Plant Proteomics in Europe. COST Action FA 0603. Abstracts of the II Meeting

Jesús V. Jorrín Novo. Responsible of the Working Group 2 and Local Organizer of the Meeting.

The II "Plant Proteomics in Europe" (COST Action FA 0603; http://www.costfa0603.com/index.php) Meeting (Working Group 2) is taking place at the University of Córdoba (Córdoba, Spain; www.uco.es), "Campus de Rabanales, from 6 to 8, February 2008.

As the local organizer, I wish to make known and acknowledge those public and private organizations, as well as individual people who have helped me with the organization of the event. First of all, the University of Córdoba, and more concretely, Prof. Enrique Aguilar Benítez de Lugo, "Vicerrector" of Research and New Technologies. My University has provided me with all the facilities I needed and requested plus financial support. Thanks are also due to FUNDECOR (Álvaro, Cristina, María Victoria) for being in charge of the Technical Secretariat; to Applied Biosystems and Bio-Rad for financial support; to Jenny Renaut (head of the Action) and Lutz Eichacker, with whose experience everything has been much easier; to colleagues and friends of the Scientific Committee for their comments, suggestions and chair duties; to people from my group, and members of the Local Organizing Committee; to our guest speakers, Setsuko Komatsu and Cristof Lenz; and, finally, to all the participants, whether they be reimbursed or not. I hope we shall have a successful meeting and, even more important, have the opportunity of hosting you again in the near future here in Córdoba, as a logical consequence of your stay here, that we intend to make as pleasant as possible.

Next, I shall summarize some data. The number of participants will be 103; out of those, 36 will be reimbursed. They come from 19 countries (only Norway and UK being omitted): Belgium, Bulgaria, Czech Republic, Denmark, France, Finland, Germany, Greece, Ireland, Italy, Luxembourg, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland, The Netherlands, There have been 74 contributions, with 23 presented as oral communications, and the rest as posters. Believe me, the selection of the oral communications is not necessarily related to the scientific quality of the work, and I hope no one be disappointed in this respect. First, it has been a choice of the authors and finally (we did get more than the 20 planned) they have been selected by the Scientific Committee. All the contributions have been organized in twelve sections according to the topic covered: model systems, plant growth and development, abiotic stress, biotic stress, symbiosis, food, genotyping, plant breeding, transgenic crops, allergens, forest trees, miscellaneous (methodology and abstracts received at the last-minute). All the contributions will be formally published in issue no. 1 of "PROTEÓMICA" (Journal of the Spanish Proteomics Society, ISSN 1888-0096).

While organizing this meeting I have borne in mind that we should try to stick to what is stated in the COST Action proposal and, more concretely, to the Working Group 2 objectives (http://www.costfa0603.com/working-groups.php)

With respect to the program, I have taken into account the excellent organization of the previous Munich meeting, and, if possible, hope to improve it, mainly based on the scientific committee's suggestions. All of us agree that the Action is just a beginning and because of that we should start taking some steps forward. For instance, although the meeting starts on Thursday 7 February, it has occurred to me to organize one more event for the 6th, a discussion session to work on the idea of a future Research proposal on Plant Proteomics to be presented to the EU.

We consider the poster sessions as important as the oral ones, and in view of this I have planned four poster sessions plus a final poster discussion session. The two lectures by Dr. Komatsu and Dr. Lenz will be given on the 7th.

The scientific program is accompanied by a social program including two dinners, for those coming on the 6th and leaving on the 8th, and a guided visit to the famous Jewish suburb. I would like to provide more visits but unfortunately there is little time and less money.

Thank you, and have a nice stay in Cordoba

LECTURES

Update and challenges on soybean and rice proteomics

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The advent of proteomics has made it possible to identify a broad spectrum of proteins in living systems. This capability is especially useful for crops as it may give clues not only about nutritional value, but also about yield, and how these factors are affected by adverse conditions. Rice is not only an important agricultural resource but also a model plant for biological research. Once the rice genome was completely sequenced, the challenge ahead for the monocot plant research community is to identify and regulate their function. Although research on rice

provides insight into many fundamental aspects of plant biology, the genome sequencing of major crops is in its infancy. Our previous research highlighted different aspects of the construction of rice proteome database, cataloguing rice proteins of different tissues and organelle, differential proteomics using electrophoresis and functional characterization of some of the proteins identified. In the recent research, challenges regarding different methodological approaches and techniques for soybean proteomics are considered along with the usefulness of bioinformatics for database and cluster analysis in the field of proteomics. The recent progress in crop proteomics and highlight the achievements made in understanding the proteomes of major crops, which are rice and soybean, is described. The major emphasis will be on crop responses to abiotic stresses. Rigorous genetic testing of the role of possibly important proteins can be conducted. The increasing ease with which the DNA, mRNA and protein level can be connected suggests that proteomics data will not be difficult to apply to practical crop breeding.

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ProteinPilot: accomodating genetic diversity in mass spectrometry-based plant proteome research

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The identification of proteins from organisms with poorly characterized genomes by LC/MS/MS analysis is challenging. Sequence databases from closely related species can be interrogated by *de novo* sequencing/ BLAST similarity searching, so far the only means to account for inherent genetic variability. BLAST results, however, are difficult to score and require a high amount of manual validation.

Recent advances in database searching algorithms now allow for genetic variability during first-pass searches. The novel Paragon algorithm embedded in the ProteinPilot software uses the concepts of Sequence Temperature Values (STVs) and Feature Probabilities to account for a large number of sequence modifications in first-pass protein identification experiments (Shilov et al., 2007). In this context the expected set of amino acid substitutions effected e.g. by Single Nucleotide Polymorphisms (SNPs) can be treated as a large set of possible amino acid modifications, without exploding the number of possible sequence hypotheses that need to be scored. As a consequence, the large search space associated with amino acid substitutions can be examined using moderate computing resources, with a robust scoring.

The approach has been tested using a set of validated LC/MS/MS protein identifications from 2D gel spots of *Quercus ilex*, the dominant tree species in Mediterranean forest. The results have been compared to analysis by *de novo* sequencing/BLAST similarity searching.

References

Shilov IV et al.. The Paragon Algorithm: A Next Generation Search Engine that Uses Sequence Temperature Values and Feature Probabilities to Identify Peptides from Tandem Mass Spectra. Mol Cell Proteomics 2007 May 29;6:e-publication ahead of print.

I

MODEL SYSTEMS ORAL COMMUNICATIONS

1. A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis* thaliana

De Jaeger, G.[1], Van Leene, J.[1], Stals, H.[1], Buffel, Y.[1], Eeckhout, D.[1], Neirynck, S.[1], Persiau, G.[1], Van Isterdael, G.[1], Van De Slijke, E.[1], Pharazyn, A.[2], Van Damme, S.[2], Witters, E.[2], Van Onckelen, H.[2], and Inzé, D.[1]

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Defining protein complexes is critical to virtually all aspects of cell biology, because many cellular processes are regulated by stable protein complexes and their identification often provides insights into their function. We developed a high-throughput tandem affinity purification/ mass spectrometry platform for cell suspension cultures to analyze cell cycle-related protein complexes in *Arabidopsis thaliana*[1]. Elucidation of this protein-protein interaction network is essential to fully understand the functional differences between the highly redundant cyclin/cyclin-dependent kinase modules, which are generally accepted to play a central role in cell cycle control, in all eukaryotes. Cell suspension cultures were chosen because they provide an unlimited supply of protein extracts of actively dividing and undifferentiated cells, which is crucial for a systematic

study of the cell cycle interactome in the absence of plant development. To map the cell cycle protein interaction network, we use an integrated approach comprising generic Gateway-based vectors with high cloning flexibility, the fast generation of transgenic suspension cultures, tandem affinity purification adapted for plant cells, matrix-assisted laser desorption ionization tandem mass spectrometry and data analysis. So far, we identified protein interactions for 100 proteins, known to be involved in the plant cell cycle. This systemic approach provides new insights into the basic cell cycle control mechanisms and is generally applicable to other pathways in plants.

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2. Proteomics of *Lotus japonicus* seeds and pod walls

Nautrup-Pedersen, G.[1], Dam, S.[1], Laursen, B.S.[1], Siegumfeldt, A.L.[1], Ørnfelt, J.H.[1], Lorentzen, A.M.[2], Roepstorff, P.[1], Stougaard, J.[1]

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The protein rich seeds and pod walls of legumes constitute a major part of human and animal diets and provide important nutrients especially in some developing countries.

Studies on seeds from legumes have mainly been done in bean or pea. However, our group is focusing on the smaller plant *Lotus japonicus*. *L. japonicus* is a suitable model plant as the genome is small and gene rich regions of the genome are sequenced. Seed development in legumes proceeds through three phases: 1) cell division/prestorage, 2) maturation/seed filling, and 3) desiccation.

In our study the seed development in *L. japonicus* is analysed from early after flowering until mature seeds can be collected. During this time course, which should cover the three developmental phases, 14 different developmental stages were harvested.

Thin sections of the seeds were made from all 14 developmental stages. Staining with toluidine blue showed the development of seed coat, embryo, and endosperm. From each of these 14 stages, proteins were extracted from seeds and from pod walls. Extracted proteins were separated by 1D SDS-PAGE. Based on these results five stages representative of the above mentioned three developmental phases were chosen for detailed analysis by 2D gel electrophoresis. Isoelectric focusing was done in the pH intervals 4-7 and 6-11. Protein spots were isolated and identified by MALDI-TOF-TOF. So far about 1100 protein spots are identified from the seeds, and about 200 spots from the pod walls.

The work in progress focus on identification of additional spots from the pod walls followed by a comparison between the proteome of the seeds and the pod walls. The results should provide information about timing and pathway regulation during seed development, and about the transfer of nutrients through the pod wall to the seeds.

I

MODEL SYSTEMS POSTERS

3. Analysis of the Arabidopsis thaliana SUMO interactome by Yeast Two-hybrid technology

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SUMO (small ubiquitin-related modifier) is a modifier polypeptide chain reversibly attached to a lysine residue of a broad spectrum of target proteins. SUMO conjugation has emerged as a central regulatory mechanism of diverse pathways such as cell-cycle regulation, DNA repair and replication, cell signalling and plant/pathogen interactions. SUMO proteins are highly conserved proteins and consist of 8 components (SUMO 1-8) in Arabidopsis thaliana.

To get insights into the functional diversity of proteins interacting with SUMO1 in Arabidopsis thaliana leaves, a Yeast Two-hybrid strategy was adopted. In these experiments, SUMO1 isoform was fused to the DNA binding domain of Gal4 yeast transcription factor and used as bait. As a prey, a cDNA library from leaves was constructed and fused to the activating domain of Gal4. To discriminate between SUMO conjugation to target proteins and protein-protein interactions with SUMO1, positive clones will be probed with SUMO1 isoform lacking the C-terminal Gly-Gly.

We anticipate that results from the yeast two hybrid screen together with previously identified SUMO conjugates or partners in leaves may provide the basis for the development of a SUMO interactome map.

4. AGRON-OMICS (Arabidopsis GROwth Network integrating OMICS technologies)

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Plant growth and development are important processes but their biochemical and molecular regulation remain poorly understood. In the European project AGRON-OMICS (Arabidopsis GROwth Network integrating OMICS technologies) a consortium of laboratories are taking a systems approach to understand complex developmental processes that are directed by spatial and temporal changes at the transcriptome, proteome and metabolome levels. As the main goal, this project will identify the molecular components and networks that drive the concerted cell cycle and growth of cells in the developing Arabidopsis leaf. This approach will provide new insights how components interact and coordinate their activities across different levels of organisation. The data obtained using different technological platforms will be integrated and analysed to create a package of integrated information together with systems biology applications and web services for modelling leaf growth.

In the frame of this project, we will provide comprehensive analysis of the Arabidopsis leaf proteome during the circadian cycle and at defined stages of plant development. We are investigating changes at the protein expression level as well as the posttranslational modifications (focusing on phosphorylation and glycosylation) that modulate protein activity and stability.

We expect that our deep proteome analysis at spatial resolution will identify new proteins and provide information on the potential function of unknown protein.

5. Evaluation of alternative tandem affinity purification tags in *Arabidopsis thaliana* cell suspension cultures

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6. *Arabidopsis* phosphoprotein profiling in response to 2-deoxyglucose

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2-Deoxyglucose (2DOG) is a non-metabolisable glucose analogue and works as a substrate for hexose transporters and hexokinase. The uptake of 2DOG is followed by a depletion of phosphorylated intermediates, changes ATP level and inhibits photosynthesis. Here we provide the first results of externally induced protein phosphorylation changes in *Arabidopsis*. In this study, total proteins were extracted from *A. thaliana* cell culture 6 and 24 hours after treatment with 2DOG. The proteins were separated by 2D gel electrophoresis and phosphoproteins were visualized with Pro-Q diamond phospho-stain. Subsequently all gels were stained with Coomassie Brilliant Blue (CBB) in attempt to directly correlate phosphoprotein with total protein expression. Reproductive protein expression profiles and nearly 300 phosphoprotein spots were resolved on 2D gels over a pH range of 3 to 11. More than 90 protein spots showed large and reproducible changes. In the presents of 2DOG they were either up- or down regulated. Subsequently, all of these differentially phosphorylated proteins were excised and subjected to in gel digestion and LC/MS. Functional category analysis showed that these phosphoproteins were grouped into energy metabolism; signalling; cell wall remodelling; cytoskeleton dynamics; protein assembly; transcriptional, translational regulation and stress response. Among the phosphopeptides found to be differentially phosphorylated upon 2DOG treatment, 20 corresponded to 19 phosphoproteins identified from 2-D gels, the fraction of energy metabolism related proteins (35%) are the biggest category in all identified phosphopeptides. These findings may provide important information to further investigate the function of phosphoproteins involved in mediating multiple signalling and energy metabolism.

II

PLANT GROWTH AND DEVELOPMENT ORAL COMMUNICATIONS

7. Using proteomics to dissect germination and seed vigor in sugar beet

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We have used proteomics to characterize germination and early seedling vigor in sugar beet. Our strategy includes (1) construction of proteome reference maps for dry and germinating seeds; and (2) investigation of the specific tissue accumulation of proteins (root, cotyledon, perisperm).

More than 1,100 sugarbeet seed proteins have been identified by LC/MS-MS mass spectrometry (albumins, globulins and glutelins have been analyzed separately). Due to the conservation of protein sequences and the quality of MS sequencing (more than 5000 peptide se-

quences have been obtained), the success rate of protein identification was on the average of 80%. This is to our knowledge the best detailed proteome analysis ever carried out in seeds. The data allowed us to build a detailed metabolic chart of the sugarbeet seed, generating novel insights into the molecular mechanisms determining the success of germination and the development of a new seedling. Also, the proteome of a seed-storage tissue as the perisperm is described for the first time. During the presentation, the data obtained with sugar beet seeds will be compared to our previous work on Arabidopsis (Gallardo et al., 2001; Rajjou et al., 2004, 2006; Job et al., 2005; Chibani et al., 2006; Holdsworth et al., 2007).

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8. Bud proteome analysis in peach (*Prunus persica* (L.) Batsch) during vegetative and floral development

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Bud formation is an important developmental process for survival in perennial trees during the winter. Peach trees (*Prunus persica*) maintain vegetative and floral primordia in separate buds. Bud break for both types of buds occurs in spring once chilling requirements have been fulfilled. Two major factors affecting peach flowering time are variety and location. Identification of genes involved in flowering time in peach can improve breeding for more compact flowering season and region specific bud break.

We have collected samples of the 'Evert' variety from two locations in Greece with different bud break periods. Vegetative and floral buds were harvested separately. Samples were analyzed by two-dimensional (2D) polyacrylamide gel electrophoresis. Comparison of vegetative and floral bud protein profiles revealed high degree of colinearity between the two bud types. Protein profiles buds from two different regions also showed high degree of colinearity. Protein spots showing differential expression as well as spots showing co-expression, between bud-type and/or location were selected and analyzed for sequence determination by MALDI-TOF mass spectrometry. High degree of co-expression between treatments indicates presence of genes involved in re-activation of the buds and bud break. Proteins showing differential expression between bud types indicate developmental specificity, while differential expression between locations may indicate advancement towards bud break. In contrast to the model species *Arabidopsis thaliana* and *Antirrhinum spp.*, in which both floral and vegetative tissues differentiate on the same meristem, peach presents a model for perennial tree species that vegetative and floral meristems develop in discrete buds. Proteomics empowered analysis allowed a more direct comparison of the processes occurring between floral and vegetative buds.

II

PLANT GROWTH AND DEVELOPMENT POSTERS

9. Phospho proteome analyses provide new insights into light induced signal transduction in rice (*Oryza sativa L.*)

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The etioplast is a non-photosynthetic plastid type that develops in leaf tissues in the absence of light. Illumination of etiolated tissues results in the rapid re-differentiation of etioplasts into chloroplasts, which is accompanied by the reorganization of a heterotrophic into an autotrophic metabolism. This fundamental reorganization of the plastid metabolism has an impact on the entire plant. In initial experiments, we have characterized the effect of light on the etioplast proteome and reported a comprehensive quantitative proteome analysis that also comprised an analysis of posttranslational modifications (Kleffmann et al. 2007). We could demonstrate that phosphorylation is involved in the early light adaptation of the organelle and expanded our analysis now to the entire plant cell. This is an important expansion of the organelle-centric analysis because the plastid is embedded in a cellular signaling system and

receives and emits signals in order to coordinate metabolic activities. We analyzed the phosphoproteome of illuminated versus non-illuminated rice seedlings and identified 1007 phosphorylated proteins from dark-grown seedlings and 737 proteins from light-grown seedlings. The identified phospho proteins are distributed among different organelles, functional categories and metabolic processes. A comprehensive analysis of phosphorylation sites confirmed the activity of different kinases and suggested the light-induced activation of a casein-kinase like enzyme. The identified phosphorylation sites are the basis for the design of targeted, kinome profiling experiments for the identification and characterization of the kinases involved in light-driven signaling events.

