabacum leaf trichomes are known to be involved in the production and secretion of interesting secondary metabolites, some of which are involved in the plant defense. However, little is known about the synthesis and transport of those metabolites. We therefore undertook a large scale analysis of the proteome of the *N. tabacum* trichomes to identify the enzymes involved in the secondary metabolism, as well as the transporters putatively involved in the secretion of secondary metabolites.

Leaf trichomes were collected, homogenized and soluble and membrane protein fractions were isolated and analyzed independently. For the analysis of soluble proteins, two complementary proteomic approaches, two-dimension gel electrophoresis (2DE) and one-dimension liquid chromatography (1DLC), were carried out to increase the number of identified proteins. Membrane proteins were analyzed through 1DLC. Both soluble and membrane proteins were then identified by tandem mass spectrometry (MALDI-TOF/TOF). This led to the identification of 726 proteins through the 2DE analysis and 858 proteins through the 1DLC analysis, representing one of the most detailed proteomic analyses of plant trichomes.

While most identified enzymes are involved in the primary metabolism and energy production, our proteomic analysis also uncovered enzymes involved in the metabolism of flavonoids and terpenes. For instance, the seven enzymes involved in the non-mevalonic pathway of terpene precursor synthesis were identified. Furthermore, enzymes involved in plant defense against both biotic and abiotic stresses were identified, confirming the defensive role commonly assigned to plant trichomes. We also identified several transporters, such ATP-Binding Cassette (ABC) transporters, a protein family known to be involved, among others, in the transport of secondary metabolites.

This analysis is expected to contribute to the elucidation of secondary metabolic synthesis and transport pathways occurring in *N. tabacum* trichomes. Taking advantage of the engineering potential of trichomes, this in turn might lead to improving the production of pharmacologically- or industrially-relevant secondary metabolites.

The possibilities of the use of dehydrins as markers of frost tolerance in barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*)

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Frost tolerance (FT) is a complex trait determined by various genes. Certain dehydrins from LEA protein family accumulate in plants under cold acclimation (CA). We have studied accumulation of WCS120 proteins in wheat and DHN5 protein in barley under CA, and tried to find some relationships between their accumulation and plant FT determined either in controlled conditions by direct frost tests or in field conditions by provocation pot method. We used 1D SDS-PAGE, immunoblots and densitometric analysis for dehydrin quantification. In barley, we found a correlation between DHN5 accumulation and acquired FT in 21 cultivars of different genetic background after a 3-week CA. When we worked with doubled-haploid (DH) lines derived from a spring cultivar and a moderately tolerant winter cultivar, we obtained a correlation between DHN5 accumulation and FT later when the spring lines were already in reproductive stage, thus the differences between the growth habits became more apparent. We have also studied WCS120 accumulation in 21 winter wheat cultivars differing in FT when grown under different temperatures. (25, 17, 9 and 4 °C). There was a good correlation between FT and WCS120 accumulation in plants grown under 9 or 4 °C. Moreover, the cultivars differed in WCS120 content at 17 °C, a temperature at which they cannot be distinguished via a frost test. It can be concluded that dehydrins seem to present valuable markers of FT in *Triticeae* and could be utilized in selection of tolerant cultivars not only at low, but also at high temperatures.

Introduction

The aim of our study was to investigate cold-inducible dehydrins - WCS120 proteins in common wheat and DHN5 in barley - in differently frost-tolerant cultivars grown either under cold (2 - 5 °C) or at higher temperatures (10 - 20 °C). We wanted to find out whether the differently frost-tolerant wheat and barley cultivars can be distinguished according to WCS120 and DHN5 accumulation, respectively, not only when grown under cold, but also at higher temperatures.

Materials and methods

Wheat and barley cultivars with different level of frost tolerance (FT) were grown either under cold (3 - 5 °C) or at higher temperatures (10 - 20 °C) in growth chambers. These plants were used for determination of dehydrin content and for determination of FT via direct frost tests. Other plants were grown under field conditions in order to determine their maximum FT as plant winter survival. Dehydrin accumulation was determined either from silver-stained 2D-gels or from 1D-immunoblots using a specific anti-dehydrin primary antibody. The density of silver-stained protein spots and bands on immunoblots was evaluated by PDQuest and QuantityOne software, respectively.

Results

I / Low-temperatures (cold)

A / Wheat
B / Barley

II / Higher temperatures (mild cold)
A / Wheat
B / Comparison of wheats and barleys

Conclusions
We were able to distinguish two winter wheat cultivars with different FT according to WCS120 accumulation after 21 days of cold (2 °C). A correlation between DHN5 accumulation and acquired FT was found in 21 barley cultivars after 21 days of cold (4 °C). A correlation between WCS120 accumulation and plant FT level was also found in wheat grown at higher temperatures (17 °C and 9 °C) while analogous relationship was not found in barley grown at 10 °C, 15 °C or 20 °C.

Changes in protein and allergen patterns are detectable in pollen from *Poa* plants grown in Cd^{2+} contaminated soils

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Allergic diseases are increasing and they are estimated to affect half of the European community by 2015, with a full financial impact in Europe of around € 100 billion per annum (www.eaaci.net; www.efanet.org/allergy/index.html).

The causes of the rising incidence of allergic disease are different and have to be searched mainly in the modern lifestyle; a decreased stimulation of the immune system ("hygiene hypothesis") and an increase of exposure to new allergens or to indoor/outdoor pollutants are some of the supposed causes.

