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P4/E4–PLANT PROTEOMICS

Organised by Professor A.R. Slabas (University of Durham)

P4/E4.1
Quantitative and versatile peptide-centric proteomics

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Modern proteome approaches gradually shift from gel-based techniques in which separations of proteins come on the first line, towards peptide-centric methods in which the proteins are not any more separated, but treated as mixture and as such subjected to proteolytic digestion. This procedure generates a highly complex mixture of peptides (mostly tryptic peptides) and the challenge is now to separate this huge number of components, of which the complexity may easily amount to 200,000. In order to deal with this problem, several groups have developed affinity-based procedures that allow specific selection of well defined subsets of peptides. Such subsets are then separated in their components, each component being representative for the parent proteins where they are derived off.
In our laboratory we have used the concept of diagonal-chromatography to sort for specific subsets of peptides. Essentially the method uses two consecutive identical chromatographic separations with a specific chemical or enzymatic modification in between the two separations. Due to this intermediate modification, the altered peptides acquire elution properties which differ in the two chromatographic runs, allowing specific isolation of the altered peptides. Our method, referred to as Combined FRActional DIagonal Chromatography (COFRADIC) can sort for any type of peptide family, provided it contains functional groups that can be specifically modified. This is for instance the case for Met- or Cys-peptides, for the N-terminal peptides of all proteins present, for phospho-peptides, and for various kinds of post-translational modifications. A COFRADIC analysis typically yields around 1500 protein-identifications and up to 2500, after repetition of the procedure. Quantitative differential analysis is performed by trypsin-mediated labeling by two ^{18}O at the COOH- ends of tryptic peptides. The procedure will be illustrated by an in-depth analysis of a proteome of T cell leukemia Jurkat cells, revealing more than 2500 different proteins. By a global study on Fas-mediated apoptosis on Jurkat cells, by the analysis of the phospho-proteome of forskolin-stimulated HepG2 hepatocytes; and finally by proteome-profiling studies on a large number of tumor tissues.
Keywords: Apoptosis, Phosphorylation, Post-translational modifications

P4/E4.2
A new way to handle complex LC-MS raw data

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One of the major challenges in proteomics is the lack of reproducibility in LC-MS experiments. The replicate analysis of the same sample results often in a different set of proteins identified. This is very pronounced with proteins, which are in low abundance. A prerequisite to overcome the problem is to have a very robust and stable nano-LC-MS system.
Up to now the data interpretation relies only on the MS/MS data. Depending on the signal intensity of a peptide in one LC-MS experiment, an MS/MS experiment is performed and in the repeat it is not. Integrating now the retention time, parent m/z and the topology of the intensity profile using a novel 2D and 3D visualisation approach it is possible to achieve repeat analysis with a very high reproducibility.
We have investigated the reproducibility of this approach by utilising tryptic digestions of integral membrane proteins from organelle enriched fractions from *Arabidopsis thaliana* and have demonstrated that we can reach very high reproducibility integrating the retention time and parent mass in the data evaluation.
Keywords: 2D visualisation of LC-MS data, Reproducibility

P4/E4.3
Protein complex and profiling studies using iTRAQ reagent technology

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A multiplexed peptide tagging chemistry–iTRAQ™ reagent technology–has been developed for quantitative proteomic analy-

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sis. This technology uses a 4-plex set of amine-reactive *isobaric* tags for peptide derivatization following digestion. In MS spectra, peptides labeled with any of the tags are indistinguishable. Upon fragmentation, signature ions are produced that give quantitative protein expression information. Significantly, ion currents for the sequence informative ions are additive (no splitting), such that the peptide backbone fragments represent the cumulative ion current from up to four samples.

The reagents derivatize the N-termini therefore all peptides in a digest mixture are labeled. Derivatization also occurs at lysine residues. The N-terminal tag contains a basic group, which enhances the abundance of b and y ions to yield greatly simplified MS/MS spectra as well as promoting ionization of lysine containing peptides. The principle features of this technology are demonstrated in the measurement of protein expression differences between control and knock-out strains of yeast (two different knock-out mutants compared in the same experiment). The advantages over mass difference labeling approaches are illustrated, as well as the absolute quantitation of target proteins using derivatized synthetic peptide standards.

Due to the advantages of multiplexing, this technology was then used to study effects of drug inhibition in a time-dependent fashion, as well as other studies involving replicate analyses of control versus stressed conditions.

P4/E4.4
Proteomics of tomato fruit ripening

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Inthispaperwediscusstheanalysisofplantandfruitproteins.Wewillshow two examples of where modern analytical techniques can be used for quantitativeandqualitativeproteinmonitoringusingmassspectrometry.Thefirstexamplewillfocusontheproteinsexpressedintomatofruit, analysedusinga2DLC-MS/MSstrategy.