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10. Translationally silent large RNP complexes in tobacco male gametophyte are associated with cytoskeleton and contain ribosomal subunits

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The haploid male gametophytes of higher plants play a vital role in plant life cycle, plant fertility and crop production. Pollen ontogeny also provides an attractive model of cellular development, in which to dissect the fundamental processes of cell growth and division, cellular differentiation and intercellular communication. In addition, tobacco male gametophyte represents a natural model for the study of translational regulation of gene expression in plants. Pollen specific gene *ntp303* and its encoded 69 kDa glycoprotein, major component of pollen tube cell wall, are excellent markers for this study. ntp303 mRNA is intensively synthesized during pollen maturation and stored in the form of EDTA/puromycin-resistant ribonucleo-protein particles (EPPs).

Here we report the first structural data on translationally inactive large ribonucleo-protein complexes in tobacco male gametophyte. These EPPs are formed in immature pollen and contain translationally silent mRNAs stored for later use during the progamic phase of male gametophyte development. EPP complexes were found to be present in growing pollen tubes for over 24 hours. Although massively activated at early phases of pollen germination, they also serve as a long-term storage transported along with the translational machinery to the tip region. Moreover, EPP complexes were shown to contain both ribosomal subunits; such pre-formation and temporal inactivation of the whole translational apparatus fits well with the demand of rapid activation of various reserves at the onset of progamic phase as well as long-term storage. To our best knowledge, it is also the first report of stored mRNP complexes containing pre-formed ribosomes in plants.

Acknowledgments

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11. Proteome alterations during grapevine berry development and under water stress conditions

Francisco, R.[1], Zarrouk, O.[1], Santos, R.R.[1], Ortuño, M.F.[1], Costa, M.[1], Santos, T.[1], Rodrigues, A.P.[2], Jenöe, P[3], Ricardo, C.P.[1,2], Chaves, M.M.[1,2]

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Mild water deficit imposed by deficit irrigation strategies is being used to grow grapevine plants with water saving. Besides, it is believed that by avoiding extreme water deficit conditions productivity is only slightly affected and quality could even be improved. Regulated-deficit irrigation (RDI) has been one of those strategies. RDI consists on the reduction or removal of irrigation during specific periods of the grape berry development. At present, the precise effects on grape berry quality of this practice are still largely unknown. The specific purpose of the present study is to characterise the grape berry skin proteome and its alterations during development and under different water supply regimes. For that, total protein skin extracts from berries of field grown grapevines of the variety Aragonez were analysed by two-dimensional gel electrophoresis (2-DE). From the analysis of the different protein maps obtained for berries at distinct developmental stages and from stressed

and control plants (fully irrigated) several quantitative/qualitative alterations of protein expression patterns were observed. The main alterations will be presented. Some of the polypeptide spots that significantly changed as a result of the experimental conditions were excised from the gels and identified by mass spectrometry. These proteins are mainly involved in environmental information processing, transcription or plant defence responses.

12. Tomato Chromoplast Proteome: challenges and perspectives

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In our work we focus on non-photosynthtic plastids (i.e Chromoplasts) which are part of the compartimentalised genetic machinery of the plant cell and originated from endosymbiosis. We report about different proteomics technology to characterize the functional genome of the organelle. Both, chromoplasts and protein extraction protocols were set up. It has been necessary to adapt a method used to extract tomato proteins, which involves a phenol-based phase separation, introducing a preliminary step during which the homogenized sample is washed with cold acetone in order to remove phenolic interfering compounds. A proteome study based on differential gel electrophoresis (2D-DIGE) was performed in order to analyze the differences between chromoplasts of transgenic and wild-type tomato fruits. The aim of this work was to study the ripening-associated proteome changes of tomato chromoplasts from two different transgenic lines (one overexpressing DXS and the other PSY), with the emphasis to monitor the overall changes in the protein pattern to identify differential proteins specifically related with carotenoids accumulation. The analysis was performed with DIGE technology with the inclusion of an internal standard. 2D-DIGE takes advantage of differential fluorescence protein labelling and sample multiplexing to improve accuracy and reliability of comparative analyses. Proteins were separated using 2-DE, using a non-linear pH 3-11 electrofocusing gradient in the first dimension and 12.5% polyacrylamide gels in the second dimension. Four replicate for each sample were obtained. DeCyder software (GE Healthcare) was used to distinguish clear statistical differences in protein expression. On the other side, since membrane proteins are under represented in 2-D gels due to their high hydrophobicity, BN-PAGE was used instead. BN-PAGE is a special case of native electrophoresis for high resolution separation of enzymatically active protein complexes from tissue homogenates and cell fractions. The separation principle relies on binding of Coomassie blue G250 which provides negative charges to the surface of the protein. BN-PAGE was performed as described by Reisinger et al. (2006). Spots for identification were excised from 2D-DIGE gels and BN-PAGE gels, tryptically digested and analysed by MALDI-TOF/TOF MS or nanoLC/ESI-Q-TOF MS. Acquired spectra were then searched with Mascot (Matrix Science, UK) against the NCBI database and blasted against the Tomato EST database. The characterization of some of these 2D-DIGE spots by nanoLC/ESI-Q-TOF MS analysis allowed the identification of proteins involved in the physiological processes such as stress, defence, carbon metabolism and energy conversion, such as different HSP, NADH-ubiquinone oxidoreductase, SOD and ripening related proteins. The identified proteins were involved in response to biotic or abiotic stresses, in cellular and in secondary metabolism. In particular, as far as it concerns the relative abundance, the dominant proteins were involved in defence mechanism and ripening. On the other side, the BN-SDS PAGE technique has been proven to be a good method to resolve highly hydrophobic integral membrane proteins from chromoplast preparations, but also challenging for this kind of sample. These membrane protein complexes are just now being analyzed by nanoLC/ESI-Q-TOF.

III

ABIOTIC STRESS ORAL COMMUNICATIONS

13. Twenty one years since Chernobyl disaster: What seed protein can tell us?

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The explosion of one of the four reactors of Chernobyl nuclear power plant (CNPP) on 26 April 1986 caused the worst environmental nuclear disaster in the history. A total amount of about 1018Bq radioactivity was released not only to the close surroundings of the power plant but also to large parts of Europe. In the present time, the Chernobyl contaminated area represents a unique area for radioecological and radiobiological research difficult to perform elsewhere. Despite the fact that since 1986 radiation levels in the affected environment have declined several hundred folds, dangerous long-living isotopes

such as ^{137}Cs and ^{90}Sr remains as main contaminants. Now, 21 years after the accident, the question how plants in contaminated Chernobyl were able to adapt is still open, and needs to be fully answered. Plants are stationary and thus must adapt to extreme conditions in order to survive. The main objective of our research is to characterize quantitative differences on protein levels between soybean (*Glycine max*) and flax (*Linum usitatissimum*) grown in contaminated (~5 km from CNPP) and control (~100 km from CNPP) experimental fields in order to elucidate molecular mechanisms plants used for adaptation. Here we will present the first step of our effort; comprehensive proteomics characterization of mature soybean seeds grown in contaminated and control experimental fields. To acquire complex proteome information about expressed proteins in the seeds grown in Chernobyl condition, the total proteins were quantitatively analyzed using two-dimensional gel electrophoresis (2-DE). The proteins that changed in their expression were excised from 2-DE gels and analyzed by liquid chromatography tandem mass spectrometry.

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14. Proteome analysis: a powerful approach to unravel the acclimation towards osmotic stress and to get insight into intercultivar differences

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The Laboratory of Tropical Crop Improvement (K.U. Leuven, Belgium) hosts, under the authority of Bioversity International, the global *in vitro* collection of banana varieties (*Musa* and *Ensete* spp.). The aim of this international gene bank is to conserve all banana and plantain genetic resources safely and to supply the germplasm to any *bona fide* users. K.U. Leuven was one of the pioneers to explore the possibilities of storing germplasm in liquid nitrogen (at -196°C). For successful storage at -196°C, cells need to survive a severe dehydration process prior to freezing. In general, dehydration tolerance is achieved by an osmotic stress acclimation. However, more than half of the collection consists of varieties that show a low survival rate. Hence, there is a need to unravel the mechanisms behind acclimation and to get insight into the genotype specific diversity. Protein separation via two-dimensional gel electrophoresis (2DE) and protein identification via tandem mass spectrometry (MS/MS) is the most informative approach for a poorly characterized organism like banana (Carpentier et al., 2005; Carpentier et al. 2007).

We are interested in intercultivar differences and the effects of 3 different specific acclimation treatments over time. Using the DIGE approach we consider 4 different time points. Experiments show that 4 days of acclimation is significantly correlated to the highest post-thaw survival. A first insight into the complex data set was obtained via principal component analysis. It confirms that the proteome at 4 days is clearly different from the other sample points. Moreover, PCA identifies multiple proteins that are correlated to this sample point and shows the correlations among several proteins. The individual differences between the sample points are validated via confirmatory ANOVA. The exploration of the biodiversity of the banana genome (AA, BB, AAB, ABB, BBB, AAA) showed already evidence for B genome specific proteins.

15. Comparative plant proteome analysis of responses to abiotic stress using LC-based approaches

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Environmental influences such as high light, drought, high or low temperature and salinity affect growth and yield of crop plants by leading to altered gene and protein expression, metabolic changes such as flavonol biosynthesis, and growth retardation. How such environmental stimuli are perceived and trigger the complex defensive and adaptive signalling networks, and how these events result in resistance/tolerance is of major practical interest. We aim to identify regulatory circuits of abiotic stress responses in plants using *Arabidopsis* and barley as model systems. In our presentation we will give examples for quantitative protein profiling of plant material to monitor responses to different abiotic stresses. We will focus on LC-based label-free techniques besides the classical 2-D approach for comparative protein analysis.

A complementary proteome study was started to analyse the response of *Arabidopsis* plants exposed to cold stress (6°C). We first carried out a comparison of protein patterns by using DIGE technology. Taking advantage of recent developments in proteome analysis fractionated samples from these studies were also analysed by a label free quantification method using a nanoLC System combined with ESI-Q-TOF MS/MS (Waters). This novel approach showed quantitative differences in response to cold treatment for an additional number of proteins. Furthermore we have started to investigate fractions of phosphorylated proteins from these experiments using gel-based as well as gel-free separation techniques prior to identification by mass spectrometry. Many of the proteins identified have been described previously in the context of cold stress responses, indicating the validity of our complementary approach for further in depth studies.

Besides we are interested in monitoring changes in protein composition during UV-stress by comparing barley plants, which were either exposed to UV-B-radiation or grown under regular conditions (control plants). Epidermal tissue was chosen for analyses because previous experiments showed that UV-B-absorbing secondary metabolites are mainly accumulated in this tissue type. The quantitative protein profiling was performed using a 2-D approach in first instance. A label free LC-based analysis was applied for complementary investigations. Therefore whole crude extracts were digested and directly analysed by LC-separation combined with ESI-Q-TOF MS. The revealed data confirmed earlier results obtained by using conventional 2-D separation technology. A number of supplementary proteins displaying quantitative differences were identified with the help of this novel approach. Among them are proteins which are hardly resolved using 2-D gel electrophoresis, like low abundant proteins and those with extreme pI-values.

Our results supply evidence that the used methods for protein separation and quantification are complementary rather than compatible for the elucidation of changes in protein patterns during abiotic stress responses. In fact, using LC-based approaches is especially advantageous when investigating regulative components such as proteins related to DNA activation/deactivation (e.g. Histons and transcription factors) or involved in signalling cascades (e.g. Kinases).

III

ABIOTIC STRESS POSTERS

16. Effects of Cd in the xylem sap of tomato (*Lycopersicon esculentum*) plants: a proteomic approach

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Cd toxicity in crops has become in a serious problem nowadays, especially in developed countries. In soils, Cd accumulation may come from different sources, including air pollutants and soil applications of commercial fertilizers, sewage sludge, manure and lime. In these polluted soils, Cd is generally present as free ions or soluble forms, and its mobility depends on the presence of chelating substances and other cations but being overall easily taken up by the roots. Once within the plant root, Cd is mobilized throughout the plant where it can reach edible parts and become a potential hazard for human and animal health. A critical step in Cd mobilization is xylem sap transport, but little information is still available about this process, including the chemical form(s) in which this heavy metal is present in this fluid. The goal of this work was to study changes induced by Cd toxicity in the proteome of xylem sap obtained from tomato plants, in order to elucidate if any proteins are involved in Cd transport and to better understand the physiological changes involved in Cd toxicity.

Tomato plants cv. Tres Cantos were grown in a controlled environment chamber (80% RH, 23ºC-16 h/19ºC-8 h day/night regime) in half-strength Hoagland nutrient solution for two weeks. After this period, plants were transferred to nutrient solution containing 0 μM Cd (control) or 10 μM $CdCl_2$, and grown in these conditions for 10 more days. Xylem sap was obtained by collecting the fluid bled by the plants after stem decapitation. Proteins were precipitated from 10 mL of pooled xylem exudates obtained from 18 plants and resuspended in rehydration buffer (Bio-Rad). First dimension isoelectric focusing was carried out on 7 cm Ready Strip IPG strips (Bio-Rad) with a linear pH gradient from pH 5-8. The second dimension SDS-PAGE was performed in 12% SDS-polyacrylamide gels, at 20 mA per gel for 1.5 h. Gels were subsequently silver stained and analysed with PDQuest 7.1 software (Bio-Rad). In a preliminary study, 13 protein spots of interest were excised from the gels, in-gel digested by trypsin and the mass spectra obtained with a MALDI/TOF-MS apparatus (Bruker Daltonics). The experiment was repeated 3 times.

Two-dimensional separation of xylem sap proteins from plants grown with 0 or 10 μM Cd resolved 204 and 209 spots, respectively. Averaged polypeptide maps analysis indicated that the 10 μM Cd treatment caused increases in signal intensity in 34 spots and decreases in 36 spots, when compared to control plants. Also, 10 and 2 spots were only detected in plants grown with 10 and 0 μM Cd, respectively. The initial batch of spots analyzed included 6 spots exhibiting signal increases and 7 newly detected spots in gels from xylem sap of Cd treated tomatoes. Six spots gave significant matches to known proteins from different species. These proteins include: 3 chitinases: a putative basal resistance related chitinase from *Nicotiana tabacum* (CAI54289), a class II chitinase (AAB96340) from *Solanum tuberosum* and a chitinase (CAA78845) from *Lycopersicon esculentum*, 2 peroxidases: one peroxidase (CAB67121) and a peroxidase precursor (CAA64413) from *Lycopersicon esculentum* and an osmotin 81 (AAP14938) from *Solanum tuberosum*.

17. Comparative proteomics of developing soybean and flax seed tissues in chronic ionizing radiation and control field conditions

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After nuclear weapon trials and several industrial disasters, land pollution with radioactive wastes became a significant common problem. For instance, vicinity of the Chernobyl Power Plant still have considerable radioactivity level due to the presence of long living isotopes, such as ^{137}Cs and ^{90}Sr. Sadly, even 20 years of research since reactor explosion did not bring clear answers on mechanisms of plant abilities to survive, grow and successfully reproduce under permanently increased level of radioactivity. To fill this hole, we started to utilize systematic approach on protein level in order to establish complex view on several plant generations grown under long-term influence of low-level ionizing radiation in the Chernobyl area. The research will elucidate changes in metabolic pathway patterns and/or their developmental/spatial dynamics which allow adaptation to such environment. During 2007 growth season *Glycine max* and *Linum usitatissimum* mature seeds were collected from experimental plots in Chernobyl and “clear” zone. After phenol based protein extraction 2-DE reference maps were created. Following Colloidal Coomassie Blue staining quantitative differences were analyzed by ImageMaster software. Differentially expressed spots were subjected to LC-MS/MS analysis for protein identification.

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18. Studies on resurrection behaviour of *Haberlea rhodopensis*

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A small group of angiosperms, known as resurrection plants, possesses unique ability to survive extreme dehydration of vegetative tissues and to restore quickly viability after rehydration. We are focused on the Bulgarian endemite *Haberlea rhodopensis*, trying to reveal the complex mechanisms behind its dehydration tolerance. Our tasks are:

- etsablishment of efficient in vitro propagation system;
- development of standartized system for desiccation and rehydration;
- complex physiological, biochemical and molecular studies at certain points of drying and recovery.

As plant material we use leaves of in vitro plants dried at controlled open air conditions. Samples for analyses were collected according to various levels of RWC during desiccation and rehydration. The reaction to oxidative stress was followed by measuring H_2O_2, SOD, MDA, phenols and glutathione.

For molecular studies we use cDNA-AFLP as an RNA imaging technique to identify transcripts that are strongly accumulated, induced *de novo* or are switch off in response to drought stress. Differential cDNAs were obtained from silver stained polyacrilamide gels. After re-amplification the subset of TDFs will be sequenced. cDNA-AFLP expression profiles will be verified by Northern blot analysis or RT-PCR.

The results from cDNA-AFLP analysis enable us to discover proteins which are involved in dehydration processes of *Haberlea*. In further experiments overexpression in *E. coli* of some differentially-expressed cDNA clones, production of specific antibodies followed by immunodetection will allow us to study the expression patterns of respective proteins.

Knowledge of RNA transcripts and proteins will highlights signalling networks and translational control during water stress in plants.

19. Protein oxidation and PCD-signaling in wheat (*Triticum aestivum* L.) mitochondria during oxidative stress

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Stress related protein oxidation has been implicated to act in the signaling cascade leading to programmed cell death (PCD) in plant tissues. It is known that carbonyl groups are formed early in oxidative stress and the oxidized proteins act as biomarkers of stress. The carbonyl groups formed are reasonably stable. As mitochondria play an important role both in the production of reactive oxygen species and in signaling the onset of PCD, we have searched for oxidized proteins in the mitochondrial proteome in wheat root tissue. One week old wheat seedlings were grown hydroponically in a nutrient solution and divided into two groups: i. without further treatment and ii. treated with 400 µM menadione for 16 hours to boost the antioxidative protection of the tissues. To choose the appropriate menadione concentration before the experiments with root tissue a range from 50 µM to 4 mM menadione was first tested with wheat protoplasts. Mitochondria where separated from root tissue and purified with Percoll-gradient fractionating centrifugation. After respiratory activity and membrane integrity assays to check the intactness of the purified mitochondria, proteins were extracted and precipitated with acetone. The mitochondrial proteome was analysed by 2-dimensional PAGE, the proteins transferred to PVDF-membranes, stained with silver

and with a primary antibody against carbonylated proteins. Carbonylated proteins were then located with a secondary antibody attached to a peroxidase (HPR) with SuperSignal® West Pico Chemiluminescent assay system. Comparisons with the silver stained membranes and the use of ExPASy protein databank Tagldent programme lead to the identification of two oxidized proteins: COX1_WHEAT, the mitochondrial cytochrome *c* oxidase subunit 1 and NU5M_WHEAT, subunit 5 of NADH-ubiquinone oxidoreductase located in the inner mitochondrial membrane respiratory chain complex 1.