Regarding environmental pollution, recent interesting findings suggest that, besides to play a role in the worsening of airway diseases, air pollutants are able to make allergens, especially pollen allergens, more aggressive through post translational modifications. No literature data is instead available concerning the effects of soil pollutants on plant allergens. It is of great concern if we consider that plants have a stationary habit and are exposed to soil contaminants during all their life cycle. The exposure to soil contaminants should induce for example the production of organs (i.e. fruit, pollen leaf etc) containing modified or differently expressed well known allergens or containing new allergenic proteins (i.e. defence proteins which are known to be cross-reacting allergens). Among soil pollutants, heavy metals are a class of contaminants useful to investigate the relationship between soil pollution and allergy, because they can modify the plant proteome and are widespread.

In our study we are assessing the effect of cadmium on pollen allergenic proteins of the annual blue grass, *Poa annua* L. This grass species has been chosen because, belonging to the *Poaceae* family, it is considered an important allergenic plant.

Poa plants were grown on soil contaminated or not (control) with 50 mg/ml Cd^{2+}. Plant leaves and pollen were collected and analysed by proteomic techniques. Preliminary results suggest that cadmium exposure induces changes not only in pollen proteome but also in allergen pattern. Specifically, the expression of a 32 kDa protein/s is promoted by the cadmium treatment.

Transformation of protein composition during cold acclimation period of oilseed rape (*Brassica napus* L.)

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In spite that during the last decade many investigations have been carried out employing various genetic approaches and model systems the molecular–biochemical mechanism of cold acclimation – preparation to wintering (frost resistance and dormancy keeping) period is not elucidated so far.

Several genes families are known to alter their expression during cold acclimation period. Knowing about gene products-proteins synthesis *de novo*, metabolism, accumulation, degradation peculiarities and specificities as well as the possible role of these changes are weasel–worded.

Evidently that proteins being various signal receptors (hormones, temperature, light and other environmental factors, etc.), signal transducers (kinases/ phosphatases, namely histidine kinases) as well as proteins–enzymes or transporters may be (are) involved in course of various adaptive processes of plant cells and can determine the responses.

In order to achieve the better notion on the relationship between the protein composition and cold acclimation degree, as a test objects the two different oilseed rape (*Brassica napus* L.) cultivars ('Casino' and 'Valesca') according to their resistance for wintering have been used.

Our study focuses on changes in composition of both soluble and solubilized by non-ionic detergents membranous structures protein fractions in significant for oilseed rape wintering organs (terminal bud and root collum) cells during cold acclimation period (from middle of September till first decade of November). The protein compositions were tested by column chromatography and gel electrophoresis methods.

Our results show that protein composition transformations are related not only with increase in accumulation or the synthesis of novel, not characteristic for growth period proteins, but also to decrease in amount or destruction of others in cells of terminal buds and root collums of both tested cultivars. The level of new proteins formation exceeds their destruction and the number of individual proteins increases during cold acclimation period. The transformation of protein composition occurs more sharply in terminal bud cells and soluble protein fractions in comparison with root collums and membrane protein fractions of both tested cultivars. Increase in protein number and transformation of proteins composition of this nature are detected in bud cells of other plants, too (Shuliakovskaja & Anisimovienė, 1985). However, comparison of changes in protein composition in different oilseed rape cultivars according to wintering success revealed, that the more number of individual proteins characteristic for autumnal growth period disappeared and more new ones (specific thermostabile proteins–dehydrins among them) were formed in better wintering cultivar 'Valesca' during the cold acclimation period. The obtained results let us to suppose, that the better wintering

of cultivar ‘Valesca’ may be related with six (6) specific proteins indistinctive for cultivar ‘Casino’. Two of them are high molecular proteins (molecular masses > 272 kDa); others have m. m. in range of 14 – 66 kDa.

According to data of model assays, the better wintering of oilseed rape may be coherent with presence of 5 – 7 individual proteins in their terminal bud cells at the end of cold acclimation period (Anisimovienė & Novickienė, 2004; Anisimovienė *et al*., 2006). 2 – 3 of them belong to specific thermostabile proteins–dehydrins. Still there should be elucidated which of these 6 proteins, characteristic to cultivar ‘Valesca’ may be related with alteration of plant development processes, hormonal system action, or with frost tolerance and dormancy keeping.

From the theoretical point of view, data of these investigations may have the significance for understanding the molecular mechanisms of various plant cold acclimations. These results may be useful for plant breeders to select agronomical perspective cultivars.

Acknowledgement

This work was partially supported by Lithuanian State Science and Studies Foundation.

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Response to AM symbiosis in leaves: two proteomic studies

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Arbuscular mycorrhizae (AM) are a widespread mutualistic symbiosis between the roots of most land plants and the soil fungi included in the phylum *Glomeromycota*. A M fungi are known to influence plant performance in many ways: they improve plant nutrition, modify root architecture, promote plant tolerance or resistance to pathogens, drought, salinity and heavy metal stresses. In addition AM fungi have been shown to trigger fungus-specific responses in the leaves of different plants, enhancing the attraction of aphid parasitoids in tomato, modifying the essential oil production in basil and restoring the leaf structure in zinc-treated poplars. However, information concerning the modifications induced by AM fungi in the leaf proteome are still scanty. Investigation on the epigeous organs of mycorrhizal plants could provide relevant information for the understanding of the mechanisms at work in the symbiosis and resulting in improved stress tolerance. Therefore, the aim of the present work was to study the protein expression profile in *Pteris vittata* fronds and *Populus alba* leaves, in plants inoculated with three AM fungi (*Glomus mosseae*, *G. intraradices* or *Gigaspora margarita*).