Protein patterns have been compared from green stage (first stage of tomato ripening) to red stage (last stage of tomato ripening). From similar extracts a detailed 2D-gel analysis and micro-array transcriptome analysis have been performed previously. Both these analyses presented a multitude of spots that were differentially expressed between different stages of tomato ripening.

The second example will focus on identification of protein changes in *Arabidopsis thaliana* in response to cold stress. We will compare and contrast the data obtained from a two-dimensional gel electrophoresis separation, followed by MS for protein identification to a non-gel based LC-MS strategy for protein identification and quantification, without the use of isotope labeling.

Keywords: Quantitative proteomics, LC-MS

P4/E4.5
2D DIGE

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Two-dimensional electrophoresis remains an important tool in proteomics. It allows thousands of protein spots to be separated and visualised simultaneously and can also indicate the post-translational modification states of proteins. Quantitative proteomics using comparison of images from different gels, where a single sample is run per gel, has been impaired by gel-to-gel variation hindering both detection and quantification of proteins. 2D difference gel electrophoresis (2D DIGE) involving the multiplexing of samples overcomes such issues. Samples are labeled with spectrally resolvable fluorescent CyDyes^{TM} (Cy2, Cy3, and Cy5;) prior to electrophoresis and are mixed before isoelectric focusing, and resolved on the same 2D gel. This approach greatly reduces variation in spot intensities due to gel-specific experimental factors, including protein loss during sample entry into the immobilized pH gradient strip, as the effects will be the same for each sample within a single DIGE gel. For a multi-gel approach, one of the CyDyes can be used to label an internal standard, consisting of a pooled sample comprising of equal amounts of each of the samples to be compared. This facilitates both inter- and intra-gel matching.

We have used 2D DIGE extensively and have investigated this technique in terms of how to determine what constitutes a significant change in abundance and approaches to statistical analysis of data.

Within the field of plant proteomics we have used this technique both qualitatively and quantitatively and examples of each approach will be given.

Keywords: DIGE, Quantitation, Power, Reproducibility

P4/E4.6
Proteomic analysis of organelle membrane proteins

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The Golgi apparatus is specialized for glycosylation reactions. In this organelle, many plant cell wall polysaccharides and proteoglycans are synthesized. These cell wall components are then trafficked in vesicles to the cell surface. To discover proteins involved in the synthesis of these glycans and in their sorting to the plasma membrane, we are analyzing the protein composition of the Golgi apparatus and of putative plasma membrane lipid rafts. Localization of Organelle Proteins by Isotope Tagging (LOPIT) is a new tool for high-throughput membrane protein localization. Organelles are partially separated using centrifugation. Proteins from the same organelle co-fractionate and therefore exhibit similar distributions in density gradients. Protein distributions can be determined through use of isotope-coded affinity tags to enable relative quantitation of protein levels by mass spectrometry. Novel proteins are then localized by using multivariate data analysis techniques to match their distributions to those of proteins that are known to reside in specific organelles. Using LOPIT we have identified

putative glycosyltransferases, transporters and other novel proteins in the Golgi apparatus in Arabidopsis. We hypothesize that some of these are involved in glycosylation processes. LOPIT also allowed us to identify putative plasma membrane proteins. These were compared to plasma membrane proteins identified by traditional Dextran-PEG partitioning, and to putative lipid raft proteins identified in detergent resistant membranes.
Keywords: LOPIT, Membrane Proteomics, Golgi apparatus, Lipid rafts

P4/E4.7
Sequencing covalent modifications of membrane proteins

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A number of strategies have successfully extended plant proteomics into the bilayer domain. Important benefits can be afforded by including a well-resolved intact protein mass spectrum alongside peptide identification experiments; recent studies of thylakoid membranes have yielded new information on the primary structure and covalent post-translational modification of many of the integral proteins. Intact mass proteomics is advancing through development of core technologies in separations and mass spectrometry, with the goal of providing comprehensive primary structure coverage that includes transmembrane domains, alongside reliable measurements of protein abundance and turnover.
To address limitations associated with separation technologies such as 2D-gel electrophoresis, alternative systems are being investigated and 2D-liquid chromatography of thylakoid membrane proteins, using both denaturing and non-denaturing first dimensions, has been successful, extending separation space and providing intact protein solutions for electrospray-ionization mass spectrometry and top-down proteomics. High-resolution conventional, and Fourier-transform, mass spectrometry is bringing increasing resolution to tandem mass spectrometry allowing for 'top-down' mass spectrometry of intact proteins. Thus the core chromatographic technologies already developed for intact mass proteomics of integral membrane proteins also allow their 'top-down' analysis. Thylakoid membrane proteins with one and two transmembrane helices have been analyzed demonstrating the ability of collision-activated dissociation (CAD) to sequence through transmembrane domains.
Quantitation has become the major challenge facing proteomics as the field matures. The latest developments in stable isotope labelling for quantitative proteomics as well as issues related to detection of background components in membrane proteins such as the cytochrome b6f complex will be discussed.
Keywords: Proteome, Proteomics, Integral membrane protein, Thylakoid, Chloroplast