The use of MALDI-TOF facility at the Biotechnical Institute of Helsinki University was tested with this material but did not give satisfactory results as wheat protein information in the databanks is still fragmentary. The data shows that protein oxidation takes place in wheat root mitochondria grown in aerated hydroponic culture conditions and that menadione treatment leads to decreased protein oxidations in wheat root mitochondria. It is probable that near lethal levels of menadione concentrations would lead to increased protein oxidation and hence to enhanced signaling leading to programmed cell death.

20. First steps of a chloroplast proteome study of two *Pisum sativum* L. lines during cold acclimation

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Both theoretical-based models and strong trends in climate change already evident presume global warming consequences supposed to lead to a significant cooling during winter in Europe. A better understanding of plant behaviours at low temperatures is now an inevitable issue, in order to address agricultural adaptation more coherently. This is especially crucial for plants of economical interest such as pea (*Pisum sativum* L.), which is a high quality source of proteins for animal feeding. Cold acclimation (commonly described as a progressive acquisition of freezing tolerance by plants subjected to low, non-freezing temperatures) has been studied in two different pea genotypes 'Terese' and 'Champagne', which are known to differ in their ability to recover after winter (freezing sensitive and freezing tolerant, respectively). We focused our study on their chloroplast proteome because i) it is the first and most severely affected organelle during chilling injury, and ii) because considering cold response at the plastid level relieve us of the usual limitations related to the complex proteomes from tissues (the broad dynamic ranges restrict detection of differentially accumulating proteins under physiological conditions). The pea chloroplast proteome was analysed by using two-dimensional electrophoresis in two out of the six different compartments of this complex organelle: the stroma and the lumen. Different protein precipitation methods, rehydration buffers, pH ranges for IEF and IEF conditions were used in order to perform good quality and reproducibility in separation patterns. Chloroplast proteins from stroma and lumen were resolved into about 450 and 360 spots respectively, in both Champagne and Terese genotypes. Among them, 23 proteins were highlighted as potentially genotype-specific, suggesting their implication in the ability of a pea genotype to differently cope with low temperatures.

21. Plant responses to abiotic stress: proteomic approach to identify LEA (Late Embryogenesis Abundant) proteins from plant seeds.

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Plants have evolved different physiological, biochemical and molecular mechanisms to tolerate environmental stress conditions. Among others, a large set of proteins named Late Embryogenesis Abundant (LEA) proteins accumulate naturally in some desiccation tolerant plant structures such as the seed and they are also produced in plant vegetative tissues during exposure to abiotic challenges (drought, salinity and cold). Their presence correlates with the acquisition of stress tolerance, but their specific physiological functions have not yet been determined (1).

The presence of LEA proteins in the seed and their abundance makes this plant structure especially useful as a source to obtain large numbers of LEA proteins for analytical studies on a proteomic scale. By heat-treating followed by acid treatment of soluble salt extracts we are able to obtain a subproteome fraction, highly enriched in LEA-type proteins and devoid of major storage protein contaminants (2).

To identify the protein content we employed two approaches suitable for protein identification in less complex protein mixtures or subproteomes: 1D (SDS-PAGE) gel-based procedure associated with MS analysis using an electrospray ionization source (LC-ESI-MS/MS) and a gel-free protocol associated with an off-line HPLC and MS analysis via matrix assisted laser desorption/ionization (LC-MALDI-MSMS).

Using these approaches we have identified several LEA proteins, belonging to different LEA subgroups, in Arabidopsis seeds and in *Z. mays* mature embryos.

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22. Proteome analysis of barley epidermal tissue

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Several stress factors can dramatically affect plant growth and crop yield. It is known that the epidermis of leaves acts as an effective protector against different stresses. Nevertheless only a marginal knowledge about proteome of epidermal tissue exists. The aim of our work is on the one hand to generate a reference map of the proteome of barley epidermal tissue. Furthermore we are interested in monitoring changes in protein composition during abiotic stresses. We focus our research on increased UV-B-radiation, because it is one of the severe abiotic stress factors most critical for plants at high altitudes and in specific ecosystems. Plants living at high altitudes are predicted to have mechanisms to prevent damage from this radiation. But till now only few information about the cellular responses of sensing and responding to this harmful radiation exist. The aim of our work is to monitor changes in protein composition during UV-stress by comparing barley plants, which were either exposed to UV-B-radiation or grown under regular conditions (control plants). Epidermal tissue is chosen for analyses because previous experiments showed that UV-B-absorbing secondary metabolites are mainly accumulated in this tissue type.

The protein profiling both for analyses of complete proteome and of UV-stressed material was done using 2-D gel electrophoresis. Subsequently mass spectrometry methods (MALDI-TOF-MS, nanoLC-ESI MS/MS) were applied for identification. An LC-based label free separation was applied for further investigations. Therefore whole crude extracts were digested and directly separated using a nanoLC System combined with ESI-Q-TOF MS/MS (Waters). The revealed data confirmed earlier results obtained by using conventional 2-D separation technology. A number of supplementary proteins were identified with the help of this novel approach. Among them are proteins which are hardly resolved using 2-D gel electrophoresis, like low abundant proteins and those with extreme pI-values.

Identified proteins obtained out of the proteome mapping were sorted due to their functional distribution. Furthermore the enzymes which were found are visualized according to the respective pathways using VANTED onto biological networks based.

23. Comparative proteomic analysis of heat stress on the metabolic seed protein fraction in the widely grown Italian durum wheat cultivar Svevo

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In Central and Southern Italy, where durum wheat is mostly grown and represents one of the most important crops, grain filling occurs between April and May, when sudden increases in temperature may take place. High temperature during grain filling has already been recognized to cause a deviation of expected properties and quality characteristics of bread wheat doughs. This was a consequence of differential accumulation of gluten proteins that resulted in an alteration of their ratios that, in turn, modify technological properties of doughs.

Wheat grain proteins are typically classified according to their solubility proprieties into albumins (water soluble), globulins (salt soluble) and prolamins (gliadins and glutenins). These latter make up the gluten, and are mostly responsible for rheological properties of wheat doughs. Non-prolamin fractions include proteins with metabolic activity or structural function. Many of these proteins may generate allergies or intolerance in sensitive individuals.

In order to verify the consequences of heat stress on endosperm protein accumulation in durum wheat, we submitted the widely grown cultivar Svevo to two thermal regimes (heat stress vs. control), by producing four biological replicas for each treatment.

Two-dimensional electrophoresis (IEF/SDS-PAGE) was carried out on the metabolic (non-prolamin) fraction. IPG strips (18 cm long) in the pH range 3-10 were used to perform three different technical replicas for each biological replica. Spots were revealed with Coomassie Brillant Blue (CBB) and analyzed with the software Progenesis SameSpots (Nonlinear Dynamics, UK), in order to identify differentially expressed polypeptides between heat stressed and control plants.

This analysis revealed 132 differentially expressed polypeptides (both up- and down regulated). These polypeptides were collected and their identification by MALDI TOF and MALDI-TOF-TOF is in progress.

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Agricultural and Forestry Politics, and by an STSM grant from the EU COST action FA0603 "Plant Proteomics in Europe".

24. Cadmium impact on the photosynthetic properties of spinach leaves: a proteomic approach

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Cadmium is reported to adversely affect photosynthesis. We exposed spinach plants to two levels of cadmium (50 and 100 mM) for 15 days to examine changes in the proteomic structure of photosystems and associated changes to pigment composition and physiological responses. Both levels of Cd reduced significantly the amount of antenna proteins of PSI, while PSII antennae were less affected, with exception of the isomeric Lhc1.1. The formation of an anomalous core PSI complex, containing PsaA and PsaB, was also observed at the end of the treatment. Cadmium reduced photosystem supercomplexes, and PSII core proteins, while did not affect cytochrome b6/f and the ATP-synthase complex. No new protein was formed and no specific protein disappeared altogether in Cd-treated leaves. Ten days of Cd nutrition reduced the concentration of chlorophyll a and, to a lesser extent, of chlorophyll b. A slight increase was observed in lutein, neoxanthin and violaxanthin but, overall, the xanthophylls cycle did not appear to be affected by Cd, even after 15 days of treatment. Photosynthesis of Cd-treated leaves was reduced by 40-70% with respect to controls at growth or higher than growth light intensities. However, Cd only slightly affected light and CO_2 response, at light and CO_2 lower than those experienced during growth, which indicates no limitations due to photochemical reactions and to Rubisco activity, respectively. Cadmium did not affect the quantum yield of PSII, measured by the ratio between variable and maximal chlorophyll fluorescence in dark-adapted leaves, again suggesting that PSII operations are not impaired by this heavy metal. Our results indicate that Cd preferentially affects PSI of spinach leaves but also reveal that damage to PSI only influences photosynthesis when light intensity drives high rates of electron transport, which in turn require large rates of RuBP regeneration.

25. *Avena strigosa* – a new crop resistant to aluminium stress

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According to the FAO criteria an underutilised crop to have a chance to be re-introduced must have a potential to contribute significantly to nutrition and food security, sustainability, the local germplasm must be available and evidences of occasional breeding must be recorded. It seems that *Avena strigosa* (bristle oat, black oat) fulfil the above criteria. It is currently under field trials in Hawaii, where it is trying like ginger in bread production. In Orkney region (Islands of Scotland), it is still under cultivation in small farms. Its excellent nutritive value champions its uses in protection of food security and prevention of civilisation illness. With 27% more proteins than *A. sativa*, 30% more than *H. vulgare* and 13% more than *T. aestivum*, *Avena strigosa* belongs to species with the highest amount of proteins among cereals. The excellent amino acid profiles of all grains with prolamins might reduce the potential risk of *A. strigosa* to coeliac patients. In addition, this crop contains one of the best balances of fatty acids for the most part unsaturated, dietary fibre, a desirable complex of carbohydrates with a high level of β-glucans and vitamins, carotenoids and tocopherols that are essential to human diet. The dietary fibre content is extremely high in *A. strigosa*, from 29% to 360% higher than in the other cereals. The eating dietary fibre helps to protect against number of diseases of the digestive tract such as constipation, haemorrhoids and possible cancer of the large bowel. It also helps to control weight because it is filling but low in calories. The consumption of dietary fibre in Europe has been estimated to be around 20 g per a day but it is encouraged to increase this level to 30 g per a day to promote health benefit.

Avena strigosa is an ideal species for sustainable agriculture because of reasonable yield in poor soil conditions and without fertiliser supplies. It prefers acid brown soils, leached brown soils and podzol soils, which are classified among the poor and very poor soil complexes with very low pH, ranging from 4.2 to 5.5, in ploughing part. In these conditions, very low yield of the major crops makes the cultivation unprofitable and furthermore leads to the abandonment of arable land by farmers. The introduction of *A. strigosa*, that has low edafic requirements, is likely to stop this process, sustain cultivation and protect arable lands from erosion. In our experimental conditions, on very good or good soil complexes (wheat complexes) and appreciate agronomy, the grain yield of *A. strigosa* was 40% lower that the yield of *A. sativa*. However, proportions were inverted if fertiliser supplies and agronomy were insufficient, *A. strigosa* yielded even 300% higher than *A. sativa*. The role of *A. strigosa* in preventing soil from erosion has been confirmed by a release a modern cultivar "SoilSaver" in the USA. Among other advantages, it role as animal forage, cover crop, green manure and weed controller due to strong allelopathic activity should be mentioned.

My recent discovery that this species tolerates extremely high doses of aluminium is another reason champions for re-introducing *A. strigosa.* Aluminium, constituting 7.5% of the earth' crust, belongs to the most abundant minerals in soil. If pH is neutral or slightly acid, Al is found as insoluble oxides or silicates that are inert to plants.

However, at pH 5 and below (acid soils), Al is releases into soil solution in the form of ion Al^{3+}, which damages roots, thus affecting the whole plant development. The problem of Al toxicity exists in many areas of Europe as a result of "acid rains" and acidity caused by fertilisers. The attempts to find Al-tolerance sources have been undertaken in all major cereals but most of them rarely tolerate Al and if, only modest doses (an average 50 µM in laboratory tests). The only tolerant cereal is rye (up to 300 µM Al), however it is of low interest for animal feed due to anti-nutritional factors. Up to now, *A. strigosa* is the most tolerant species, being able to grow at a concentration up to 600 µM in laboratory conditions while rye exhibited growth inhibition and grey root tips stained by haematoxylin. Such a high tolerance is exhibited by Polish ecotypes nevertheless; the majority of world *A. strigosa* accessions collected in gene banks tolerate at least 300 µM of Al. None ecotype was sensitive. Some bigger diversity is observed in our mutant collection (two master theses are going on under my supervision). Hence, *A. strigosa* can also serve as a source of genes controlling tolerance to aluminium as well as can be a good model for studying Al-tolerance in plants.

26. WCS120 and DHN5 – similarities and differences in the pattern of accumulation of two cold-regulated dehydrin proteins in wheat and barley

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WCS120 and DHN5 are both cold-inducible K_n-type dehydrins which accumulate in vegetative tissues under the conditions of cold acclimation (CA). They are orthologues, *i.e.*, they are of the same structural type and they are located at the homologous positions on the long arm of group 6 chromosomes in wheat and barley. It has been known for more than fifteen years that the level of the accumulation of the WCS120 protein corresponds to the acquired level of frost tolerance (FT), thus it was suggested that the WCS120 could be a marker of FT in wheat. In contrast, analogous relationship between the level of accumulation of DHN5 and the acquired level of FT in barley has not been published yet. Moreover, no researcher has published any information on the dynamics of WCS120 or DHN5 accumulation under a wide range of growth temperatures (4 – 25 °C). Therefore, our work was aimed to answer the following questions:

1/ What is the relationship between dehydrin protein accumulation and the acquired level of FT when WCS120 protein or DHN5 protein are considered? Can the WCS120 protein be used for distinguishing of two wheat cultivars with relatively moderate differences in the acquired FT, *e.g.*, two differently frost-tolerant winter wheats? Can a relationship of correlation between the level of DHN5 accumulation and the acquired FT be found in barley?

2/ What is the dynamics of WCS120 accumulation under different growth temperatures? Can it be used for distinguishing of differently frost-tolerant wheat cultivars? Can differently frost-tolerant wheat cultivars be distinguished on the level of WCS120 accumulation at higher temperature than 4 °C? What are the similarities and differences in the temperature-dependent kinetics of accumulation between WCS120 in wheat and DHN5 in barley?

To answer these questions, we have employed the techniques of determination of FT (direct frost-tests) and the techniques of 2D- SDS-PAGE and 1D- SDS-PAGE in combination with immunoblots to determine the level of dehydrin protein accumulation.

1/ We have found out that the level of WCS120 accumulation can be used not only for distinguishing of highly frost-tolerant winter wheat cultivars versus frost-sensitive spring ones, but also for distinguishing of two differently frost-tolerant winter wheat cultivars Mironovskaya 808 (higher FT) and Bezostaya (lower FT) after three weeks of CA at 3 °C. Thus we have confirmed that the level of WCS120 accumulation corresponds well with the level of acquired FT, *i.e.*, the WCS120 protein can be considered a reliable biomarker of FT in wheat. In order to find any analogous relationship between the accumulation of dehydrin protein and the level of acquired FT in barley, we have investigated the accumulation of DHN5 after a three-week CA (when maximum FT is acquired) in 21 (twenty-one) differently frost-tolerant barley cultivars belonging to different growth habits (intermediate, winter, spring). We have found a statistically significant linear correlation between DHN5 accumulation and the acquired FT, but we have obtained this correlation only when the data obtained on the cultivars with contrasting levels of FT (*i.e.*, winter versus spring cultivars) were used for the calculations. No significant correlation was observed on a narrower scale, *e.g.*, when only differently frost-tolerant winter cultivars were used for the calculations. These results indicate that DHN5 in barley does not seem to be as reliable biomarker of FT as the WCS120 in wheat; however, it should also be taken into account that in barley, not so contrasting differences in the level of acquired FT can be found in the wide-spread cultivars as it is common in wheat.

2/ We have found a temperature-dependent accumulation of WCS120 proteins in five differently frost-tolerant wheat cultivars which has corresponded with the results described for the expression of *Cor14b* mRNA in wheat, *i.e.*, the highly frost-tolerant winter wheat Mironovskaya 808 began accumulating the WCS120 protein at higher temperature (17 °C) than the less frost-tolerant winter wheat Šárka, Bill and Zdar (9 °C), and analogously, these medium-tolerant winter wheats accumulated the WCS120 protein at higher temperature (9 °C) than the frost-sensitive spring wheat Sandra (4 °C). Therefore, the WCS120 protein can be used as a marker of the cultivar's specific FT not only under cold, but already at higher temperatures (17 °C or 9 °C). When comparing the temperature-dependent kinetics of expression of WCS120 and DHN5 in two cultivars with contrasting levels of FT (a winter

cultivar versus a spring one), we have found out that in wheat, the winter cultivar Mironovskaya 808 and the spring cultivar Sandra differ in the threshold temperature for WCS120 expression (Mironvskaya 808 17 °C, Sandra 9 °C) whereas in barley, the highly frost-tolerant winter cultivar Luxor and the relatively low frost-tolerant spring cultivar Atlas 68 had the same threshold temperature for DHN5 expression (17 °C) and they began to differentiate at lower temperature (9 °C) according to the level of DHN5 accumulation (Luxor exhibited higher level of DHN5 accumulation than Atlas 68). The experiments demonstrated that the wheat and barley cultivars do not differ in their maximum FT capacity alone, but also in the threshold temperatures for dehydrin accumulation. This result might possibly be explained by a generally higher FT, as well as a wider FT range in wheats than in barleys; and / or by a greater sensitivity to lower temperatures of wheats than barleys. It also indicated that the high threshold temperature for the accumulation of dehydrins could be used for a pre-screening program for highly frost tolerant wheat cultivars, and also could distinguish these cultivars much more rapidly than by LT_{50} analyses.