Fern pinnae and poplar leaves were ground in liquid nitrogen and proteins extracted with TCA/acetone precipitation. All the samples were separated by two-dimensional electrophoresis (2-DE), using linear pH 3-10 or 4-7 IPG strips. 2-DE gels were Coomassie stained and comparative analysis of the differentially expressed proteins was performed with PD-Quest software. Results were compared by one-way ANOVA statistical analysis. Identifications were carried out by nano-LC ESI Q-TOF MS/MS peptide sequencing on QSTAR-XL mass spectrometer, followed by searching the NCBInr database with the Mascot algorithm.

A wide range of leaf proteins were up- or down-regulated in the presence of AM fungi. In particular, the three AM fungi modulated proteins belonging to photosynthesis, photorespiration, glycolysis, protein folding, electron transport, reductive pentose-phosphate cycle and other important biological processes. Both in *P. vittata* pinnae and in *P. alba* leaves, many differentially expressed spots belonged to the RuBisCO complex (LSU and SSU) and to the RuBisCO activase (RCA).

Proteome analysis showed that in the AM symbiosis: i) modifications of leaf protein expression are induced demonstrating that the whole plant metabolism (not only root metabolism) is affected; ii) the main metabolic pathways involved during the symbiosis were the photosynthetic and sugar metabolisms.

Impact of homogenization conditions and protein extraction on the obtained proteomic spectra of tobacco pollen

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The male gametophyte of angiosperm plants (including tobacco), mature pollen, carrying genetical information has to withstand environmental stresses as well as suboptimal conditions. Its desiccated cytosol and extremely hard exine (outer part of cell wall) make it resistant also to common extraction protocols. Moreover, *Arabidopsis thaliana* pollen proteomic datasets from three known resources (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Sheoran *et al*., 2006) differed from each other rather than being similar (only 18 proteins out of 267 overlap, i.e. 7%). Such variability was caused mainly by the low coverage of traditional proteomic methods, as demonstrated by recent study of Grobei *et al*. (2009) introducing the shotgun proteomics to the tiny world of pollen.

All the above-mentioned facts pinpoint the importance of properly performed homogenization to obtain as broad protein spectra as possible. In this work, we have shown that not only thorough manual homogenization but also automated homogenization using MagNA Lyser Instrument (Roche) leads to satisfactory results. But using automated homogenization one has to choose the optimal homogenization conditions – too hard homogenization leads to protein fragmentation whilst too mild homogenization does not break down the tough pollen wall properly.

Not only homogenization conditions but also extraction protocol was shown to have an impact on the resulting proteomic spectra. So we compared four extraction protocols using different reagents. All used protocols included thorough washing steps in the final stages of the extraction since the purity of the extract is critical for subsequent isoelectric focusing of 2D-GE.

Acknowledgement

Authors gratefully acknowledge the financial support from ESF (COST Action 0603), Ministry of Education of the Czech Republic (MSMT grant No. OC08011).

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Anatomic differences in adult plant organs of *Medicago truncatula* under drought- and salt stress: Symbiotic nitrogen fixation leads to stress alleviation

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Plants are able to adapt to environmental changes of their habitat. However, perturbations may result in a physiologically and/or anatomically visible reaction. *Medicago truncatula* is a plant which belongs to Legumes and can establish symbiosis with the bacteria *Rhizobium meliloti*. These bacteria colonize the meristematic tissue of the root tip, whereby nodule development is induced. This bacteroid – plant interaction plays a key role enabling nitrogen fixation of the plant. Comparison between nitrogen fixing and assimilating plants show differences in phenotype such as in leave size and color. Significant anatomical variations were found in the number of stomata in the leaves as well as the grade of development of the vascular system of the stem between symbiotic and non symbiotic plants. After exposure to stress they exhibit physiological changes such that e.g. under salt stress plants show remarkable changes in color and growth. However, at cell level there are slight changes in the single plant organs, like shrinking of the parenchymatic cells. Furthermore, cells of root nodules show an increase of vacuole size simultaneous with shrinking of the cell. During re-watering, stressed plants show a clear temporal difference during cell regeneration between the symbiotic and non-symbiotic groups. Thus, there is anatomic evidence that nitrogen fixing *Medicago* plants may accomplish the different stress situations much better than those without root nodules. Proteomic and Metabolomic studies are under progress.

Proteomics of Holm oak (*Quercus ilex*)

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Holm oak (*Quercus ilex* subsp. *ballota* [Desf.] Samp.) is one of the dominant tree species in natural forest ecosystems over large areas of the Western Mediterranean Basin. In Spain, it is widely used for conservation forestry systems and sylvicultural practices, covering an approximate surface of 3 MHa, with estimated values over 120 M€ in fruit acorn production per year. The Holm oak acorns are major component in the feeding systems of many Mediterranean wild and livestock species; and, at the same time, the basic feed ingredient for domestically-bred high quality meat pigs. Forest restoration and reforestation is a high priority objective nowadays, and in the light of the changing forest management scheme, Holm oak is acquiring more interest for Mediterranean forestry. This activity has increased the demand for Holm oak seedlings and favoured their nursery production. As natural, non-domesticated plant species, with great plasticity and phenotypic variability, a key challenge prior massive clonal propagation is the establishment of techniques for the selection of elite genotypes among provenances with high survival percentage and productivity under specific environmental conditions. In this direction, we do pretend to determine the amount of variation which exists between natural Andalusian populations of *Quercus ilex*, in terms of growth, productivity and tolerance to stresses, and the same time to develop propagation protocols and cataloguing techniques. For that we are using a multidisciplinary approach, including ecophysiology, classical biochemistry, microscopy, and the modern –omic techniques, including transcriptomics and proteomics (Valero *et al*., 2009).