P4/E4.8
Proteomics opens new doors for thioredoxin function

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Thioredoxins (Trxs) are small ubiquitous proteins with a conserved redox active site, WC[G/P]PC. In plants, the disulfide (S-S) between the two Cys is reduced either (1) photosynthetically by ferredoxin via an iron–sulfur enzyme, ferredoxin–thioredoxin reductase (FTR), or (2) heterotrophically by NADPH via a flavin enzyme, NADP–thioredoxin reductase (NTR). By reducing S-S groups, Trxs function as hydrogen donors for the reduction of biochemical substrates or, more broadly, the regulation of enzymes. The Arabidopsis genome encodes19 different Trxs that can be grouped in 6 subfamilies. Chloroplasts contain four types of Trx—*f*, *m*, *x* and *y*. Trx *o* is located in mitochondria. The *h* isoforms are distributed in multiple cell compartments: cytosol, nucleus, ER and mitochondria. The advent of proteomics together with new isolation protocols has broadened the role of Trx to include processes beyond imagination at the time of the original chloroplast work. Recent studies with chloroplasts, mitochondria, seeds and seedlings have led to the identification of approximately 200 Trx-linked proteins. The work has uncovered new types of regulation–e.g., oxidative regulation in chloroplasts–as well as previously unrecognized modes of communication between organelles. Thus, we can now visualize how mitochondria respond to light processed by photosynthesis in chloroplasts. The field is such that, as concluded in a recent review, it now appears that redox, in many cases by way of Trx, regulates processes functional at virtually every stage of plant development (Buchanan, B.B. and Balmer, Y. 2005 Redox regulation: A broadening horizon. Annu. Rev. Plant Biol. in press.).
Keywords: Thioredoxin, Redox regulation, Organelle communication

P4/E4.9
Comparative proteomics—towards a systems view of plastid differentiation and functions

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During plant development, tissue specific programs control the differentiation of progenitor plastids into functionally specialized organelles. The mechanisms that regulate these differentiation processes are still poorly understood. In addition, little information is available about the proteomes and metabolic capacities of specialized plastids. We have performed a large scale analysis of the proteomes from Arabidopsis chloroplasts, rice etioplasts and the undifferentiated proplastids from a tobacco BY2 cell culture and together identified more than 800 plastid proteins. An extensive proteome analysis of the light-induced development of dark-grown rice etioplasts to mature chloroplasts at various time points after illumination revealed an initial up-regulation of proteins 2 h after the onset of illumination. The phosphorylation status of plastid RNA-binding proteins increases rapidly suggesting the light-dependent activation of a protein kinase. Phosphorylation of RNPs is thought to alter their RNA binding properties, which is important for RNA stability and regulation of gene expression. Our data suggest that the initial events of light-dependent chloroplast development are associated with a stabilization of mRNAs that encode for photosynthetic proteins. We have expanded our proteome analyses to include functional proteomics characterization of the proteins involved in the regulation of plastid mRNA stability and turnover and provide a comprehensive sketch of the molecular mechanisms involved in these processes.

P4/E4.10
Arabidopsis membrane proteomes

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Plant proteomics has become a complementary technology for functional genomics. In particular, sub-proteomes dedicated to plant cell organelles (mitochondria, chloroplasts, vacuoles...) led to relevant identification of proteins associated to a cell compartment and enhanced our understanding of plant organelle functions. We have set up a general strategy for membrane proteomics and we analyzed the proteomes of different *Arabidopsis* membrane systems, namely the chloroplast envelope membranes (Ferro et al., 2003, *Mol. Cell Proteomics* 2, 325–345), the plasma membrane (Marmagne et al., 2004, *Mol. Cell Proteomics* 3, 675–691) and membranes from mitochondria (Brugière et al., 2004, *Phytochemistry* 65, 1693–1707). Such membranes types were taken as models because they can be prepared in a reasonable stage of purity from different types of tissues: the plasma membrane and mitochondria from cultured cells and the chloroplast envelope membrane from whole plants. The interest to combine several complementary extraction procedures to take into account specific features of membrane proteins will be discussed in the light of our recent proteomics data (Ephritikhine et al., 2004, *Plant Physiol. Biochem.* 42, 943–962). These examples also illustrate how on one hand proteomics can feed bioinformatics for a better definition of prediction tools and, on the other hand they can give valuable information for biological investigations. In particular, membrane proteomics brings new insights over plant membrane systems, on both, the membrane compartment where proteins are working and their putative cellular functions.
Funding by Génoplante is acknowledged.
Keywords: Plasma membrane, Chloroplast, Mitochondria, Hydrophobic proteome, Functional genomics