27. Proteome response of germinating soybean *(Glycine max* L.*)* seeds to cold stress

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Low temperature limits productivity and geographical distribution of many important crops. Chilling temperatures that range from 0 to 12°C are common in temperate regions during the growing season and can substantially decrease plant productivity. To defend against the stress, plants use several strategies, for example the regulation of gene expression. The hypothesis that cold-responsive proteins are likely to be involved in cold tolerance has led to great efforts to study the gene expression profile during cold stress. Identification of novel response genes, determination of their expression patterns, and understanding their function will provide the molecular basis for effective engineering strategies leading to greater stress tolerance. In the present research, soybean (cv. Progres) seeds were subjected to two different germination conditions in a 96h long experiment: optimum temperature germination (+25°C, control) and low temperature germination (+10°C, cold stress). Protein expression changes in seedlings in response to cold stress were studied by two-dimensional electrophoresis (2-DE). Total protein extracts were prepared using thiourea/urea lysis buffer. Proteins were first separated by electrophoresis according to charge. Isoelectrofocusing was carried out with 200 mg of proteins of the various extracts using gel strips forming an immobilized nonlinear pH gradient from 3 to 10. Proteins were then separated according to size in 12% polyacrylamide gels. After electrophoresis, acrylamide gels were stained with silver nitrate according to the manufacturer's instruction from Bio-Rad. Developed gels were scanned using an ImageScanner (Amersham) with Labscan 5.0 programme and analyzed using appropriate software (PDQuest by Bio-Rad and 2D Platinum by Amersham). The proteins whose expression was up-regulated were identified by enzymatic digestion, LC-MS/MS analysis, *de novo* sequencing and sequence similarity search. The identified proteins, whose expression was up-regulated more than two-fold in response to cold treatment were: enzymes (glyoxalase I, chalcone isomerase), glycine-rich RNA-binding protein, stress-induced protein SAM22-like, low temperature-responsive RNA-binding protein, dehydrin, late embryogenesis abundant protein, seed maturation proteins (seed maturation protein: PM24, PM25, PM32 and 18 kDa seed maturation protein), lectin precursor, eukaryotic translation initiation factor, Bowman-Birk type proteinase isoinhibitor D, trypsin inhibitor, storage proteins (alpha' subunit of beta-conglycinin, beta-conglycinin alpha' subunit, napin-type 2S albumin 1 precursor, glycinin) and oleosin isoform. The proteomic analysis enabled us to identify 20 different proteins implicated in a variety of cellular functions. This study provides an initial insight into the proteome response of the embryonic tissue of germinating soybean seeds (cv. Progres) to cold stress.

28. Relative and absolute protein quantification of *Medicago truncatula* root nodules: involvement of N and C metabolism in drought response

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Legumes are important in agriculture as they are able to establish symbiotic relations with nitrogen-fixing soil bacteria. One of their major limitations is their inconsistent production due to their sensitivity to abiotic stresses such as drought. In the present work a liquid chromatography mass spectrometry-based approach (LC/MS/MS) has been applied to analyse proteomic changes occurring in root nodules under drought. Model legume *Medicago truncatula* cv. Jemalong A17 plants, grown in symbiosis with *Sinorhizobium meliloti* 2011, were subjected to water deprivation for six days under controlled environmental conditions. Subsequently, plants were recovered by watering to field capacity for two days. Nodule samples were harvested at three time points, representing two stages of drought and a partial recovery situation.

LC/MS/MS analyses led to the identification of 172 bacterial origin proteins and 140 plant proteins. For the semi-quantitative analysis of the data spectral count was performed. Proteins were grouped into five major categories based on their relative variations during the treatment.

For a more detailed analysis, a number of enzymes involved in nodule C and N metabolism were selected and absolute quantification of proteins was carried out (Wienkoop et al., 2006). Proteins were trypsin-digested in the presence of synthetic peptide standards of known concentration with an incorporated stable isotope (^{13}C, ^{15}N). As stable isotope-labelled and unlabelled peptides co-migrate during chromatography, absolute quantification is achieved by comparison of the peak area abundances of the internal standard peptide with the corresponding native counterpart. Regarding enzymes involved in N assimilation, asparagine synthetase, glutamine synthetase, aspartate aminotransferase and glutamate dehydrogenase were targeted. About C metabolism, sucrose synthase and isocitrate dehydrogenase were studied. Sucrose synthase appeared to be the most abundant protein of all, showing a clear decline under severe drought conditions and a partial recovery after rewatering. Among the N assimilation enzyme, asparagine synthetase showed one of the strongest decline under water deficit, which was not ameliorated during recovery.

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29. Proteome analysis of plasma membrane proteins in barley cultivars with different tolerance towards salt stress

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Among the cereals, barley (*Hordeum vulgare*) is considered to be one of the most salt tolerant even if cultivars display considerable variability in tolerance towards salt stress. Hydroponic salt stress experiments using the parent lines of the Steptoe-Morex mapping population revealed a more elevated tolerance of the Morex parent towards salinity treatment than the Steptoe parent. In order to identify proteins conferring salt tolerance and to understand the regulation of protein expression during salt stress, we initiated a proteome analysis of both cultivars. We found more proteins differentially regulated upon stress in root tissue than in leaf tissue, indicating the importance of ion uptake, transport and regulation of water status in the root. In this context, especially proteins embedded in the lipid bilayer of plasma membranes are of great biological importance as they control the uptake of Na^+ from the soil and the transport within the plant. In a subcellular proteomics approach for the characterisation of proteins embedded in or attached to plasma membranes, we employed aqueous two-phase partitioning method for the enrichment of plasma membranes. This highly selective method for subfractionation of the microsomal fraction takes advantage on the different surface properties of endomembranes and plasma membranes. In the analysis, control and salt stress treated samples from roots of Steptoe and Morex were processed and the quality of preparations was verified using western blot analysis of marker proteins for cytosolic, endomembrane and plasma membrane fractions. Preparations were used for further enrichment for integral membrane proteins by reverse-phase chromatography. Protein identification as well as quantification between treatments and cultivars will be performed using label-free LC-based separation methods. Latest results of this analysis are presented here.

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IV

BIOTIC STRESS ORAL COMMUNICATIONS

30. Plant defense response against pathogens: identification of AtCPK1-interacting proteins as components of the defense signalling pathway in *Arabidopsis thaliana*

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In order to survive the continuous threat of diverse pathogenic microorganisms, plants have developed efficient defense mechanisms. Upon pathogen recognition, a highly coordinated process is initiated in which different signal transduction pathways operate for the activation of the plant defense responses. Studies in various plant/pathogen systems have demonstrated that activation of defense responses involves Ca^{2+}-regulated protein phosphorylation processes. Calcium-dependent protein kinases (CPKs) represent an independent group of plant protein kinases. Within a single polypeptide, CPKs contain an N-terminal serine/threonine kinase domain fused to a carboxy-terminal calmodulin-like domain. CPKs are therefore ideally structured for sensing changes in intracellular calcium concentration and translating them into kinase activity and subsequent downstream signalling events. Results obtained in our group indicate that a specific *CPK* gene from Arabidopsis, the *AtCPK1* gene, is functionally involved in the defense response against fungal and bacterial

pathogens. In order to understand the biological function of AtCPK1, it is essential to characterize the components of the AtCPK1-mediated signalling pathway. To identify AtCPK1-interacting proteins, two different proteomic approaches have been followed, including the immunoprecipitation of protein complexes in which AtCPK1 participates and the purification of the AtCPK1 protein complexes by using the tandem affinity purification system (TAP). Components of these protein complexes were analyzed by high resolution bidimensional electrophoresis and the selected proteins were identified by mass spectrometry. Among the identified proteins were an ATPase and an ATP synthase. Results here obtained provide new insights in the AtCPK1-mediated signal transduction pathway associated to the defense response of Arabidopsis plants.

31. Proteomic studies in potato-PVY-Colorado Potato Beetle interaction

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The main research topic of our group is study of biotic stress. One of the plant models we use is potato (*Solanum tuberosum* L.). In the past few years we have gathered data on the interaction with pathogens and pests on the level of transcriptomics. We are now introducing 2DE analysis in combination with identification with LC-MS-MS to complement the knowledge also on the protein level.

We have developed procedure to analyse potato leaf proteome: extraction of proteins with Tris/CHAPS buffer, cleaning with 2-D clean up kit, isoelectric focusing, SDS-PAGE, staining with fluorescent dye Sypro Ruby, image analysis with Dymension 2-D analysis software programme and statistical analysis of results using R and MEV software. The procedure was first evaluated for technical performance. Then, proteome between biological replicates (3) and between different potato varieties (Igor, Sante, Desiree) was compared to assess natural variability of protein expression. On average 500 spots were detected on a single gel. 46 identical spots between gels were reliably defined with selected methodology and used in further evaluations. Average coefficients of variation (CV) in spot intensity between technical replicates was 14% for the selected spots, while between different biological replicas average CV was always below 30%. It has to be however noted that some individual proteins showed variability of more than 50%. As a test example leaf proteome of several transgenic Igor lines was also analysed and variability compared to variability occurring in natural cultivars. In that way potential of proteomics as a tool to assess safety of genetically modified (GM) plants was elaborated. Several statistic approaches were used to assess variability between varieties and between transgenic and non-transgenic variety of potato, linear models for identification of differentially expressed proteins, PCA, clustering. Variability of transgenic plants did not overcome variability between varieties, nor could we identify any protein that would be specifically up-regulated in transgenic plants. This results show that, besides targeted methods, proteome profiling can be additional method to assess safety of GM plants. Further improvements of the system are now in progress to separate and detect larger number of proteins in leaf proteome. Rubisco is abundant protein in leaf samples constituting of more then 50% of total protein. Therefore it significantly impairs separation and detection of less abundant proteins in the sample. We have introduced affinity chromatography procedure to remove it.

Similarly, we have also introduced a system to analyse proteome of Colorado Potato Beetle guts. It is generally known that plants respond to insect attack through synthesis of defence compounds. But it has been only recently shown that insect react to this and that this adaptation enables them to remain efficient pests. Using the same approach as in analysis of potato leaf sample we were able to detect 500-850 spots per gel. Technical repeatability and variability between biological replicates were in the same range as well. We have studied process of adaptation in different time points. Using linear models we have detected 11 proteins that were differentially expressed and identify them using LC-MS-MS. Most proteins will require more detailed analysis to allow functional annotation as no hits were obtained using Mascot Search Tool. Three proteins were however identified as digestive cystein proteinases, enzymes belonging to the family of proteins already shown to have role in adaptation on the gene level.

32. Changes induced by the *Pepper mild mottle tobamovirus* on the chloroplast proteome of *Nicotiana benthamiana*

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Introduction

Plant proteomes are highly dynamic; for this reason is of high interest their study under different stress conditions, in order to know which metabolic pathways are activated or repressed. Proteomic studies on plant responses to biotic and abiotic stresses are very recent (reviewed in Rosignol, 2006; Jorrín et al., 2007).

Studies on the chloroplast proteome, a powerful environmental sensor, offer a clear picture from the metabolism of this organelle, expecting to contain 2000-3000 proteins (1243 already identified, see Plant Proteome data base, PPDB, http://ppdb.tc.cornell.edu/subproteome.aspx; van Wijk, 2004). Some efforts have been done to identify the changes on the proteomic profile of chloro-

plast from plants suffering under stress factors, such as high light (Phee et al., 2004; Giacomelli et al., 2006), iron deficiency (Andaluz et al., 2006) and cold stress (Cui et al., 2005; Goulas et al., 2006). Proteomic studies carried out in pathogen-infected plants have showed that chloroplasts and, especially, PSII may be key players in plant defence (Zhou et al., 2005; Jones et al., 2006).

We had investigated by two dimensional electrophoresis (2-DE) the changes on the protein pattern of the oxygen-evolving complex (OEC) of photosystem II (PSII) during the infection of *Nicotiana benthamiana* plants with the Spanish strain of the *Pepper mild mottle tobamovirus* (PMMoV-S) (Pérez-Bueno et al., 2004). In the present work, we go further insight in the analysis of the chloroplast proteome of *N. benthamiana* by 2-DE and mass spectrometry (MS) followed by database searching. In addition, changes in this chloroplast proteome induced by the infection with PMMoV-S were studied after 14 days post-inoculation (dpi).

Material and methods

Plant growth and the experimental infection were carried out according with Pérez-Bueno et al., (2004). Chloroplast-enriched preparations from virus-infected plants and their corresponding controls were isolated according to Reche et al., (1997) at 14 dpi. Sample solubilisation was carried out according to Schuster and Davies (1983) with minor modifications. 2-DE was performed according to Pineda (2007). Protein identification was achieved by MALDI TOF/TOF mass spectrometry and further database searching.

Results

Chloroplastidic proteins from both control and PMMoV-S infected *N. benthamiana* plants were separated by 2-DE and the gels were analysed to study the changes induced by the viral infection. To improve the gel resolution, we made separate 2-DE maps for the low (4-7) and high (6-11) pH range.

More than 200 spots could be visualised in the 2-DE gels from chloroplast preparations isolated from control plants: 150 spots in the pH range 4-7, most of them in the 20-43 kDa M_r region and 53 spots in the pH range 6-11. From the analysis by MALDI-TOF/TOF mass spectrometry, 71 proteins were identified (35.5%); in addition, several LHCII proteins were visible in the gels and identified by Western Blot. It was very remarkable the high resolution grade reached in the case of the different isoforms from PsbO and PsbP proteins, extrinsic proteins of the OEC.

Comparative analysis of the chloroplastidic proteome from control and PMMoV-S infected plants revealed that the expression levels of some proteins implicated in Benson-Calvin cycle, electron transport chain and nitrogen metabolism decreased during the PMMoV-S infection.

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33. Proteomic identification of S-nitrosylated proteins in *Arabidopsis thaliana* in response to pathogens

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Nitric oxide (NO) is a highly reactive gas produced by plants under normal growth conditions, as well as under stress situations. It is now recognized as a key signaling molecule in plants but still little is known about the way in which NO regulates different events [1,2]. Analyses of NO-dependent processes in animal and plant systems have demonstrated that the biological effects of NO are mostly mediated through S-nitrosylation of cysteine thiols. The very transitory nature of this posttranslational modification constitutes an important redox-based regulation mechanism for many proteins. During the last few years an increasing number of reports have implicated NO in the regulation of many plant physiological processes, and several proteins potentially regulated in this manner have been identified [3]. In particular NO plays a crucial role in plant resistance to pathogens by triggering hypersensitive resistance-associated cell death and by contributing to the local and systemic induction of defence genes [4,5].

The aim of this study was to identify protein candidates for S-nitrosylation in Arabidopsis upon infection with the bacteria *Pseudomonas syringae*. By identifying the NO-targets we hope to get insights into the physiological functions of protein S-nitrosylation during plant defense responses. For that purpose we have used the "biotin switch method" converting S-nitrosylated Cys to biotinylated Cys. Biotin-labelled proteins were then be purified by affinity chromatography and analysed by means of proteomic methodology using nano-HPLC coupled to a LTQ mass spectrometer [3,6].

This approach allowed us to identify a number of proteins from Arabidopsis cell culture extracts and leaves treated with the NO-donor S-nitrosoglutathione (GSNO) or infected with and the bacterial pathogen *Pseudomonas syringae,* which represent targets for S-nitrosylation in plants. Among the proteins identified are proteins involved in defence-and stress-related responses, redox- related proteins, cytoskeleton proteins, metabolic enzymes and signalling/regulating proteins. Some of these proteins have been already described in Arabidopsis and in animal systems suggesting they might be important under oxidative and nitrosative stress conditions.

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IV

BIOTIC STRESS POSTERS

34. Proteomic studies of legume responses to infection by *Orobanche crenata*

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Broomrapes (*Orobanche* spp.), root parasitic weeds, infecting important crops like *O. crenata* is a major constraint for legume production in Mediterranean environments (Rubiales, D., 2003). Control is difficult due to its characteristic biological cycle and the scarcity and complexity of genetic resistance in most crop species.

We have used a proteomic approach to study the molecular basis of the incomplete or non-host resistance observed in pea crop (*Pisum sativum*) or in the model legume *Medicago truncatula* against *O. crenata*. Two accessions of *M.* truncatula showing early or late resistance and two from pea, one susceptible and other partially resistant, have been utilized. Proteins differentially expressed between accessions and in response to the inoculation were resolved by 2-DE and identified by mass spectrometry.

Most of the proteins identified belong to the functional categories of energetic metabolism, and of defence/stress-related proteins. Part of these results has been published (Castillejo et al., 2004; Jorrín et al., 2006; Rispail et al., 2007). Interestingly, 55% of identified proteins in *M. truncatula* corresponded to defence/stress category, being most of them proteases inhibitors. We speculate that protease inhibitors are related to resistance by inhibiting parasite proteases which are secreted by the parasite in an attempt to break host physical barriers (cell wall), hence stopping parasite invasion.

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35. *Medicago truncatula* resistance to powdery mildew (*Erysiphe pisi*): a proteomic study

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In order to characterize the mechanisms of resistance of *M. truncatula* to powdery mildew (*E. pisi*), we had carried out a proteomic approach, in which the classical platform 2-DE/MS (MALDI-TOF/TOF) has been utilized. We have compared the leaf proteome of either control (non-inoculated) and inoculated plants from susceptible (Parabinga) and resistant (SA 1306) genotypes. Proteins were extracted by using the TCA-acetone precipitation protocol and resolved by 2-DE, with IEF in the 5-8 pH range. Gels were Coomassie stained, images captured by using a densitometer (GS-800, Bio-Rad) and analyzed with the PD-Quest software. Around 380 resolved spots were detected in the 7–98-kDa range. Forty five spots showed differential protein expression between genotypes in non-inoculated plants. 41 and 61 spots were differentially expressed between control and inoculated leaf extracts from Parabinga and SA1306 plants, respectively. Proteins were identified from PMF or MS/MS spectra by interrogating NCBI and *M. truncatula.*

(ftp://ftp.tigr.org/pub/data/m_truncatula/OLD/) databases. From the 147 differential spots 60 proteins were identified, being them grouped in the following categories: a) enzymes of the photosynthesis and carbohydrate metabolism: i.e. RubisCO activase (gi|23320705); b) stress and defence related proteins: i.e. chaperonin (gi|806808); HSP70 (gi|20835); L-ascorbate peroxidase (gi|7484752); c) enzymes of the secondary metabolism: i.e. S-adenosylmethionine synthetase (gi|21593291); isoflavone reductase (gi|19620), d) signal transduction: i.e. villin (gi|4938492); e) protein synthesis and degradation: i.e. endopeptidase (gi|419773).