In this meeting we will present data obtained by using a proteomic approach, this with the following specific objectives: i) Analyze intra- and inter-population variability; ii) Characterize populations through proteotyping; iii) Study plant responses to stresses, including drought and fungi. Proteomic analysis has been performed with leaf tissue, acorns, and pollen, collected from both field- and greenhouse-grown trees. Data obtained by using leaf tissue have been previously published (Jorge *et al*., 2005, 2006; Echevarría-Zomeño *et al*., 2009). The workflow included: i) experimental design; ii) protein extraction; iii) one- and two-dimensional gel electrophoresis; iv) mass spectrometry; v) statistical analysis of the data; v) protein identification.

Despite the great variability in the 1-D and 2-D protein profile within the same populations, it has been possible to discriminate and establish phylogenetic relationships among then as well as correlate the profile with edafoclimatic characteristics and morphometric parameters. Despite being a recalcitrant species, the main difficulty is related to the absence of *Q. ilex* sequences in databases, which limits the number of proteins identified.

Acknowledgement
This work is part of the Financed Research Project: “Cultural and biological control of root rot caused by *P. cinnamomi* in *dehesas*; variability, cataloguing, stress response and clonal propagation of Holm oak (*Quercus ilex L*.) (DECOVA). AGL2009-12243-C02-02. Spanish Ministry of Science and Innovation, Spain.

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Valero *et al*., 2009. Population variability and mother tree selection in holm oak (*Quercus ilex* L. subsp. ballota [Desf.] Samp) based on acorn morphometry and analysis by near infrared reflectance spectroscopy (NIRS). Submitted.

Comparative proteomics of seed development of soybean in Chernobyl area

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With each passing year since Chernobyl accident, more questions arise about the potential for organisms adapt to radiation exposure. The explosion of one of the four reactors of Chernobyl nuclear power plant on 26 April 1986 caused worst environmental nuclear disaster in the history. It transported vast amounts of radioactive material into the atmosphere, much of which was subsequently deposited not only in the immediate vicinity of power plant in but over the large parts of Europe. Nowadays, 23 years after the accident, the soil in the close vicinity of the CNPP is still significantly contaminated with long-living radio isotopes, such as ^{137}Cs. Despite this fact, the plants in the Chernobyl area were able to adapt.

The aim of our research is to investigate plant adaptation mechanisms toward permanently increased level of radiation using quantitative high-through-put proteomics methodology. During the first year of the project, soybeans of local variety (Soniachna) were sown to the contaminated and control fields of Chernobyl region and mature seeds were analyzed. Following year, developing seeds of secod generation were harvested at 14, 28 and 42 days after flowering in addition to mature seeds and analysed using two-dimensional gels electrophoresis (2-DE) based proteomics approach. In total, 466 protein expression profile pairs were established using ImageMaster 4.9 software between plants grown in control and contaminated fields. All spots representing the pairs with statistically signifficantly changed expression profiles between those two fields were excised from the 2-DE gels and subjected to tandem mass spectrometry for protein identification.

Acknowledgement

This investigation was supported by Seventh Framework Program of the European Union – International Reintegration Grant (MIRG-CT-2007-200165) and MVTS-COST FA0603.

A systematic proteomic study of flax seed development

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Flax (*Linum usitatissimum* L.) is an important industrial crop. It is grown for high quality fibres and for oil production. In adition, flax fibre is a valuable component of modern composite materials used in the automobile industry. Nowadays his imporance as functional food is growing because of its high content of fatty acids, mainly α-linolenic acid, and lignan oligomers. The aim of our investigations was to determine the expression profile of proteins during seed development. Highthroughput proteomic approach based on two-dimensional gel electrophoresis (2-DE) was applied (followed by tandem mass spectrometry). Total protein was extracted from four developmental stages of seeds, 14-16 days after flowering (DAF), 20-22 DAF, 8-30 DAF and from mature seeds. The 2-DE was perform using strips with immobilized pH 5-8 gradients. Total of 478 2-DE spots were quantified using ImageMaster 4.9 in biological triplicate, excised from gels and subjected to tandem mass spectrometry for protein identification.

Acknowledgement

This investigation was supported by Seventh Framework Programme of the European Union – International Reintegration Grant (MIRG-CT-2007-200165) and MVTS-COST FA0603.

A method for identification of proteins involved in the regulation of plant transpiration

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We have recently established a method for finding open stomata mutants in *Arabidopsis thaliana*. The method consists of three different ways of measuring water-loss of cut (rosette) leaves. Each step is narrowing down the number of mutant candidates until the individuals with strong phenotype remain. A mutant of SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) is used as a positive control in the screen. The *slac1* mutants have no obvious visible phenotype under normal or dry growth conditions. However, leaves cut from the *slac1* plants loose 50-80% of its weight whereas wildtype plants loose only 15-30%. We have found two putative individuals with a strong water-loss phenotype. One of these, *cb5* (*cool breath 5*) displays an interesting phenotype. Grown at normal conditions *cb5* is having fused leaves, a character found in many cuticle-affected mutants. Toluidine blue treatment revealed a strong staining in mutant rosette leaves further suggesting an impaired cuticle. Interestingly, both toluidine blue staining and water-loss phenotypes are only observed in older (2-3 weeks) rosette leaves, but not in younger true leaves, cotyledons or cauline leaves, suggesting that the responsible protein is only active or necessary after a switch in leaf cuticle development. Further characteristics of *cb5* are currently being analyzed by using our gas-exchange equipment. *cb1* also displayed a strong water-loss phenotype in excised leaves but also had an extremely delayed bolting phenotype. Since we were screening a 35S insertion line library, the affected genes are now being identified by TAIL-PCR.