P4/E4.11
14-3-3 Phosphoproteomics for linking signalling pathways to cellular processes

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14-3-3 Proteins exert a widespread influence on cellular processes in all eukaryotes. They bind to specific phosphorylated sites on diverse target proteins, thereby forcing conformational changes or influencing the interactions of their targets with other molecules [1,2]. Hundreds of phosphoproteins can bind to 14-3-3s [3], so 14-3-3s must connect signalling pathways to the control of many cellular processes, including metabolism, actin dynamics, trafficking and proliferation. Which signalling pathways promote the interactions of which targets with 14-3-3s? In plants, we are dissecting multiple roles for 14-3-3s in a dark-induced signalling pathway that prevents dangerous metabolic imbalances when photosynthesis is suddenly inhibited [1]. In humans, several proteins (in translation, vesicle trafficking and other processes) bind to 14-3-3s when cells are stimulated to proliferate by a growth factor [4]. Finding such arrays of phosphoprotein targets downstream of common kinases makes us wonder whether the relative strength of signalling through different target processes is influenced by the spectrum of 14-3-3 isoforms in the cell. Do cells give kinetically different responses to activation of a particular kinase depending on their complement of 14-3-3 isoforms? In some cases, a 14-3-3 dimer will only bind after targets are phosphorylated by two different kinases, suggesting that 14-3-3s can be ‘signalling integrators’ that engage and trigger a response when dual signals are received.
Keywords: 14-3-3, Affinity chromatography, Growth factors, Nutrients, Phosphorylation
[1] MacKintosh, C (2004) Biochem J. 381:329–342.
[2] Kulma et al. (2004) Plant J. 37:654–667.
[3] Pozuelo Rubio et al. (2004) Biochem J. 379:395–408.
[4] Pozuelo Rubio et al. (2003) EMBO J. 22:3514–3523.

P4/E4.12
Phosphoproteomics in *Arabidopsis* from databases to signalling networks

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We recently developed a method for enriching phosphopeptides from complex mixtures to sequence in vivo protein phosphorylation sites from Arabidopsis proteins by LC-MS/MS (Mol Cell Proteomics 2: 1234). Besides yielding potential insights into mechanistic regulation of specific proteins of known function, interrogation of 300 sites from putative plasma membrane proteins allowed the generalization of rules for phosphorylation in plants (Plant Cell 16: 2394). Among these are that (a) phosphorylation occurs outside known functional domains and (b) phospho-sites are more conserved between orthologues than paralogues.
Extrapolating from this concept, we reasoned that evolutionary divergence of residues surrounding the phosphorylation site must be constrained amongst orthologues in order to maintain kinase-substrate specificity. If true, we can use this information to train a bioinformatics search of Arabidopsis to identify additional proteins that may be targets of the same kinase and, therefore, may be co-regulated during specific responses. We tested this hypothesis using two MAP kinase substrates we identified from a phosphoproteome analysis of responses to the bacterial elicitor, flagellin, to search for other Arabidopsis proteins containing the target sequence. Most of predicted substrates we tested were excellent substrates for the MAPKs using in vitro kinase assays. We have confirmed in vivo phosphorylation on the predicted site for one of these proteins and are currently investigating the others. Thus, in conjunction with our on-going work to create a phosphorylation site database for Arabidopsis, this approach will accelerate the identification of signalling networks in silico.
Keywords: Phosphoproteomics, Protein phosphorylation, Signal transduction, Arabidopsis

P4/E4.13
Organellar proteomics unravels novel signalling pathways in *Arabidopsis thaliana*