36. Comparison of 2D patterns of mild and lethal pathotypes of *Verticillium albo-atrum*

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Verticillium wilt, caused by the phytopathogenic fungus *Verticillium albo-atrum*, is a serious threat to hop production in Slovenia since 1997, when a new, more virulent pathotype of *V. albo-atrum* was first observed. Before Slovenia, the lethal pathotype of *V. albo-atrum* was known only in England. 2D patterns of the two Slovenian and two English pathotypes were analyzed and compared. Major differences were observed and identified by LC-MS/MS. Our results indicate that the main differences between mild and lethal pathotypes include proteins involved in interfering with plant defence like peroxiredoxine and proteins which are the building blocks and regulating factors of the cytoskeleton. The cytoskeleton is thought to be of great importance concerning fungal ability to penetrate the plant surface, hyphal growth inside the xylem vessels and especially conidiation rate at trapping sites. Some proteins from carbohydrate and protein metabolism pathways were also up-regulated in lethal pathotypes. These results reveal some of the differences between the two pathotypes at molecular level, which could explain a considerable portion of the difference in virulence.

37. Proteomic and genomic approaches for the study of in-root interaction between nematode (*Meloidogyne artiellia*) and fungal (*Fusarium oxysporum* f. sp. *ciceris* race 5) chickpea pathogens

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Chickpea, the most important food legume in the Mediterranean Basin and the Indian subcontinent, can be severely affected by more than 50 diseases of diverse aetiology that occur worldwide. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (*Foc*), is the most important soil borne disease of chickpeas and ranks as the major yield-limiting factor for the crop. Control of the disease is primarily by the use of chickpea cultivars with resistance to specific races of the pathogen. However, valuable *Foc*-resistance can be annulled by joint infections of resistant roots with *Foc* and the root-knot nematode *Meloidogyne artiellia*. Infections by the nematode begins with penetration of root tissues by second-stage juveniles (J2) at the zone of root elongation; thereafter, the J2s individuals establish a permanent feeding site and induce formation of several (usually 4-6) multinucleate giant cells that support growth and reproduction of the sedentary reproductive females.

Co-infections of chickpea by *M. artiellia* and race 5 of *F. oxysporum* f. sp. *ciceris* (*Foc* 5) increased the severity of Fusarium wilt in genotypes partially resistant

to *Foc* 5, and overcame resistance to the fungus in some genotypes with complete-resistance phenotype. However, the underlying mechanisms seem be more biological and/or biochemical than mechanical. The objective of this research was to determine whose mechanisms are involved in the plant defence response when the plant is co-infected by these pathogens. We used two chickpea genotypes whose resistant phenotypes remained either stable (ICC14219K) or unstable (CA 336.14.3.3) in co-infections with both pathogens. Plants were root-inoculated by either one or the two pathogens and their root proteomes were analysed by 2-DE and MALDI-TOF MS in sectioned galls induced by the nematode. Several proteins whose expression was differentially affected with respect to equivalent root samples from non-inoculated plants could be revealed. These results suggest that root-gall-segments constitute the better choice for studying defensive root proteins in these plant-pathogen interactions at the proteomic level. For to study defensive mechanisms developed at earlier infection steps a second approach has been performed by analysing by Real-Time QRT-PCR the expression of genes related with the pathogenesis. Thus, the use of proteomic and genetic tools allowed us to study localized and systemic plant responses in different periods of time.

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V

SYMBIOSIS ORAL COMMUNICATIONS

38. A proteomic approach to follow RubisCO expression in arbuscular mycorrhizal and metal-stressed plants

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The influence of arbuscular mycorrhizal (AM) fungi in the plant-responses to metal stress is a very complex multi-factorial mechanism. We employed a proteomic approach to gain deeper insight into the expression of RubisCO in two phylogenetically distant plants: *Pteris vittata*, which hyperaccumulates As *and Populus alba*, grown in a Cu and Zn polluted soil. In both cases, the plants have been inoculated with the AM fungus *Glomus mosseae*.

From land plants to green algae, RubisCO is a holoenzyme composed of eight large subunits (LSU) and eight small subunits (SSU); it is well known that oxidative stress is responsible for LSU fragmentation, but no information exists on the effect of AM fungi in modulating the RubisCO expression in the presence or not of metal stress.

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 and 4-7 IPG strips. 2-DE gels were Coomassie stained and comparative analysis of the differentially expressed proteins was performed with PDQuest software; results were validated by two-way ANOVA statistical analysis. Identifications have been carried out by nano-LC ESI Q-TOF MS/MS peptide sequencing on QSTAR-XL mass spectrometer, followed by searching the NCBInr database in the Mascot algorithm (www.matrixscience.com).

Many differentially expressed spots belonged to the RubisCO complex (LSU and SSU) and to RubisCO activase (RCA). Thanks to the high resolution of 2-DE, multiple forms of LSU and SSU were identified, many spots corresponding to LSU fragments, while some others corresponding to aggregation of LSUs. In *P. vittata* a drastic reduction in LSU abundance has been detected when the fern was treated with As, while the co-presence of *G. mosseae* and As brought LSU expression to control values. In *P. alba* a characteristic LSU fragmentation pattern was evidenced in control soil, while the mycorrhizal fungus mainly influenced the expression of different forms of RCA in the presence of metals. These results indicate that both the metal/metalloid and the AM fungus affected the mature expression and assembly of RubisCO. We can suggest that the proteomic analysis (2-DE *plus* mass spectrometry data) of RubisCO profile could be employed as a protein signature for biotic and abiotic events in plants.

39. The arbuscular mycorrhizal symbiosis, a modulator of cadmium stress

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Ecosystems are submitted to various abiotic stresses, among which heavy metals represent major industrial pollutants. Cadmium (Cd), that has damaging effects on plant metabolism, occurs in agricultural environments through industrial pollution and human activities, including phosphate fertiliser and sewage sludge applications. Metal availability to plants can be modulated by soil microorganisms, such as arbuscular mycorrhizal (AM) fungi. In the present work, Cd effects on the model legume *Medicago truncatula* inoculated or not with the AM fungus *Glomus intraradices* have been studied at 3 levels: (1) plant biomass production

together with green part chlorophyll quantification and root isoflavonoid accumulation, (2) *G. intraradices* development inside roots and (3) root and shoot profiles of total proteins. A Cd concentration of 2 ppm caused a reduction of root growth, which was alleviated in colonized plants. Cd application led to an increased accumulation of some isoflavonoids [ononin, malonylononin and medicarpin-3-O-(6'-malonylglucoside)] in nonmycorrhizal roots compared to mycorrhizal roots. Metabolic markers of mycorrhization (apocarotenoids) were partly reduced in Cd treated plants. Global changes in root and shoot protein accumulation of Cd-stressed *M. truncatula* during the symbiotic interaction with *G. intraradices* were monitored by differential protein display (2-dimensional electrophoresis). Cd provoked changes in protein accumulation in root and shoot tissues, some of them being reverted by the AM symbiosis. The proteins whose abundance was modified in Cd and/or G. *intraradices*-treated roots and shoots were identified by LC-MS/MS and MALDI-TOF mass spectrometry, respectively. In roots, most of the proteins whose amount was increased by the mycorrhizal association belonged to metabolism category (38%), signal transduction (9%) and miscellaneous ones (9%). Interestingly, 44% of the proteins that were identified in response to Cd stress, and whose volume was reduced by the AM symbiosis, belonged to defence and cell rescue processes. RNA expression profiling was performed for the gene encoding a thaumatine-like, showing a good correspondence between transcript and protein expression profiling. In shoots, both Cd and mycorrhizal colonisation affected mainly proteins involved in the photosynthetic pathway, including several isoforms of the rubisco diphosphate carboxylase. A phosphoglycerate mutase enzyme mainly characterized the response to Cd stress of mycorrhizal plants. Results will be discussed in relation to a possible role of AM symbiosis in detoxification and/or resistance mechanisms towards Cd in *M. truncatula* plants.

V

SYMBIOSIS POSTERS

40. Alterations in the root proteome of rice plants during the establishment of an arbuscular mycorrhizal symbiosis

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Arbuscular mycorrhizal (AM) symbiosis is one of the most widely distributed root-microbe interactions (Smith and Read 1997). This mutualistic association is characterized by a bilateral exchange between the two symbionts: plants benefit from an improved mineral nutrient uptake from the soil (mainly phosphorus) while, in turn, AM fungi are supplied with the organic carbon forms essential for achieving their full life cycle. The process for the establishment of the AM symbiosis can be divided into different stages: i) the presymbiotic phase; ii) contact and entrance of the fungus into the root tissue; iii) intraradical fungal proliferation; and iv) cell invagination and nutrient transfer. The establishment of the AM symbiosis in the whole root is, however, highly asynchronous with the occurrence of the different stages, once the fungus has initiated root penetration. Application of genomic tools to studies the AM symbiosis has revealed a multitude of potentially relevant plant genes that respond to the development of the symbiosis. Genomic approaches have mainly been initiated in dicotyledonous plant species, such as the model legume system *Medicago truncatula* (Journet *et al*. 2002). Arabidopsis is refractory to colonization by AM fungi. Among monocotyledonous species, rice is a host for AM fungi. A microarray analysis of rice after colonization by a symbiotic fungus, *Glomus intraradices*, revealed conserved mechanisms in the response of mono- and dicotyledonous plants to AM. This study also showed conservation of the transcriptional response of rice to colonization by symbionts and pathogens (Güimil *et al.* 2005). Now, proteome analysis can be used to characterize components required for the development of AM symbiosis in plants.

We initiated a project to investigate changes in the proteome of rice roots associated to the establishment of the AM fungi *G. intraradices*. High-resolution 2D-PAGE of rice roots identified 1.250 root proteins. By using a comparative proteomic approach, statistically significant changes in protein abundance were recorded between inoculated and non inoculated roots of rice plants. Progress in the study of proteins associated with the development of mycorrhizal symbiosis in rice plants will be presented.

VI

FOOD ORAL COMMUNICATIONS

41. Barley thioredoxins, thioredoxin reductases and quantitative cereal disulfide proteomics

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Thioredoxin (Trx) is a ubiquitous redox protein involved in key life processes. Trx reduces disulfide bonds in target proteins, thereby providing reducing equivalents or modulating enzymatic activities. Trx has been extensively studied during the past 30 years but the target protein recognition mechanism is still poorly understood. We

study the molecular mechanisms of two barley h-type Trx isoforms (HvTrxh1 and HvTrxh2) identified in barley seeds[1]. A range of target proteins of HvTrxh1 and 2 have been identified using proteomics techniques[2]. We have recently established a quantitative proteome analysis of the extent of Trx (or other "reagents") disulfide reduction and apply this to identify the status for individual protein disulfides in cereal seeds and used for extracts on germinating embryos. Specific Trx target disulfide bonds are identified[3], e.g. the C144-C148-disulfide in barley α-amylase/subtilisin inhibitor (BASI). We determined the first three-dimensional structure of a Trx-target protein complex (HvTrxh2-BASI) as a disulfide bonded covalent reaction intermediate[4]. The crystal structure shows a conserved hydrophobic motif in Trx having van der Waals' and backbone-backbone hydrogen bonds contacts residues from BASI. This mode of binding suggests that recognition of features around protein disulfides plays an important role in Trx target specificity. The quantitative disulfide bond proteome analysis is validated in the light of structural motifs. The new information will have specific impact on cereal food technology. Recently we have expanded this area of research by the first characterisation of interaction of NADPH dependent thioredoxin reductase isozymes (HvNTR1 and HvNTR2) prepared by cloning and heterologous expression with the two thioredoxin isozymes. Our results suggest that different isoforms are differentially regulated but may have overlapping roles, with HvNTR2 and HvTrxh1 being the predominant isoforms in the barley seed aleurone layer.[5]

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42. Proteomics approach for the development of celiac-safe wheat

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Celiac disease is an inflammatory response in the small intestinal mucosa caused by prolamins (gluten) from wheat, rye and barley. Gluten is diverse proteins encoded by large gluten gene families in hexaploid bread wheat and only a subset of these proteins contains immuno-toxic peptides. Recent research based on detection of CD-toxic elements (epitopes) through specific T-cell, antibody and DNA tests has demonstrated that the CD-toxicity varies across gluten proteins within a single cereal variety, and among different wheat varieties and species.

To analyze biological variation for toxicity and to identify individual proteins containing the immuno-toxic peptides, a large number of modern and old hexaploid and tetraploid wheat varieties were selected with allelic variations based on LMW glutenin and gliadin loci. Prolamin extracts from these varieties were analyzed by Differential in Gel Electrophoresis (DiGE) to identify variety-specific gluten proteins. Antibodies raised against immuno-toxic peptides were used to identify proteins containing these immuno-toxic peptides by SDS-PAGE and 2-dimensional electrophoresis (2-DE). The antibodies were also used in competition assays to identify high and low toxic wheat varieties. By combining the results from different analyses we are able to identify individual gluten proteins which contain immuno-toxic peptides which are harmful to CD patients. With this knowledge we will be able to aim for the development of safer foods, by selecting non- (or low) toxic wheat cultivars, or by developing a new non-toxic wheat cultivar by marker-assisted breeding.

VI

FOOD POSTERS

43. Proteomic characterization of Low Molecular Weight Glutenin subunits associated to the *Glu-A3* locus in durum wheat

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Qualitative differences, such as pasta-making and bread-making properties associated to different bread and durum wheat cultivars, are due to gluten proteins (the viscoelastic mass remaining after washing the dough with salt solutions), their composition and their interactions with other seed components.

Gluten proteins are mainly composed by two protein fractions, termed gliadins and glutenins. This latter group of proteins is formed by large polymeric structures whose constituent subunits are linked through intermolecular disulfide bonds. Viscoelastic properties of both durum and bread wheat doughs are positively correlated with the molecular size of glutenin polymers, that is highly variable and can reach millions of dalton. The size of glutenin polymers depends on the capability of the constituent subunits to form intermolecular disulfide bonds that give rise to polymeric chains of different length. Number and position of cysteine residues present in glutenin subunits are one of the major structural features determining dough rheological properties. Glutenin subunits are classified into high (HMW-GS) and low (LMW-GS) molecular weight.

The latter group of subunits has been less characterized due to their great number and heterogeneity. LMW-GS have been subdivided into B, C and D groups but only subunits included in the B group are considered typical LMW-GS. In fact, C and D groups correspond to mutated gliadins. Typical LMW-GS have been classified into three major groups, (LMW-s, LMW-m and LMW-i types) according to their first amino acid residue (Met, Ser, and Ile respectively) of the mature protein. The LMW-i type has been detected more recently than the others and seems to be mainly encoded by genes present at the *Glu-A3* locus. Additionally, this last group of subunits shows striking structural differences compared to the LMW-m and LMW-s groups since they lack an N-terminal region and all the cysteines are localized in the C-terminal region, though sharing the same number of cysteine residues (eight) with the other LMW-GS types. This difference in cysteine distribution might impact glutenin polymer formation and, more in general, gluten interactions, and be responsible for quality differences observed in different durum and bread wheat cultivars.

In order to gain more information on this particular group of LMW-GS we have used a proteomic approach for their study using a durum wheat line carrying a 1BL.1RS translocation, in which the short arm of the chromosome 1B is replaced by the short arm of the chromosome 1R of rye, and therefore leaving only the LMW-GS associated to the 1A chromosome. Comparative electrophoretic and mass spectrometry analyses carried out on LMW-GS prepared from the durum wheat cultivar Svevo and the line carrying the 1BL.1RS translocation have provided further information on these complex group of proteins.

44. Identification of differentially expressed proteins in the flesh of blood and common oranges

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In Italy the sweet orange production is characterized by red pigmentation, due to the anthocyanin content. The anthocyanins are natural red, purple and blue pigments, mostly present in plant epidermal cells, into the vacuoles, in which give colour to different tissues. They can act as antioxidants, phytoalexins or as antibacterial agents. The antioxidant activity of anthocyanins gives cause for a variety of medicinal usage: prevention of cancer, anti-inflammatory activity and anti-arteriosclerosis activity. On citrus mature fruits anthocyanins are exclusively expressed in blood oranges and its hybrids. Anthocyanin greatly varying content is strictly related to genotype and environmental conditions, moreover expression levels vary considerably among flesh and rind, these facts causing trouble to marketing of the product.

Taking advantage of these knowledge, we analyzed the citrus proteome. Using 2D-gel electrophoresis separation we analyzed differentially the flesh proteome of Moro nucellare 58-8D-I, one of the cultivar with the highest anthocyanins content, and of Biondo, a common orange. The tryptic digest of each spot was characterized by LC-MSMS and the protein was identified by searching both protein and EST databases.

This work is to refer on preliminary results from analysis of some protein related to anthocyanins biosynthesis.

45. Analysis of the protein content of melon juice and of commercial Extramel® melon extracts

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Bionov Ltd company produces and sells melon extracts rich in antioxidant enzymes like superoxyde dismutase (SOD) and catalase (Extramel® micro-balls commercial product). This extract is known for its protective effect against free radicals and oxidative stress diseases. The majority of the proteins of melon juice and of the Extramel® commercial product are unknown and remain thus to be identified.

A research contract between Bionov Ltd and INRA (Joint research unit UMR n°1083 "sciences for enology") aimed to compare the protein contents of melon juice and their corresponding Extramel® extracts.