New insights into the proteome of the transcriptionally active chromosome from chloroplasts

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Chloroplasts as semiautonomous organelles possess their own DNA (ptDNA), which encodes proteins of the photosynthetic apparatus and components of plastidic transcription and translation machinery. The coordination of plastid transcription by nucleus-encoded proteins is only poorly understood and of growing interest. To get new insights into the protein composition of the transcriptional apparatus in plastids we compared the protein composition of a highly purified transcriptionally active chromosome (TAC-II) from spinach (*Spinacia oleracea* L.) chloroplasts to the proteome of the conventionally prepared transcriptionally active chromosome (TAC-I) by two-dimensional gel electrophoresis. The purified TAC-II fraction is enriched in 85 proteins of the 132 polypeptides identified in the TAC-I fraction. The protein composition of the TAC-II fraction was further examined with tandem mass spectrometry whereby 45 new proteins could be identified. Fourteen of them possess domains and motives known for binding to DNA or RNA like MYB- and SWIB-domains or PPR/TPR repeats.

One of newly identified TAC-proteins is AtSWIB-1 (*Arabidopsis thaliana* SWIB domain-1 protein). An AtSWIB-1-GFP fusion protein was shown to be localized both in plastids and in the nucleus of the same cell. In chloroplasts AtSWIB-1 was found to co-localize with the PEND (plastid envelope DNA-binding) protein in nucleoids. Immunological experiments with a polyclonal antibody raised towards an AtSWIB-1 peptide confirmed the dual localization of the protein in nuclei and plastid extracts from Arabidopsis leaves. Moreover, the antibody recognised a protein in the transcriptionally active chromosomes fraction isolated from spinach leaves. Hence AtSWIB-1 is a novel plant protein associated to DNA both in plastids and the nucleus it might have a function in coordination of the plastid and nucleus genome.

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Accumulation of dehydrins during desiccation of resurrection plant *Haberlea rhodopensis*

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Integrity of photosynthetic apparatus is essential for survival of plants at desiccation. Forming of photosynthetic supercomplexes is a strategy to supress degradation of basic photosynthetic proteins. The aim of our research is to investigate formation of such structures by BN/BN PAGE and their stochiometry during desiccation of the resurrection plant *Haberlea rhodopensis.* Accumulation of dehydrins and their role in protecting the photosynthetic system will be discussed.

Proteome analysis of flooding response in root and hypocotyl of soybean seedling at emergence stage using DIGE technique

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Flooding injury is one of the major constraints for cultivation of soybean. Improvement of flooding tolerance in soybean is important for increase in yield. A proteomic approach was used for clarifying the mechanism of flooding injury of soybean. Two-day-old seedlings were flooded with water for 12 h and 24 h, and whose roots and hypocotyls were used for two-dimensional difference gel electrophoresis (2D-DIGE) analyses using CyDye fluorescence label with the comparative analysis between untreated and flooding treated samples. Out of detected 464 protein spots, 14 and 16 protein spots were up- and down-regulated, respectively. These protein spots were identified by nanoliquid chromatography -tandem mass spectrometry. The identified proteins spots contained the proteins involved in primary metabolism, energy production, secondary metabolism, disease/defense and cell structure. Among these proteins, most up- and down-regulated proteins were glyoxalase II and xyloglucan endotransglucosylase / hydrolase, respectively. These results suggest that up-regulation of glycolysis and glycolysis related detoxification system and down-regulation of cell wall metabolism are primary responses in soybean seedling under flooding.

Proteomics of a potato cell line adapted to a high salinity environment

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Salinity is a major constraint to crops, limiting both plant growth and productivity. It is estimated that more than 6% of the world´s land and 30% of the world´s irrigated areas already suffer from salinity problems (UNESCO Water Portal, 2007 - www.unesco.org/water). Potato (*Solanum tuberosum* L.), one of the most important food crops in the world is described as moderately salt sensitive (Katerji *et al*., 2000). In order to analyse potato responses to salt stress at cellular level we make use of *in vitro* established salt tolerant cell lines. The NaCl-tolerant cell line that was selected show a good cell proliferation in spite of decreased growth rate (Queirós *et al*., 2009). Here we report the challenges as well as the progress achieved with 2DE based protocols on the study of proteins of such potato cell line growing in a highly saline environment. Briefly, we tested 2 different extraction protocols, several solubilisation solutions and desalting methods. For the several protein fractions considered we consistently detect more than 500 spots (Progenesis Same Spots, Nonlinear dynamics). To find out about differently expressed proteins we applied both parametric and non parametric statistical tests, here presenting the similarities and differences found. The complete characterization of the potato cell line proteome by mass spectrometry is currently being undertaken, comprising both the constitutive proteins as well as the differentially expressed ones.

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Stilbene synthase genes in transgenic pea and their evolution

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A set of stilbene synthase genes were identified in plants. These genes are related to a family referred as the phytoalexins, which have critical functions in the protection of plants to many stresses including biotic-stresses, such as fungi, bacteria and viruses. Fungal diseases are among major factors limiting plant production worldwide. For instance, in pea, losses related with fungi can reach up to 75%. Although many efforts have been made to breed resistant cultivars, traditional approaches have not overcome the problem due to limited resources and possibilities of manipulations. Stilbene synthase is frequently used to modify plant secondary metabolism with the aim of producing the self-defence phytoalexin resveratrol. Transgenic pea expressing stilbene synthase from *Vitis vinifera* (*Vst1*) is a good alternative to traditional methods providing stable expression and lack of undesirable effects. We developed such pea, but expression of the *Vst1* transgene in greenhouse experiments was variable between individuals and generations as well as other changes at genome and phenotype level were observed. On the other hand, disease resistance is often encoded by major plus minor genes, thus expressing quantitative inheritance. Moreover, each plant has a repertoire of evolutionary related resistance genes and the recombination between a transgene and host homologues can influence heterologous expression. The aim of the research was to identify homologues of *Vst1* gene in transgenic pea, compare them with the transgene and other sequences deposited in the NCBI database to model molecular evolution.