Native proteins were extracted from two melon juices (from two different melon varieties) and from their corresponding Extramel® extracts. After a global quantification of protein contents (Bradford method), proteins were separated by bidimensional electrophoresis (Isoelectric focusing (IEF) with a non linear pH gradient 3-10 in the first dimension, and sodium docedyl sulfate polyacrylamide gel (SDS-PAGE on 12% acrylamide) in the second. Gels were stained with colloidal blue, and scanned at

600 dpi before image analysis in order to reveal proteins differentially expressed in the four different analysed samples. These protein spots were further identified after nano LC-ESI-MS/MS mass spectrometry by exploring the UNIREF100 non redundant database. Some protein spots were identified by *de novo* sequencing, in order to obtain amino acid sequences of non identified peptides. Such tags were further analysed by Blast query against UNIprot and NnDB databases, with the help of OVNIp software.

Image analysis made possible to detect an average of 750 to 800 spots on the 2-DE gels. The first results confirmed that the protein contents of melon juices and their corresponding Extramel® extracts were similar, but that significant differences occurred between the two melon hybrid varieties studied. Secondly, on 41 proteins submitted to identification, only 35 were truly identified, mainly by nano LC MS/MS methodology. Amongst these proteins, one superoxide dismutase isoform (Mn dependent SOD) was identified.

VII

GENOTYPING ORAL COMMUNICATIONS

46. Proteome variations and coding SNPs in barley cultivars: towards integration of the barley genetic and proteome maps

Finnie, C.[1,2], Bagge, M.[3], Steenholdt, T.[2], Østergaard, O.[2], Bak Jensen, K.S.[2] , Backes, G.[4], Giese, H.[4], Larson, J.[5] , Roepstorff, P.[6] , Svensson, B.[1,2]

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Two-dimensional gel electrophoresis was used to screen spring barley cultivars for differences in seed protein profiles. In parallel, 72 microsattellite (SSR) markers and 11 malting quality parameters were analysed for each cultivar. More than 60 protein spots displayed cultivar variation. The most similar cultivars differed in four spots and many differed in over 30 spots. A clustering algorithm grouped cultivars based on the spot difference matrix. Cultivars with superior malting quality grouped together indicating malting quality to be more closely correlated with seed proteomes than with SSR marker profiles. Proteome analysis of doubled haploid progeny lines derived from a cross between two of the cultivars resulted in the genetic localisation of specific protein phenotypes in relation to markers on a barley chromosome map. Identification of proteins by mass spectrometry showed some spot variations to be due to amino acid differences encoded by single nucleotide polymorphisms. The coding SNPs could be validated by peptide mass spectrometry in combination with expressed sequence tag and 2D-gel data. Coding SNPs can alter the function of the affected protein, and thus represent a direct link between cultivar traits, proteome and genome. This study demonstrates that proteomic studies of cultivar differences and natural variation can be used to identify potential functional markers for quality traits.

VII

GENOTYPING POSTERS

47. Quantitative analysis of seed storage proteins in four quasi-isogenic varieties of *Brassica napus* by mass spectrometry and iTRAQs reagents

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Our studies are focused on the biosynthesis and assembly of storage proteins in developing grains (cereals, oilseeds, legumes seeds). The aim is to gain knowledge into factors that influence the technological and nutritional values of crops in order to improve their usage in both food and non-food industries.

Rape (*Brassica napus*) is widely produced in Europe, mainly for oilseed industry. However, the protein fraction remaining after oil extraction – termed meal – is used only for animal feed. The nutritional value of meals is strongly influenced by the composition in storage proteins; these proteins account for 60% of the total protein amount of seeds, and belong to two families : 12S globulins and 2S albumins.

In order to improve the nutritional quality of meals, it is necessary to set up methods that monitor the expression level of these two protein families in seeds. With recent advances in analytical methods and the ever-growing number of sequences in protein databanks, proteomics has emerged as a powerful method to investigate protein expression in planta.

In the common approach, proteins are first separated by two-dimensional gel electrophoresis. Yet, this technique fails to resolve the 2S albumins, due to their low molecular mass and the basicity of some isoforms. Moreover, 12 globulins scatter into numerous spots, mainly

due to gene polymorphism and modifications. This results in a hazardous quantification by image analysis.

In this context, we set up an alternative strategy, named "shotgun proteomics", which is based on the enzymatic hydrolysis of the entire proteome, followed by a multi-dimensional chromatographic separation of the resulting peptides and their analysis by tandem mass spectrometry. This method was found powerful to detect all isoforms of both 2S albumins and 12S globulins. In order to compare the expression level of these proteins between two samples, peptides were isotopically labeled with iTRAQs reagents (Ross et al., 2004) prior to mass spectrometry.

The method was used to investigate and compare the storage proteins into four quasi-isogenic varieties of B. napus with differing amount of erucic acid and glucosinolates. Results show that the ratio between the 12S globulins and the 2S albumins in seeds is correlated to the amount of glucosinolates.

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VIII

PLANT BREEDING ORAL COMMUNICATIONS

48. Wheat kernel proteomics: studies carried out at INRA for future quality improvements

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To illustrate the usefulness of the proteomics tool, we report on experiments aimed (1) to analyse the proteins in wheat developing kernel, (2) to study impacts of environmental factors on wheat grain development and protein synthesis, (3) to identify and map mature wheat kernel albumins globulins in a segregating progeny, and (4) to characterize starch granule associated proteins in wheat.

These informations will be helpful for predicting the functions of many proteins in response to developmental or environmental changes; knowing where and when proteins are expressed can provide key elements about genes which could be used for future breeding programs.

(1) Developing wheat kernel

Proteomic analysis during kernel formation from the ovule stage to 15 days after anthesis allowed many albumins and globulins to be tracked during cell and tissue formation.

Quantitative and qualitative analysis evidenced five types of protein expression. Four were found among the spots that were present at all sampling stages but with different expression profiles. A fifth type was composed of proteins expressed for a short period of time that were, in our case, new proteins present only in the analysed sample stage and disappearing in the next following stage. A major part of this fifth type was composed of proteins involved in the regulation of the metabolism such as transcription factors and also proteins having short life duration in the developing endosperm. Many major spots were identified by mass spectrometry (MS). This effort to identify the majority of the proteins expressed in the developing endosperm will make possible several approaches:

- For the identification of the cascade of protein expression to be linked with the morphological changes that occur in the developing tissues of the wheat kernel.
- For the association between the proteome to the transcriptome.

(2) Impacts of environmental factors on wheat grain development and protein synthesis

High temperatures during grain filling are one of the major factors that can affect the dough properties and quality characteristics of wheat. To determine the influence of high temperatures during grain filling on the protein composition of bread wheat three experiments were conducted. In all experiments plants were grown in the field and transferred to cabinets soon after flowering from grain development to full maturity. In each experiment, samples were harvested at different post-anthesis stages and endosperm proteins were extracted, then the proteomic approach allowed the characterization of many heat-induced and heat-decreased spot proteins. Of these, in addition to Heat Shock Proteins, many enzymes involved in different metabolic pathways of plants were identified.

These studies enable us to better understand the cascade of protein expression linked with heat stress or heat shock during wheat grain filling.

(3) Mapping the albumins globulins from mature wheat kernel

Proteomic analysis of the albumins globulins extracted from wholemeal kernels of Synthetic and Opata, hexaploid parents of the International Triticeae Mapping Initiative (ITMI) progeny, evidenced 226 significantly different spots. These 226 spots were segregating in the ITMI progeny when 104 lines were analysed by 2-D PAGE and image analysis. Among the 226 spots, 120 showed an unbiased segregation and were successfully mapped on the 21 chromosomes. The segregating spots

were located on both short and long arm of the different chromosomes and several spots were identified using MS.

Such proteomic approach is innovative and important for several topics:

- The mapping of proteic markers that were differing between the two parents.
- New candidate genes for understanding kernel differences that were not investigated up till now.

(4) Diversity and mapping of starch granule associated proteins in wheat

Starch is the major constituent of the wheat endosperm. It is composed of two components: amylose and amylopectin. These components are synthesized in amyloplasts in form of distinct granules.

We presented a protocol for extraction and 2-D PAGE of wheat starch granule associated proteins. Up to 200 spots were visible in silver-stained gels. Identifications of all different proteins by MS are still in progress. But most of the first analyzed proteins matched wheat proteins of carbohydrate metabolism (more specially starch synthesis) with known function.

The granule binding starch synthase (GBSS) - the key enzyme responsible of the amylose synthesis - appeared as charge variants with minor differences in molecular weight corresponding to the 3 wheat genomes.

Thus, a starch granule protein map will be highly valuable in our ongoing studies aimed to:

- Characterize starch associated proteins of some ancient wheat and related species.
- Describe wheat lines carrying different null waxy genes and their granule proteome differences at both qualitative and quantitative levels.

In wheat, to gain a comprehensive understanding of the grain development process, comparison of proteome of different classes of proteins (storage proteins, albumin-globulin proteins, amphiphilic proteins, ..) or of different sub cellular compartments (starch granules, aleurone layer, endosperm, embryo, ..) at different stages of growth is now essential. The four above examples illustrate the usefulness of the proteomics tools to better monitor kernel composition and characteristics of future quality wheat.

VIII

PLANT BREEDING POSTERS

49. Protein markers in phylogenetic and applied studies

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Due to simplicity and obvious genetic basis protein markers are useful both in phylogenetic and applied studies. They can be used to study the nature of processes that occur at the early stages of speciation, to estimate genetic variation induced by mutagenesis or *in vitro* cultures and to monitor changes in gene expression resulted from stress, culture or habitat distortions. Proteins are also valuable markers of quantitative characters in marker-assisted selection. Moreover, if a suitable number of markers are located on genetic maps, they can be used to map alignments more easily than anchor RFLP probes. The aim of this overview is to summarize own examples of different uses of protein markers.

The nature of processes at the early stages of speciation can be understood by investigating the patterns of non-Mendelian inheritance of molecular markers in linkage mapping populations. Reproductive isolation barriers are often indicated by more alleles from one parent than expected under Mendelian rules in interspecific populations. In contrast, such deviations are rarely observed in intraspecific populations. In four mapping populations of two botanical species, *L. multiflorum* and *L. perenne*, the proportion of distorted protein loci did not differ in intra- and interspecific crosses. Surprisingly, the highest fraction of distorted protein markers was observed in the intraspecific cross, derived from two genotypes of *L. multiflorum*, whereas no distorted segregation was found in the interspecific cross between *L. perenne* and *L. multiflorum.* Thus, distortions in segregation of protein markers in four mapping populations of *L. multiflorum* and *L. perenne* have provided support for the lack of species boundaries between both taxa and stated for their classification as subspecies according to the Integrated Taxonomic System of the USA. We have also used protein markers for identification of cryptic species within the genus *Pellia*, analyses of genetic structure of invasive mollusc, *Dreissena polymorpha*, monitoring changes of gene expression during *in vitro* cultures of *A. thaliana* as well as in transgenic plants of *Pisum sativum*.

The heritable variation resulted from *in vitro* cultures is more frequently called somaclonal variation if a culture is derived from somatic tissues or gametoclonal variation if a culture is derived from gametophytic tissues. Genetic variation resulting from *in vitro* cultures may be a risk associated with applications of *in vitro* techniques and its controlling is a challenge. On the other hand, somaclonal variations could be useful for production of new commercial genotypes. Many strategies can be used to evaluate genetic structure of plants derived from *in vitro* cultures.

Owr studies have proved that proteomic approaches can be a simple, inexpensive and appropriate method to evaluate somaclonal and gametoclonal variation. Genetically determined, stable changes in protein patterns have been detected from nearly 30% to nearly 40% loci in the *A. thaliana* callus culture and derived regenerants. The level of somaclonal variation depends on a type of culture and it is significantly lower in a system, in which the callus phase is limited (30% vs. 40%). It is worthy to note that the mutation at *Idh* locus has been lethal in a homozygous stage. Lines carrying this mutation can only be maintained as heterozygotes. In contrast, results have

indicated that no gametoclonal variation is observed androgenic barley plants developed from anther cultures using BAC3 (Barley Anther Culture induction medium).

Mapping and tagging of agriculturally important genes have been greatly facilitated by an array of molecular markers. Molecular marker assisted selection (MAS) involves selection of plants carrying genomic regions that are involved in an expression of a specific trait. With the development of DNA and protein markers as well as dense genetic maps MAS has become possible for characters governed by quantitative trait loci (QTLs). For instance, location of protein markers on a genetic map of *L. multiflorum* and *L. perenne* has been an important step in finding protein markers flanking QTLs responsible for such characters as crown rust resistance, basal leaf width, leaf colour, flag leaf length, flag leaf area, green and dry weight of tillers, days to ear emergence, spikelet length, number and floret number in a spikelet. An important outcome from the map constructed in the Department of genetics is the identification of some protein loci for the first time. They include loci encoding aconitase hydratase, alcohol dehydrogenase, cytosol aminopeptidase, isocitrate dehydrogenase, malate dehydrogenases, peroxidases and shikimate dehydrogenase.

Despite a number of success stories the comparative mapping is not as straightforward as previously thought. The analysis of comparative maps in Poaceae shows that the probability of two adjacent markers being syntenic can be as low as 50% when heterologous RFLP probes are used. The emerging approach of using expressed sequence tags (EST) or sequence specific tags also encounters difficulties with reproducibility and specificity of amplified products. If a suitable number of protein markers are mapped, they can be an easy approach to compare genetic maps. For example, *Lolium* genetic map can be aligned with previously published using several protein markers. Apparent co-location was observed between *Lolium* and barley in a case of three markers.

Integrating genomic and proteomic technologies into the fields of evolutionary studies and molecular breeding would be crucial to recognize the complementarities between morphological characters, genome and proteome level. This vital synergy would harness comprehensive research strategies towards better understanding of diversity of living creatures, its protection and more efficient and friendly to environment breeding strategies.

50. Barley plasma membrane proteomics: identification of protein targets for improvement of crop plants

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Almost all contact with the surroundings of a plant cell is initially perceived via the plasma membrane embedded proteins that are known to act as sensors and facilitators of transport. Knowledge of the plasma membrane protein profile is therefore needed, if we are to use targeted breeding or gene technology to develop crop plants that tolerate soils of poor quality, have enhanced seed quality and nutritional value or to breed plants that are resistant to pathogenic attacks.

We have optimized the aqueous polymer two-phase systems for isolation of plasma membranes from barley seeds, roots and leaves. By employing an efficient reversed-phase chromatography strategy, which combined with SDS-PAGE and tandem mass spectrometry has proved valuable for enrichment of integral membrane proteins (Hynek et al., 2006), we were able to characterize the plasma membrane of the aleurone layers and embryos from barley seeds.

At the moment, we are focusing on isolation and characterization of plasma membrane proteins from barley leaves, because plasma membrane proteins play an important signaling role in the response to pathogens. Pathogens such as the powdery mildew fungus have been suggested to induce both expression and activation of a plasma membrane proton pump in barley epidermis (Finnie et al., 2002). Furthermore, mediators of resistance to the fungus have been identified in the plasma membrane.

In order to compare the plasma membrane proteome of infected and non-infected barley leaves, we are currently optimizing and implementing a 2-DE method based on benzyldimethyl-n-hexadecylammonium chloride (16-BAC) electrophoresis in the first dimension and SDS-PAGE in the second, for obtaining a much improved separation of membrane proteins. This 2-DE gel method, when combined with tandem mass spectrometry is a powerful tool for plasma membrane proteomics.

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IX

TRANSGENIC CROPS
ORAL COMMUNICATIONS

51. Identification of allergenic proteins in foods (transgenic *vs* non-transgenic) by means of proteomics

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The safety issues regarding foods derived from genetically modified (GM) plants are central to their acceptance into the food supply. The potential allergenicity of the newly introduced proteins or the possible occurrence of either altered or de novo expression of endogenous allergens after genetic modification are major safety concerns. In this study we sought to monitor, in potentially sensitive human populations, the allergenicity effects of 5 GM materials obtained from sources with no allergenic potential and under commercialization in the European Union: soya Roundup Ready and maize MON810, Bt11, T25, and Bt176. We have, also, characterized the proteome of soya Roundup Ready, specifically its IgE-reactive proteins and have compared the IgE response of soya-allergic individuals to this genetically modified soya *versus* the non-transgenic control.

For the allergenicity studies we have performed skin prick tests with protein extracts prepared from the transgenic maize and soya samples and from non-transgenic control samples in 2 sensitive groups: children with food and inhalant allergy and individuals with asthma-rhinitis. We have also tested IgE immunoblot reactivity of sera from patients with food allergy, to soya and maize samples as well as to the pure transgenic proteins CryIA(b) (conferring insect resistance) and CP4EPSPS (conferring gliphosate resistance).

For the characterization of soya proteome we have performed two-dimensional gel electrophoresis of protein extracts from 5% GM Roundup Ready flour sample and its non-transgenic control followed by Western blotting with plasma from 5 soya-sensitive individuals. We used peptide tandem mass spectrometry to identify soya proteins (55 protein matches), specifically IgE-binding ones, and to evaluate differences between transgenic and non-transgenic samples. We identified 2 new potential soybean allergens: one maturation associated protein that seems to be part of the late embryogenesis abundant proteins group and a cysteine proteinase inhibitor. However, no differences were observed as a consequence of the genetic modification. None of the individuals tested reacted differentially to the transgenic *versus* the non-transgenic samples under study.

52. Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications.

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In order to improve the probability of detecting unintended side effects during maize gene manipulations by bombardment, proteomics was used as an analytical tool complementary to the existing safety assessment techniques. Since seed proteome is highly dynamic, depending on the species variability and environmental influence we analyzed the proteomic profiles of one transgenic maize variety (event MON 810) in T5 and T6 generation with their respective isogenic controls (F5 and F6). Thus, by comparing the proteomic profiles of F5 with F6 we could determine the environmental effects, while the comparison between F6 and T6 seeds from plants grown under controlled conditions allowed us to investigate the effects of DNA manipulation. Finally, by comparing T5 with T6 seed proteomes it was possible to get some indications about similarities and differences between the adaptations of transgenic and isogenic plants to the same strictly controlled growth environment. Approximately 100 total proteins resulted differentially modulated in the expression level as a consequence of the environmental influence (F6 *vs.* F5), whereas 43 proteins resulted up- or down-regulated in transgenic seeds with respect to their controls (T6 *vs.* F6), which could be specifically related to the insertion of a single gene into a maize genome by particle bombardment. Transgenic seeds responded differentially to the same environment as compared to their respective isogenic controls, as result of the genome rearrangement derived from gene insertion. To conclude, an exhaustive differential proteomic analysis allows to determine similarities and differences between traditional food and new products (substantial equivalence), and a case-by-case assessment of the new food should be carried out in order to have a wide knowledge of its features

53. Alterations of gene and protein expression in a transgenic bread wheat line over-expressing a low molecular weight glutenin subunit gene

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Recent efforts to increase the quantity of specific wheat gluten proteins, directly correlated with the quality of end-use products, have focused on the introduction of additional gene copies by means of genetic engineering. We have thus produced and characterized a transgenic bread wheat line over-expressing a low molecular weight glutenin subunit (LMW-GS).

In order to define the consequences of transgene(s) insertion/expression and the effects of genetic transformation on the global endosperm gene expression, we carried out a parallel transcriptional and proteomic com-

parison of seeds from a transformed bread wheat line that over-expresses a transgenic low molecular weight glutenin subunit gene relative to the corresponding non-transformed genotype.

Proteomic analyses showed that, during seed development, several classes of endosperm proteins differentially accumulated in the transformed endosperm. As a result of the strong increase in the amount of the transgenic protein, the endogenous glutenin subunit, all sub-classes of gliadins, and metabolic as well as Chloroform/Methanol soluble proteins were diminished in the transgenic genotype.

The differential accumulation detected by proteomic analyses, both in mature and developing seeds, was paralleled by the corresponding changes in transcript levels detected by microarray experiments. Microarray analysis showed that, during the seed development, 250 unigenes were significantly differentially expressed. Those genes for which a reliable annotation was available have been classified, according to their putative functional category, to provide an overview of the genome responses to genetic transformation and transgene(s) expression. Most of the differentially expressed genes encode various classes of storage proteins, as well as putative transcription/translation-related proteins or proteins involved in plant defence responses.

Our results suggest that the most evident effect of the strong over-expression of the transgenic glutenin gene consists in a global compensatory response involving a significant decrease in the amounts of polypeptides belonging to the prolamin superfamily. It is likely that such compensation is a consequence of the diversion of amino acid reserves and translation machinery to the synthesis of the transgenic glutenin subunit.

X

ALLERGENS POSTERS

54. Characterization of PR-10 genes from eight *Betula* species and proteomics analysis of PR-10 (Bet v 1) isoforms in birch pollen

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Background

Bet v 1 is an important cause of hay fever in northern Europe. Bet v 1 isoforms from the European white birch *(Betula pendula)* has been investigated extensively. The allergenic potency of other birch species is unknown. Cloning and sequencing of PR-10 genes was performed on eight birch species to establish the presence of these genes. Proteomics procedures like Q-TOF LC-MS^E were applied to identify which of the isolated PR-10/Bet v 1 genes are actually expressed in pollen and to determine the relative abundance of individual isoforms in the pollen proteome.

Results

PR-10 genes were found in all examined birch species. In total, 134 unique sequences were recovered. Sequences were attributed to different genes or pseudogenes that were subdivided into seven subfamilies. Five subfamilies were common to all birch species. Protein analysis at peptide level of pollen from five birch species revealed that the genes of two subfamilies were expressed in pollen, while each species expressed at least 4-5 different isoforms. Isoforms that were similar to isoforms with a high IgE-reactivity (Bet v 1a =PR-10.01A01) were abundant in all species except *B. lenta*, while the hypoallergenic isoform Bet v 1d (=PR-10.01B01) was restricted to *B. pendula* and close relatives of *B. pendula*.

Conclusion

Q-TOF LC-MS^E allows fast screening of Bet v 1prt in pollen by determining the presence and relative abundance of individual isoforms. *B. pendula* contains a BetV1 mixture in which both isoforms with a high and low IgE-reactivity are abundant. The presence of isoforms with high IgE-reactivity is apparently of determining influence for the allergenicity of this species. Other birch species express variants that are similar to Bet v 1a and are predicted to be allergenic as well. This excludes *B. lenta* in which isoforms of intermediate IgE-reactivity predominate and which represents the most promising candidate for further screening of allergenicity.

55. Characterization of allergens present in the seed soluble protein fraction of transgenic and untransformed wheat lines

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Wheat is one of the most important crops in the word in terms of human nutrition. Besides its great interest in food, it is noteworthy that a significant number of patients suffers from wheat allergy.

As in other crops, many strategies are explored to produce genetically modified wheat lines for research purposes aimed at increasing technological and nutritional quality. A specific transformed bread wheat line strongly over-expressing a low molecular weight glutenin subunit, in comparison with the corresponding untransformed genotype, showed a strong decrease of the soluble protein fraction, likely as a compensatory response to transgene over-expression, involving, in general, a significant decrease in the amounts of polypeptides belonging to the prolamin superfamily (see abstract of Scossa et al, this meeting). The objective of this work is to get information on the impact of such a gene transfer event on the expression of endogenous allergens.

Wheat proteins are composed of three classes according to their solubility : the water/salt-soluble albumin/globulin (A/G), the ethanol-soluble gliadins (Gli) and the glutenins (Glu). Among these fractions, the albumin/globulin fraction is involved in food allergy to wheat as well as in baker's asthma. In this study, sera from children and adults with clinically documented wheat allergy are used. Our investigation focuses on the comparison of the A/G fractions obtained from this particular genetically modified wheat (GM-Wheat) and the untransformed line, along with other wild-type bread wheat genotypes. Comparisons will be completed with 1D or 2D immunoblots followed by the identification of allergenic polypeptides by mass spectrometry and by analysis of IgE reactivity to different lines in ELISA. This work should lead to the characterization of the potential allergenic risk of this GM-wheat compared to other non-transgenic lines.

XI

FOREST TREES ORAL COMMUNICATIONS

56. Overwintering in Trees: A Proteomics Approach

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Trees growing in northern latitudes have evolved dormancy mechanisms that together with those that bring about increased freezing tolerance allow survival through extreme temperature and light conditions during winter. Regulation of dormancy is known to involve complex interaction between environmental and cellular factors, securing proper timing of dormancy induction and alleviation. One of the key cellular factors controlling dormancy is the communication ability between cells in shoot apical meristem (SAM) through plasmodesmata. During endodormancy plasmodesmata between SAM cells are closed by callose (beta-1,3-glucan), resulting in morphogenetic inactivation of the SAM. During chilling these callose plugs are opened by the enzyme, 1,3-beta-D-glucanase. Our aim is to elucidate the molecular mechanisms and signal processes that regulate the activity of the specific callose synthase and 1,3-beta-D-glucanase genes involved in the regulation of dormancy development and the alleviation in hybrid aspen. Since both types of gene belong to multigenic families our first task was to select the genes that are involved in dormancy regulation at the SAM. One of our approaches to identify the right candidate genes was to use western blotting to detect the 1,3-beta-D-glucanase proteins from the SAM and analyse these proteins by using different proteomics and informatics tools. We detected by western blotting several glucanase proteins that were differentially regulated in the active and dormant SAM. Corresponding protein bands were cut from coomassie stained 1D gel and analysed by DeNovo sequencing with CAF. We were able to identify at least one glucanase protein by using mascot peptide mass fingerprint search that gave good hit also in poplar genomic and EST databases. Identity of this protein and its possible participation in cellular communication in dormancy will be studied further by using bioinformatics and molecular genetics tools.

XI

FOREST TREES POSTERS

57. Stress proteins as indicators of *Pinus halepensis* MILL. stablishment under drought conditions

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In the Mediterranean semiarid climates, drought stress is the main cause of seedling mortality in forest plantation (Ceballos *et al*., 2004). *Pinus halepensis* Mill. is a forest specie that it can growth on sites with a yearly rainfall of 350 mm. (Díaz and Roldán 1999). In Spain, restoration activities during the twentieth century have been mainly based on conifers plantations, mainly *Pinus halepensis* (Pemán and Navarro 1998).

The use of biochemical and molecular biology techniques in forest ecophysiological studies is gaining

interest. It is necessary to know the biology of species in order to predict species behaviour in the field and to select genotypes with high survival percentages under adverse environmental conditions (i.e. water stress).

Proteomic have been shown an efficient tool to establish the phisiologycal status and characterize key proteins that can be used as markers of quality attributes involved in responses to stresses (Canovas *et al.*, 2004., Rossignol *et al*., 2006).

The aim of this study was to analyze the influence of date planting in growth and survival of *Pinus halepesis* afforestation in Spanish south-east. Two date planting was compared (october 05 and january 06). Measurements of growth, survival, water potential, and chlorophyll fluorescence were taken to evaluate seedlings water stress status. Protein expression changes in needles in response to water stress were studied using Damerval protocol extraction (1986) and by two-dimensional electrophoresis (2-DE) (Jorge *et al*., 2006).

Planting date affect significantly to growth and water potential but not about survival percentage and chlorophyll fluorescence. Protein profile was determined and some proteins was specific of a planting date in a stress situation.

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58. Differential expression patterns in the proteomic analysis of somatic and gametic *in vitro* culture derived embryos of *Quercus suber* L.

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Cork-oak (*Quercus suber* L.) is an important forest tree from the southern Europe ecosystems. Cork production from cork-oak supports an industry of economic and social relevance in Mediterranean countries. Breeding programs have been developed in order to obtain the vegetative propagation of *Q. suber* elite trees through somatic embryogenesis (Bueno et al 2004) and the production of pure lines through doubled-haploid plant regeneration from gametic embryos induced in anther culture (Bueno et al 1997). Both haploid and doubled-haploid materials have an added value for genetic studies. Although cork-oak propagation through somatic and gametic embryogenesis can offer the additional benefit of ensuring consistent tree quality, this approach is relatively recent and there are numerous biological unknowns regarding this complex developmental pathway.

Proteome analysis is a powerful tool for the identification and characterization of differentially expressed proteins. In this sense, a proteomic analysis of somatic and gametic derived embryos of cork-oak from the same parent tree would not only provide a repertoire of differentially expressed proteins in both materials, but also the foundations to investigate the biology of this complex process. The proteome analysis of *Q. suber* somatic and gametic *in vitro* culture derived embryos was conducted using DIGE and MALDI-MS/MS, reporting for the first time proteomic data on this species. Diverse protein expression patterns have been detected between gametic and somatic *Q. suber* L. embryos. These proteins are involved in a variety of cellular processes, most of which had neither been previously associated with embryo development nor identified in the genus *Quercus*.

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59. Differential protein profiles associated to needle maturation in *Pinus radiata* D. Don.

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Although the morphological steps of *Pinus* needle development are well described, very little is known concerning the physiological variations between developmental stages. Here, we present a proteomic study of needle development. We examined 857 proteins in two different needle developmental stages, one and thirteen months old. After the estimation of missing values (employing sequential k-nearest neighbor algorithm), normalization and transformation of the data, 279 differentially expressed proteins were determined (FWER, Bonferroni correction for α =0,05). Principal component analysis and hierarchical clustering analysis allowed the recognition of four main differential expressed protein groups. Comprehensive investigation of the functions associated with clusters resulted in a consistent picture of the developmental coordination of cellular processes in each developmental stage.

60. Proteomics in *Quercus ilex* and its application to the study of variability between populations and drought stress responses

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Holm oak (*Quercus ilex* L.) is an allogamous species slightly manipulated by man, what confers great phenotypic variability related to its origin and environmental conditions (Jiménez et al., 1999). In Spain, holm oak is widely used in reforestation programs (Navarro and Corrales, 2006, in press). Although it is considered a drought tolerant species, water stress represents the first cause of seedling mortality after transplantation (Navarro et al., 1998). Our groups are developing a multidisciplinary research project in which proteomics is being used as a key approach. Preliminary studies, where holm oak leaf proteome was analyzed, evidenced the great variability both between and within populations and individuals (Jorge et al., 2005) and the existence of changes in the protein profile as a consequence of drought stress (Jorge et al., 2006). New experiments have been carried out with a double purpose: i) to characterize the genetic variability between populations for future cataloging, using organs with a proteome more stable than leaves; and ii) to elucidate the molecular mechanisms responsible for their response to drought stress. Holm oak acorns from four Andalusian populations were used in the studies of variability. Leaves from *Q. ilex* seedling subjected to three watering treatments: i) well water, ii) 14 days of drought and iii) 7 days of drought plus 7 days of rewatering, were used to study the response to drought stress. Protein extractions were proceeded with TCA/acetone (Damerval et al., 1986). IEFs were performed on 17 cm IPG 5-8 stripes, and SDS-PAGE were made in 13% polyacrylamide gels. Gel images were captured with a densitometer (GS-800, Bio-Rad) and analyzed with QuantityOne or PDQuest (Bio-Rad). Differentially expressed spots were cut from the gel, digested and analyzed by MALDI-TOF/TOF or LC-MS/MS. Identification was performed using MASCOT or ProteinPilot (Applied-Biosystems). *Q. ilex* populations showed characteristic and differential SDS-PAGE and 2-DE profiles what allowed to establish phylogenetic distances. Some of the differentially expressed spots belong to the legumines family. In response to drought stress, a decrease in the expression of photosynthetic (PSII OEC 1 y 2) and glycolitic (triosephosphate isomerase, fructose biphosphate aldolase) proteins could be observed. In recovery treatment, photosynthetic enzymes recovered and showed an expression level similar to that of control plants, something that did not happen to glycolitic enzymes at the time assayed.

61. Quantitative changes in the leaf proteome of cadmium-exposed poplar plants

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Objectives

This work focuses on cadmium stress responses in poplar plants. Poplar trees are well known to be hardy plants, able to grow on hostile substrate accumulating significant quantities of cadmium. A better understanding of the mechanisms underlying heavy metal accumulation in poplar could be used for phytoremediation purposes. The innovative aspect of this work is the multidisciplinary approach that integrates characterisation studies of the induction of morphological symptoms and physiological disorders by cadmium in *Populus* spp. with a proteomic analysis of the plant response to this type of stress. This will allow a better understanding of the

mechanisms responsible for tolerance of this species to significant level of heavy metals and to correlate these mechanisms to differential protein expression.

Methods

Different poplar clones are exposed to cadmium by growing the plants in hydroponic cultures under controlled environmental conditions. Actively growing plants of *Populus* are divided in two sets. The first acts as a control, while in the second set the hydroponic solution is enriched with 20 μM $CdSO_4$. Treated and control plants are sampled after 0, 3, 7 and 14 days upon cadmium exposure. Various morphological and physiological parameters were recorded during the treatment. Growth of shoots, chlorophyll fluorescence parameters and electrolyte leakage are monitored. Proteins are extracted from the sampled leaves. Proteomic changes induced by the cadmium treatment are assessed by 2D-gel electrophoresis using fluorescent dyes (CyDyes, GE Healthcare). Differentially expressed proteins, determined by statistical analysis, were identified by MALDI-TOF-TOF analysis.

Results and conclusion

After 3 days a significant reduction in growth was observed, although visual symptoms (necrotic spots) of cadmium toxicity appeared only after 7 days of cadmium treatment. Proteomics changes in leaves followed a similar dynamic, with only 3 proteins showing a differential expression after 3 days, but 118 at day 14. In particular cadmium induced a decreased protein abundance for important oxidative stress regulating proteins, whereas pathogenesis-related proteins (PR-2, PR-3, PR-5) showed a drastic increase in abundance with the most marked fold-increase,with average ratios up to 19.9. Furthermore, a large number of proteins involved in carbon metabolism, particularly proteins from the electron transport chain and from the Calvin cycle, showed a decrease in abundance. On the other hand, proteins involved in remobilizing carbon from other energy sources were upregulated. Glutathione biosynthesis could be stimulated by the means of an increase in abundance of proteins from the glutamine and cysteine biosynthesis pathway. In conclusion, the strong reduction in growth and the appearance of necrotic spots on youngest leaves could be explained by a deleterious effect of cadmium on protein expression from the primary carbon metabolism and from the oxidative stress response mechanism. The accumulation of cadmium in stems of poplar, coupled with a low impact of cadmium on physiological parameters, promote the use of poplar trees for phytoremediation purposes. Further analyses of gluthathione and phytochelatin content, as well as the carbohydrate content, including starch could validate these findings.

62. Proteomic changes in leaves of poplars submitted to zinc or cadmium constraint

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Response of plants to heavy metals draws a growing attention, as metal pollution still increases worldwide. It is important to determine how plants are able to handle high amount of metals, especially when the metal ions are massively taken up inside the organism. Indeed, vegetables are the principal source of human metal contamination. We aim to understand what molecular actors support metal intake. This knowledge would allow the design of crops that prevent deleterious metal accumulation along food chain (like cadmium), or, in the contrary, crops that could solve problems of essential metal deficiency (like zinc).

The model tree Populus is known to accumulate the toxic metal cadmium and to thrive on contaminated soil. With a small genome that is sequenced, the most rapid growth among trees of temperate area, and the capacity to be genetically transformed, poplar is a very appropriate tool for deciphering tolerance mechanisms.

As a perennial species, poplar not only provides information on metal uptake and tolerance, but also gives the opportunity to shape the «*ideal phytoremediator*" which will be able to clean up polluted and contaminated soils.

In this study, young poplars (*P. tremula* x *P. alba*) were submitted to cadmium or zinc (1mmol per litter of soil) during 60 days. Poplars exposed to cadmium exhibited phytotoxicity symptoms (chlorosis, growth inhibition, CO_2 assimilation inhibition). Metal quantification showed a significant accumulation of cadmium in aerial parts of the plant (85, 93 and 84 $\mu g.kg^{-1}$). Theses values are close to the Cd 100$\mu g.kg^{-1}$ hyperaccumulation threshold set up by Brooks (1998). Quantification of others minerals showed modification of K, Mg and Ca content in Cd-treated plants, especially in leaves.

A proteomic approach was realised on leaf tissue. Proteins were separated and quantified by 2-dimensions gel electrophoresis using the DIGE technology. A Decyder analysis assigned about 170 proteic spots which expression levels were significantly modified by cadmium or zinc. The MALDI-TOF-TOF identification of these spots is on progress, it will be realised before the Cordoba conference.

Results will give insights on the molecular process that occurs in the leaves in the presence of an excess of Cd^{2+} or Zn^{2+}. Soon, the same analysis will be performed on cambial tissue, to improve the description of the trees response and help to specify the crucial genes that could make Poplar a valuable phytoremediator.