In total, two products were amplified while primers designed on the ends of stilbene synthase from *Vitis vinifera* (*Vst1*) were used on GM pea template. The first, faster product corresponded to the transgene (*Vst1*) and was not observed in non-transgenic control while the second, slower product corresponded to the pea homologue observed in both GM and non-GM plants. Identified pea homologues shared high similarity with stilbene synthases of *Triticum aestivum* and *Secale cereale.* All stilbene synthases formed a distinct group with 38-74% of similarity with chalcone synthase. Comparison of sequences identified from GM and non-GM pea demonstrated that several point mutations in genes encoding pea homologues of *Vst1* arose during development of transgenic lines. For example, transition of thymine into cytosine was observed in a position 100 of the stilbene synthase homologue in a line with *Vst1* gene inserted. The transversion of adenine into thymine occurred in a position 161 of a line with two transgenes, *Vst1* from *Vitis vinifera* and *pgip* from *Rubus idaeus*. The data demonstrated that transformation can induce point mutations in host homologous sequences and these mutations can drive further evolution of disease resistance.

A partial secretome analysis of embryogenic suspension cultures of *Dactylis glomerata* L.

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Somatic embryogenesis is a unique phenomenon in the plant kingdom. Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages. The transition of somatic cells into cells that are capable of forming an embryo is the most important and the part of somatic embryogenesis least understood. Somatic embryogenesis in cell suspension cultures provides a good model system for investigating early plant development. Conditioned medium of cell suspension cultures harbors a complex array of molecules, mainly derived from the cell walls, which may exert a promotive or inhibitory effect on embryo development. The process of somatic embryogenesis in *D. glomerata* L. suspension cultures marks three phases – single cells divide to form microclusters (phase 1); microclusters develop to form proembryogenic masses (PEMs) (phase 2) and embryos differentiate from PEMs (phase 3). Single cells from a non-embryogenic suspension culture with the same origin divide to form microclusters whose further development is blocked. Proteins from the medium of embryogenic suspension culture during defined stages of somatic embryogenesis were compared with those of non-embryogenic suspension culture during unorganized cell proliferation. The proteins were analyzed using 2D electrophoresis under a pH gradient 3-10 (flat bed IEF) x uniform SDS-PAGE and stained with Colloidal Coomassie. Approximately 40 polypeptides with a wide range of molecular masses (10-120 kDa) and pIs (3.5-9.5) characterized each developmental stage. The differential analysis of phase 1 between the highly embryogenic (E1) and the non-embryogenic (NE1) cell line of *D. glomerata* L. showed the presence of proteins specific for the embryogenic line. Twenty-five spots from E1 were excised, in-gel digested with trypsin and proteins were identified using MALDI-TOF-TOF. Fourteen proteins were identified among them cysteine proteinase C1A, cathepsin B and the cysteine proteinase inhibitor cystatin. The presence of these proteins in the medium is of great interest since their role in plant development is well known and the cysteine proteinases are candidates for apoplastic protein processing. Data about their role and the fine balance between proteinases and their inhibitors during somatic embryogenesis are scarce. Cloning and expression of these proteins are in progress in order to evaluate their structure and function in early plant development.

Acknowledgement
The work was supported by grant BU-B-202/06 from the Bulgarian Ministry of Education and Science and grant 077/09 from Sofia University St. Kl. Ohridski.

Mass Accuracy Precursor Alignment (MAPA) strategy for the comparative analysis of *Chlamydomonas reinhardtii* under different growth conditions

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The Mass Accuracy Precursor Alignment (MAPA) enables peptide identification, analysis und validation with no previous sequence knowledge or dependency to a genomic DB (Höhenwarter et al. 2008). Due to the high mass accuracy of Orbitrap mass spectrometers, it is possible to recognize and differentiate putative tryptic peptides just by their mass. With the ProtMAX algorithm, the identified peptides can be aligned on a matrix, each peptide being ranked to the sum of all related spectra (spectral count). The spectral count can consequently be used as a parameter for peptide (and subsequently also protein) relative quantification. Here, MAPA was used successfully in a comparative study on *Chlamydomonas reinhardtii* under differential growth conditions (photoautotrophic/heteroautotrophic). 356 proteins could be identified by matching all the validated peptides, from which 68 proteins were significantly different between both metabolic steady states. Furthermore, several peptides could be fished out using MAPA, suggesting posttranslational regulation and steady state differentiation. Thus, a clear separation was achieved, leading to the detection of several new regulatory relevant marker proteins that will be presented here.

Proteins differentially expressed in *Lupinus albus* roots due boron deficiency

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Boron (B) deficiency in plants greatly limits growth and development. This fact has been known for a long time, but there is still little information on the processes where this microelement participates. It is considered that B plays some role in the cell wall assembly, due to the capacity it has to form diester bridges between adjacent *cis*-hydroxyl-containing molecules of carbohydrates. However, this observation by itself does not seem to explain all the dramatic effects that result from B deficiency in plants. The root system is the primary nutrient sensing organ in the plant and, so, when mineral imbalance occurs signals are sent to the shoots that modify the whole plant growth and development. Since the root system is greatly affected by B deficiency we have been studying root metabolism under this stress. In the present work we use the two-dimensional gel electrophoresis technique (2-DE) for the analysis of the protein patterns of the *Lupinus albus* roots. After image analysis and statistical treatment we found that 2% of the polypeptides were differently expressed due to B deficiency, while 5% seemed to have appeared *de novo* and 26% to have been suppressed. Despite the fact that we are studying a non model plant, we could identify by MS/MS techniques circa 75% of the polypeptides selected for analysis. These data are currently being interpreted and will be discussed.