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XII

MISCELLANEOUS ORAL COMMUNICATIONS

63. Regulation of Plasma Membrane-bound Redox Systems by Iron Deficiency

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Iron availability and toxicity are one of the major problems in crop cultivation limiting productivity. On the one hand, production of active oxygen species by free iron (Fenton reaction) may cause oxidative stress on acid soils. On the other hand, iron availability in aerobic and neutral soils is weak and caused iron deficiency. Consequently, the evolution was dependent upon the development of efficient antioxidant systems and iron uptake strategies. Nowadays it appears clear that plasma membrane-bound redox systems are involved in these processes (Lüthje, 2008).

Analyses of plasma membranes isolated from control and iron deficient plants by 2-D electrophoresis (BN-PAGE, CN-PAGE, etc.) suggest that some metallo proteins were up-regulated, whereas others were down-regulated by iron deficiency. These proteins appear to be organized in high molecular mass protein complexes. At least iron chelate reductase activity and NAD(P)H oxidoreductase activities were up-regulated by iron deficiency. Besides these enzymes, plasma membrane-bound superoxide dismutase, peroxidases and malate dehydrogenases were investigated.

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64. Biomarker discovery by omics-data integration – coping with the complexity by dimensionality reduction

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In recent years "genomics" has been extended to "functional genomics". Towards the characterization of organisms or species on the genome level, changes on the metabolite and protein level have been shown to be essential to assign functions to genes and to describe the dynamic molecular phenotype (Glinski and Weckwerth, 2006). With combined computational simulation and experimental measurements we have demonstrated that biochemical regulation is reflected by metabolite correlation network dynamics measured in a metabolomics approach (Weckwerth, 2003; Weckwerth et al., 2004a; Morgenthal et al., 2006). For the integration of metabolite profiles with quantitative protein profiles we have implemented novel mass spectrometric techniques. Multivariate statistics are applied to examine pattern recognition and biomarker identification. The integration of the data reveals multiple biomarkers giving evidence for an synergistic increase of information in such holistic approaches (Weckwerth et al., 2004b; Morgenthal et al., 2005).

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XII

MISCELLANEOUS POSTERS

65. Membrane proteomics

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In-gel digestion of membrane protein complexes has been standardised using the poly(propylene) disposable OMX-S. We designed a rapid and simple in-gel digestion protocol which is carried out in one self-contained reaction tube. In order to quantify the efficiency of in-gel digestion, we use rapid on-column peptide acetylation. We show that highest quality mass spectrometric analysis of membrane proteins is possible with regard to spectrum quality, peptide yield and sequence coverage.We utilise 2-D PAGE separation of protein complexes from the thylakoid membrane of barley to show how the protocol facilitates identification of highly hydrophobic membrane intrinsic proteins. We present new insight into the assembly of membrane protein complexes.

66. A Non-targeted high throughput approach for phenotype-specific protein marker discovery featuring high accuracy mass spectrometry and unbiased statistical analysis

Hoehenwarter, W.[1], van Dongen, J.[1], Wienkoop, S.[1], Hummel, J.[1], Erban, A.[1], Sulpice, R.[1], Regierer, B.[2], Kopka, J.[1], Geigenberger, P.[1], Weckwerth, W.[1,2]

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The dynamics of a proteome can only be addressed with large-scale, high throughput methods. To cope with the inherent complexity, techniques based on targeted quantification using proteotypic peptides are arising. This is an essential systems biology approach; however for the exploratory discovery of unexpected markers non-targeted detection of proteins and protein modifications is indispensable.

We present a rapid non-targeted shotgun proteomics approach that extracts statistical relevant phenotype-specific tryptic peptide product ion spectra in an automated workflow without prior identification. These product ion spectra are subsequently sequenced with *de novo* prediction algorithms and database search. We analyzed 6 potato tuber cultivars grown at 3 locations of 2 geographically distinct regions in Germany aligning 1.5 million spectra in 107 analyses. A dominant protein polymorphism not detectable in the available databases was assigned exclusively to a specific potato cultivar. The method was crossvalidated by GC-TOF-MS metabolite profiling and revealed similar potato cultivar classifications. The approach is applicable to organisms with unsequenced or incomplete genomes and to the automated extraction of relevant mass spectra which potentially can not be identified by genome/EST-based search algorithms.

67. ProMEX: an exchange platform for systems biology studies by interconnecting a proteomics mass spectral reference library to other databases

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In the course of the feasibility of performing large scale experiments and thus producing vast amounts of data concerning the protein, RNA, and transcription level, databases have become an essential tool to manage this information. One example is ProMEX, which was recently developed as a proteomics mass spectral reference library (Hummel et al., 2007). It aims to make generated data broadly available and to interconnect the different molecular levels of a biological system. These initiatives offer an exchange platform for data interpretation and will become an increasingly important part of current and future systems biology. Initially ProMEX contained experimentally derived tryptic peptide product ion spectra, generated based on liquid chromatography coupled to ion trap mass spectrometry (LC-IT-MS). In addition to the four species from which ProMEX contained protein information at the beginning, *Arabidopsis thaliana*, *Chlamydomonas reinhardii*, *Medicago truncatula,* and *Sinorhizobium meliloti*, it now comprises information on *Solanum tuberosum* as well as *Lycopersicon esculentum*. We extended the database so that it now offers an access to 6715 manually validated spectra corresponding to 6283 unique peptides from 2131 proteins. The mass spectral library can be searched through with unknown spectra or complete LC/MS runs in mzData-format thereby improving the true positive rate for protein identification, especially for phosphorylated peptides. Mass spectra and annotated product ions can be visualized. This helps further in designing multiple reaction monitoring experiments for subsequent targeted quantification of proteins in complex samples (Wienkoop and Weckwerth, 2006). ProMEX includes posttranslational modifications such as oxidation and methylation and is interconnected with PhosphAT, a database on *Arabidopsis thaliana* protein phosphorylation sites (http://phosphat.mpimp-golm.mpg.de). The cross-linking of ProMEX to databases such as NCBI, CSB, DB, KEGG, PPDB, UniProt and Arabidopsis Interaction Viewer strengthens its role as a platform providing data of different levels of biological systems. Further cross-linking of ProMEX is highly appreciated. The ProMEX mass spectral library is available at http://promex.mpimp-golm.mpg.de/ .

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68. Quantification of proteins in complex samples of high dynamic range: Comparison of targeted isotope-dilution multiple reaction monitoring using triple quadrupole versus high accuracy/resolution mass spectrometry

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Large scale absolute quantification of complex protein mixtures using high throughput techniques is at present one of the greatest challenges. High degrees of complexity but also huge dynamic ranges, exceeding up to ten orders of magnitude in biological samples, so far allowing only for the analysis of the highest abundance proteins. It has been shown that absolute quantification of a limited amount of targeted low abundance proteins can be monitored using multiple reaction monitoring (MRM). Recently, fast ion trap hybrid mass spectrometer combined with high resolution and ion capacity is available. Here we tested whether these instruments are competitive to high sensitivity standard triple quadrupoles (tripleQps). Plant tissue samples of high complexity including 29 isotope labelled standard peptide targets were used to compare the efficiency of a sensitive MRM experiment using a tripleQp versus a high capacity and resolution experiment using a hybrid instrument.

69. Preliminar proteomic analysis supports the identification of sunflower extracellular vesicles

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We have recently demonstrated the presence of diverse phospholipids in the extracellular fluid (EF) of sunflower (*Helinathus annuus*) seeds. Since phospholipids are insoluble in aqueous solutions we have hypothesized on the existence of vesicular structures in the apoplast. The EF was then subjected to fractionation by centrifugation steps at 10000g, 40000g and 100000g and the pellets analyzed by transmission electronic microscopy. These observations revealed the presence of vesicles of around 100 nm with apparent membrane organization in the 40000g pellets. SDS-PAGE allowed the detection of several proteins in the vesicles fraction. However, using tryptic fragment fingerprinting only two proteins could be identified, as most of the bands displayed score values under the acceptable limits. A lectin belonging to the jacalin family was unequivocally identified (score 206) in the vesicles, while a small GTP binding protein from the Rab subfamily was putatively identified (score 63, 48 % coverage). This protein shows 78% of identity with human Rab11A, involved in both constitutive and regulated secretory pathways and localized in recycling endosomes as well as exosomes. The later are vesicular structures of endosomal origin secreted to extracellular fluids in animal systems. It is becoming clear that exosomes have conserved functions in evolution. We are now presenting microscopic evidence suggesting the existence of exosome-like vesicles in the apoplast of plants, also supported by the presence of Rab11A-like protein, a typical protein involved in vesicular traffic in mammals. Although proteomic analyses have proven to be a valuable tool for assessing identity in sequenced genomes it imply a harder interpretation in non-sequenced species. Despite this limitation, this work proves that proteomics could be extremely useful as a complement of physiological data.

70. Genetic and ecophysiological parameters modify the proteome expression of tomato affected by Yellow Shoulder Disorder

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Yellow Shoulder Disorder (YSD) is a physiopathology affecting tomato fruit, appearing as a yellowish area in the stem-end region (“shoulder”) in ripe fruit while the rest of the fruit appears red. Its origin remains unclear, being thought by nutritional, environmental and genetics factors. Our previous studies showed that covering fruits with aluminium foil at 1 dpa (day post anthesis) avoided almost totally the presence of yellow or green shoulder in red and green fruits respectively. Then we decided to obtain a couple of near-isogenic lines contrasting for YSD, to minimize the genetic factor: 177R4 (sensitive) and 177R6 (resistant) derived from a cross between *S. lycopersicum* L. cv. Moneymaker and *S. pimpinellifolium* L, acc. TO-937 (germplasm bank Experimental Station La Mayora-CSIC. EELM); and make a differential treatment of covering and not covering fruits. In this frame proteomics was the best tool to find differences between both lines, resistant and sensitive; to distinguish the effects caused by a covering treatment, what is “light effects in YSD”; and try to identify what happens in shoulder

and apical end to make a so well-defined dividing line between both parts of the same fruit.

Plants were grown in a polyethylene greenhouse during spring-summer in EELM. Collected fruits colour was measured in areas (Colorimeter, Minolta CR-400), shoulder and apical end were separated, frozen in liquid nitrogen and stored at -80ºC. By colorimeter data we selected fruits to be pulled to prepare the sample, according to the data of the control fruits. Samples from fruit pericarp were analyzed at two different stages: mature green (28-30 dpa) and red ripe (46-47 dpa); from shoulder and apical end areas; in genotypes: 177R4 and 177R6; from covered and not covered fruits, with 3 replicates. Tris-phenol extraction and 4-7 pH strips were used. 2D-gels were stained with Commassie Blue and analyzed with Progenesis Samespot V.2.0.

Several spots present in resistant genotype had qualitative differences with sensitive, like monodehydroascorbate reductase and actin-depolymerizing factor 2, suggesting ascorbic acid protection mechanism is affected and problems in ripening pathway. Comparing shoulder area between covering and no covering treatment in sensitive genotype fruits, acid invertase and polygalacturonase-2 are not detected in yellow shoulder tissue, which explain its hardness and abnormally high content in sucrose (metabolomic studies not shown) and agree with the idea that YS area shows several characteristics of immature fruit. Next proteomic analysis of parental lines and integration of the results with metabolomic and transcriptomic data of the same samples will be the key steps to give a hypothesis of why YS appears.

71. Combined profiling and integrated analysis of transcript, protein and metabolite data" in Poplar

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Much of the recent attention in functional genomics studies has been given to extended sample characterization using multiple profiling techniques, e.g. for parallel monitoring of transcript, protein and metabolite abundances. Extracting valuable information from such a system is a non-trivial task which requires careful experimental planning, powerful statistical methods and cautious evaluation approaches to assure that the biological conclusions are generally valid. We describe a strategy for informative data generation and integrated analysis using three profiling techniques: transcriptomics, proteomics and metabolomics measured in parallel from a xylem extract of hybrid aspen (*Populus tremula* × *P. tremuloides*). Here we will focus on the proteomic method to obtain data for this experiment. In essence; the proteomic data was generated by analysis of a total digest of the soluble protein extract. Peptide profiles were analyzed by ESI-MS and differences in samples were identified by multivariate analysis and a subsequent peptide fragmentation. Furthermore, a peptide database is constructed of peptides identified in tandem mass spectrometry proteomics experiments performed in Populus; mainly consisting from stem tissues.

72. Integration of metabolomics and proteomics in molecular plant physiology

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In recent years "genomics" has been extended to "functional genomics". Towards the characterization of organisms or species on the genome level, changes on the metabolite and protein level have been shown to be essential to assign functions to genes and to describe the dynamic molecular phenotype (Glinski and Weckwerth, 2006). With combined computational simulation and experimental measurements we have demonstrated that biochemical regulation is reflected by metabolite correlation network dynamics measured in a metabolomics approach (Weckwerth, 2003; Weckwerth et al., 2004a; Morgenthal et al., 2006). For the integration of metabolite profiles with quantitative protein profiles we have implemented novel mass spectrometric techniques. Multivariate statistics are applied to examine pattern recognition and biomarker identification. The integration of the data reveals multiple biomarkers giving evidence for an synergistic increase of information in such holistic approaches (Weckwerth et al., 2004b; Morgenthal et al., 2005). Examples in molecular plant physiology are presented to substantiate the approach.

73. Proteomic analysis of transgenics pineapple [*Ananas comosus* (L.) Merr.] plants.

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Pineapple is one of the most important tropical fruit and therefore intensive genetic improvement programs are being carried out in many countries, including Cuba (1). Our research team has previously introduced the *bar* gene, along with *chitinase* and *ap24* genes, into the pineapple ge-

nome (2). Gene recombinant technologies supply agriculture product with great vitality. But the public perception of genetically modified crops can not be ignored (3).

The safety of transgenic crops should be thoroughly evaluated based on "substantial equivalence" principle. The relevant strategies including: substantial equivalent analysis, toxic tests, protein allergenic study, nutritional assessment, etc. With the development of new technologies, the approaches of genomic, metabolomics, and proteomics would be applied to detect the unintended effect.

The present study is focused on the evaluation of the biochemical changes caused by transformation. Transformed and non-transformed plantlets were compared. Statistical significant changes, were recorded in levels of malondialdehyde, other aldehydes, chlorophyll (a, b, total), phenolics (free and cell wall-linked) and proteins. Two-dimensional gel electrophoresis and western analysis has been used to address the *in vivo* proteomic changes in transgenic pineapple plants exposed to Finale sublethal doses harvested after 0, 24, 48, 72 hours and 7 days.

Results indicate that pineapple genetic transformation caused several side effects at the biochemical level during early stages of plant hardening. These changes can promote ulterior modifications such as: 1) in the tolerance to stress because levels of malondialdehyde and other aldehyde were different between transformed and non-transformed plantlets, 2) in the efficiency of photosynthesis as levels of chlorophyll pigments varied and 3) in the fruit quality as contents of free phenolics were modified. Proteins, 100μg, were resolved by 2-DE, with IEF in the 5-8 pH linear range and 12% polyacrylamide SDS-PAGE. Proteins were visualized by Sypro staining. The subsequent image comparison and statistic analysis performed showed changes in protein profiles. Furthermore, about 18 diferential spots in transgenic plants were analized by matrix-assisted laser desortion ionization time of flight (MALDI-TOF). showed differential levels of expression between methods, being some of them absent or unique for a given sample preparation protocol.

At present, pineapple transgenic plants are being studied at the Bioplant Center's Field Experimental Station in order to know if market/public concerns regarding genetically modified organisms are scientifically justified or not. These findings offer interesting perspectives to study the effect of the herbicide Finale on transgenic pineapple plants and for pineapple defense programs.

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74. Modifications of *Arabidopsis thaliana* nuclear proteome in response to environmental cues

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Plant proteomics is far from reaching the standards of yeast or mammalian systems. Therefore, any new information about plant sub-proteomes has the added value conferred by the scarce knowledge we have and the technical difficulties involved in data acquisition (Chen and Harmon, 2006). Because plants are sessile organisms, rather than moving away from changing external conditions like animals do, they respond by modifying their physicochemical characteristics. This ability confers them a very plastic physiological behaviour (Casal et al., 2004). For this reason, plant nuclei are packed with molecules that bind to nucleic acids or are involved in the transfer of incoming and outgoing signals, making plant nuclear proteome a complex system to work with (Bae et al., 2003).

In our laboratory we are interested in the signals that modify gene expression in response to changes in environmental conditions, particularly those dealing with alterations in sugar availability. We have developed a robust and reproducible protocol to isolate nuclei, extract the proteome and enrich it in proteins that bind nucleic acids. It involves chromatographic purification steps, 2DGE and MS identification of tryptic digests. Using this protocol on nuclei extract from plants with or without sucrose addition, we have identified several transcription factors and other nuclear proteins involved in sugar binding and signalling that validate our approach. These proteins will then be analyzed applying other molecular biology and biochemical tools in an attempt to situate them in the hierarchical pathway of sugar signalling.

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75. Proteomics analysis of *in vitro* culture pineapple (*Ananas comosus* (L.) Merr.) shoots

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Bromeliaceae family plants usually contain high concentration of thiol proteases. Pineapple plants *(Ananas comosus)* contain several cysteine proteinases (1). In the literature, these enzymes are called bromelains. Stem bromelain, in particular, exhibits therapeutic effects: anti-inflammatory, digestive, anti-metastasis and anti-tumoral activities (2, 3). In recent years, it is including in the drug group modifiers of the biological answer (4). Plant tissue culture techniques have provided many solutions to basics questions and practical problems in plant biology. Therefore, considerable attention has been focused on the possibility of applying efficient plant tissue culture methods to physiologically active enzymes isolation. In the present study, we found proteolytic activity in pineapple shoots cultured *in vitro*. The highest specific protease activity was recorded in shoots cultured in temporary immersion bioreactors (TIB). Multiplication phase *in vitro* did not cause a remarkable protease production in shoots. Proteome of shoots cultured in different *in vitro* phase were compared by 2D- electrophoresis. Molecular mass of some protein spots were between 21 500- 31 000 Da. This parameter was similar to those indicated for cysteine proteases from *Bromeliaceae*. A protease was detected in TIB culture media. Retention time in RP-HPLC and molecular mass of the major protein detected in TIB culture media showed high similarity to stem bromelain.

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