Complementary proteomic and cell biology approaches reveal that cytoskeletal protein profilin 2 is involved in the formation of BFA compartments

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The growing importance of vesicular trafficking during plant development and stress responses requires the exploitation of novel experimental approaches. Among them the employment of diverse pharmaceutical drugs specifically inhibiting transport processes was emphasized in several genetic and cell biological studies. Here we have used comprehensive proteomic and cell biology approaches to study the effects of the vesicular transport inhibitor brefeldin A (BFA) on the proteome of Arabidopsis roots.

Seven days old Arabidopsis (Col 0) seedlings were treated with 50 µM BFA for 2 hours. After it, the root proteome was analysed using gel based (two-dimensional electrophoresis on 7cm long IPG strips followed by MALDI-TOF MS) and gel free (label free 2-D LC linked on-line to LCQ (THERMO) electro spray ion trap mass spectrometer as described by Nanduri et al., 2005; and Bridges et al. ,2007) proteomic approaches.

Our results revealed that the expression of several important proteins associated with vesicular trafficking (such as reversibly glycolysated polypeptide1, vacuolar ATP synthase catalytic subunit B), protein folding (such as luminal binding proteins and chaperonins), and calcium signalling (calmodulins) was significantly altered by BFA. Except for these proteins, BFA induced also some complex physiological responses in Arabidopsis roots including alleviation of protein synthesis, downregulation of pathogenesis related proteins and simultaneous upregulation of proteins associated with abiotic stress.

Importantly, actin binding protein profiling 2 (PRF2) was shown to be upregulated after BFA treatment in gel free proteomic approach. Further investigation approved the prevalent abundance of PRF2 in BFA treated samples as shown by immunoblot analysis with profilin antibody. *In vivo* imaging using *35S::GFP:PRF2* construct (Wang et al. 2009) showed that instead of reticulate pattern observed in control cells, GFP-PRF2 strongly accumulated in BFA-induced compartments in Arabidopsis epidermal root cells. Since profilins are known to regulate polymerization rates and dynamics of the actin cytoskeleton, we have examined the status of the actin cytoskeleton in stably transformed Arabidopsis root cells carrying actin *in vivo* reporter construct *35S::GFP:FABD2* (Voigt et al. 2005) before and after BFA treatment. These experiments revealed that filamentous actin (F-actin) was less defined

while general cytoplasmic background increased in BFA-treated cells. Interestingly, remaining actin was partially accumulated around BFA-induced compartments which were visualized by vital stain FM4-64. Next, time-laps imaging provided evidence that these BFA-compartments moved along remaining F-actin tracks.

Acknowledgement
This work was supported by a post-doctoral fellowship from the DAAD (Programme „Modern Applications of Biotechnology“) to T.T. and by DFG grants Nr. SA 1564/1-2 and SA 1564/2-3 to J.Š.

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Quantitative analysis of proteome change after saturation of vernalization requirement in winter wheat Mironovskaya 808

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The winter cereals such as wheat must experience a prolonged exposure to cold before flowering occurs. The process, called vernalization, develops for weeks and months during winter. The transition from vegetative phase into reproductive phase of the plant individual development is generally associated with a decline in plant potential to resist the impacts of cold and frost. In addition to cold, the developmental transition into the reproductive phase is regulated photoperiodically in some plants. The aim of our research is to bring a new insight into plant vernalization process using quantitative proteomics method two-dimensional difference gel electrophoresis (2D-DIGE).

Crowns of winter wheat cultivar Mironovskaya 808 were used to study the quantitative changes in crown proteome during long-term cold treatment (0, 21, 84 days at 6 ºC; three biological replicates). The crowns of wheat plants were used in this study because shoot apex is an organic part of this organ which is basically formed by non-photosynthetic tissues with a low level of RubisCO.

In total, 15 % of 2013 quantified spots were found to be the spots of interest (i.e., differentially expressed polypeptides up- and down regulated; absolute abundance variation of at least 1.5-fold, *p*, 0.05). All spots of interest were analyzed by MALDI-TOF/TOF and identified as proteins by searching against the *Viridiplantae* protein database or wheat EST database using a MASCOT server. In total, 22 % of the identified spots of interest belong to known stress-associated proteins as HSPs, dehydrins, cold shock protein domain, ascorbate peroxidase, etc. In comparison with 21-day cold-acclimated plants, the most of the proteins showed decrease of accumulation in crowns after 84 days of cold acclimation. However, other proteins (e.g., chopper chaperone) showed increase of accumulation after 84 days compared with accumulation after 21 days of cold acclimation. Moreover, we were able to distinguish by PCA analysis not only non-acclimated plants vs. cold acclimated ones, but also the partially vernalized plants (21 days at 6 ºC) plants from the plants with saturated vernalization requirement (84 days at ºC).

Mapping of the *Arabidopsis* N-linked glycoproteome

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N-glycosylation is one of the most important post translational modifications in eukaryotes. In mammals N-glycosylation of proteins and final structure of the N-glycan has been proven to be critical for survival; in plants the role of N-glycosylation is less clear. Only for a limited number of plant proteins the N-glycosylation has been experimentally confirmed and in most cases the site of N-glycosylation has not been mapped. First, a protocol for high throughput isolation and identification of glycoproteins from plants has been developed. A selective affinity capture procedure with hydrazide resin beads was used for isolating glycoproteins from leaves of *Arabidopsis* WT and the *cgl* mutant (which lacks the activity to modify high mannose glycans to complex glycans). After tryptic digestion of the protein extracts the glycopeptides were oxidized and subsequently bound to hydrazide. The bound glycopeptides were released by either PNGaseF, which is only able to cleave the high mannose type not complex type of N-glycans from peptides, or PNGaseA which can release both types of glycans from peptides. Analysis of the resulting peptides by LC-MS, using two different approaches for sequence analysis, i.e. DDA and MS^E, resulted in the identification of >300 glycoproteins with a confirmed N to D signature resulting from PNGase treatment.

The obtained results can give insight into the effect of certain mutations in enzymes of the glycosylation pathway on the glycoproteome in plants.

A proteomic analysis of long-term continuous stress response in roots of germinating soybean seeds

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Germination is a complex process, highly dependent on various factors, including temperature, water content and others. Germinating seeds are especially sensitive to unfavorable environmental conditions and their exposure to severe stress may result in considerably diminished of the yield and - most importantly – restrained germination. Soybean is a crop of multiple usage and hence unraveling mechanisms regulating its growth and development under stress conditions seems crucial for its future. Proteomic analysis of protein profiles during germination in optimal conditions and under long-term stress should bring important information regarding stress response in roots of germinating soybean seeds. The aim of our study was to compare expression profiles of a number of proteins showing differences in abundance between control samples and samples treated with cold stress, osmotic stress and cold stress combined with osmotic stress. Roots isolated from seeds germinating in optimal conditions and stress conditions were subjected to quantitative proteomics investigation based on two-dimensional electrophoresis and LC/ESI mass spectrometry combined with ion-trap system for protein identification. In total, 1272 proteins were quantified on the gels. Applying p-value ≤ 0.01, 923 proteins were found to be differentially expressed under long-term cold stress, 956 proteins under long-term osmotic stress and 869 proteins under long-term cold stress combined with osmotic stress. Of all differentially expressed proteins, 59 proteins present in all compared gels were excised from the 2-D gels and identified by MS. Among the identified proteins, 9 were involved in plant defense, 8 proteins were classified as responsible for protein destination and 10 proteins were involved in various tracks of carbohydrate metabolism. Furthermore, we identified 2 proteins involved in energy/electron transport, 4 proteins thought to be regulating various metabolic pathways, 2 proteins participating in secondary metabolism, 3 proteins taking part in process of protein synthesis, 14 proteins linked to development and embryogenesis, one protein taking part in signal transduction, 2 proteins related to cell transport, 2 storage proteins and 2 proteins belonging to the family of trypsin inhibitors. Analyzing differences in expression of the identified proteins, it was possible to determine soybean response to long-term stress as well as to distinguish similarities and differences between soybean roots response to cold and osmotic stress conditions.

Targeted and non-targeted quantitative drought stress proteomics of *Medicago truncatula* root nodules

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A primary goal of modern plant biology is to understand the complete assortment of plant biological processes in enough detail to permit accurate, quantitative predictions about the functions of these biological systems from the level of the gene-to-gene networks to metabolism to physiology and ultimately growth and development. Thus, one promise of systems biology is that it will greatly enhance our understanding of individual and collective functions and thereby provide a more holistic view of plant stress responses. It is clear that integrated analysis will be necessary to maximize understanding of metabolic networks and their regulation under optimal steady state conditions and most certainly during stress responds. Here, a targeted and non-targeted approach for the quantitative proteome analysis will be demonstrated. It enables rapid and undirected screening for pattern recognition and absolute quantification for stoichometric steady state analyses.

Evolution of peroxidases in selected plant species

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Notwithstanding peroxidases belong to important enzymes catalyzing red-ox reactions, their main activity is connected with defend reaction especially by reducing active form of oxygen. The enzymes are common in all plants thus providing a powerful marker system to understanding the evolution of plants. The peroxidises
have been used to identify cryptic species, in population genetic studies as well as the different Isoenzymes were mapped in barley, wheat and ryegrasses. We proved that peroxidases of *Lolium perenne* and *L. multiflorum* are mapped in close proximity to DNA markers generated by primers designed on bacterial catalase-peroxidase gene, *KatG*. This entailed the probability that some forms of plant peroxidases evolved from bacterial sequences and still share some similarity. The studies were aimed at elucidation of evolutionary relationships of peroxidases-like sequences from several selected plant species. Primers designed on *KatG* gene of *M. tuberculosis* of were used to amplify genomic DNA of 12 plant species representing both dicots and monocots as well as conifers. The reproducible amplification products were observed in seven species including *Pinus sylvestris*, *P. mugo*. *Arabidopsis thaliana, Rubus idaeus, Vitis vinifera, Medicago sativa* and *Lolium multiflorum*. Alignment of these sequences confirmed the closest relationships between two *Pinus* species and different structure of peroxidases-like sequences in the Angiosperms. The biggest differences were observed between *A. thaliana* and *V. vinifera*. Surprisingly the sequence of *L. multiflorum* was more similar to those from *Pinus* species than other Angiosperms. Although the highest similarity was observed between *P. sylvestris* and *P. mugo,* differences in as many as 10 amino acid residues confirmed our earlier data that both species are not so closely related as originally thought. Alignment of identified sequences with *KatG* gene from *M. tuberculosis* showed relatively low similarity except of primer binding sites. However, searches of NCBI data base demonstrated the similarity of all identified sequences with plant peroxidises.

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