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Previous experimentation in our laboratory focussed on identification of protein components of the plant extracellular matrix (Chivasa et al. (2002) Electrophoresis 23, 1754–1765; Ndimba et al. (2003) Proteomics 3, 1047–1059). These initial studies revealed a number of proteins not expected to reside in the extracellular matrix due to their classical function previously thought to be restricted to the intracellular compartments. This raised the possibility of the existence of hitherto undiscovered signalling pathways in plants. In this presentation, recent results from our laboratory will point to a central role of the extracellular matrix in the maintenance of plant cell viability via the coordinated control of highly conserved extracellular metabolites. We also show a novel role of the extracellular matrix in the mediation of fungal toxin-induced death in *Arabidopsis thaliana*. This constitutes an example of novel function discoveries enabled by the formulation of hypotheses based on organellar proteomic studies.
Keywords: Extracellular matrix, Cell wall, Cell death, Cell viability, Arabidopsis

P4/E4.14
TAP-tagging of fatty acid synthetase components in plants

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Our laboratory has a long-standing interest in the biosynthesis of fatty acids in plants and their incorporation into complex lipids. De novo synthesis of fatty acids occurs in plant plastids and is catalysed by a type II fatty acid synthetase (FAS) composed of separate proteins responsible for individual enzymatic steps. Genes encoding all of the components of plant FAS have now been identified following enzyme purification or by homology to *E. coli* FAS genes and the structure of a number of them determined. Attention is now turning towards analysis of the stoichiometry and organisation of FAS component enzymes within the plastid. There is clear evidence that FAS enzymes form a metabolon [1] and association between some of the component polypeptides is demonstrated by co-purification after several chromatographic steps. Experiments using tandem affinity purification (TAP)-tagged FAS enzymes are currently underway, with the aim of understanding interacting partners in a FAS complex and the nature of these interactions. Initial experiments have used *Synechocystis* PCC6803 and these are being followed by stable transformation of TAP constructs into *Arabidopsis*. Progress in this area will be presented, together with discussion of the vectors and promoters used.
Keywords: TAP-tag, Fatty acid biosynthesis, *Synechocystis*, *Arabidopsis*

[1] Roughan PG, Ohlrogge JB (1996). Evidence that isolated chloroplasts contain an integrated lipid-synthesizing assembly that channels acetate into long-chain fatty acids. Plant Physiol. 110(4): 1239–1247.

P4/E4.15
Proteome analysis to define cellular functions of tobacco leaf trichomes

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The leaf surface of most terrestrial plants is covered with trichomes. These epidermal appendages are thought to participate in many aspects of defence responses against biotic and abiotic stresses. Trichome development has been intensively studied in *Arabidopsis*, and the phytochemical composition of trichomes was analysed in a range of plant species. However, comparatively little is known on the protein complement. We therefore initiated a proteome approach to better define the cellular mechanisms operating in plant trichomes using 2-D gel electrophoresis to separate proteins of whole leaves and isolated trichomes. Tobacco was chosen as a model due to the presence of glandular trichomes secreting defence compounds. Comparative image analysis of the protein patterns indicated a number of spots highly enriched in trichomes relative to leaves. Components of stress defence responses were strongly represented among the proteins specifically enriched in trichomes. The high expression of stress-related proteins was verified by Western analysis and activity staining. Our results demonstrate the feasibility of the proteome approach to elucidate the cell biology of plant trichomes. In parallel to the analysis of the protein complement, we also investigated the metabolic composition of isolated trichomes with a focus on secondary compounds likely to be involved in stress defence. HPLC analysis of phenylpropanoids and alkaloids demonstrated elevated levels of several compounds in trichomes relative to leaves, such as rutin and nicotine. The proteome approach initially performed on the variety Samsun NN is now extended to other tobacco varieties with modified profiles of secondary compounds.
Keywords: 2-DE, Proteomics, Stress defence, Trichomes, Secondary metabolites

P4/E4.16
Shotgun proteomics and metabolite profiling in *Arabidopsis thaliana*—a systems level approach

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Qualitative analysis of proteomes has reached a novel level of comprehensiveness using shotgun proteomics or “non-gel proteomics”. Our recent studies comprise cell-specific protein analysis with Arabidopsis trichomes and epidermal cells as well as complex leaf proteome characterisation with the identification of more than 1000 proteins. Besides this a more challenging task remains for the quantitative analysis of a multitude of samples

enabling statistical analysis of dynamic processes in the proteome. Here, we extend our qualitative approach to quantitative analyses thereby visualizing protein dynamics of mutant plants or under different physiological conditions. To embed protein dynamics into metabolite dynamics the data are complemented with metabolite profiling and multivariate statistical analysis such as principal components or independent components analysis.
Keywords: Proteomics, Metabolomics, Plant systems biology, Quantitation

P4/E4.17
Cyanobacterial transcriptomics and proteomics under stress response

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When cells of the cyanobacterium *Synechocystis* sp. PCC6803 are exposed to various environmental stress, they perceive the changes in the growth conditions and regulate expression of genes to acclimate to the new conditions. Histidine kinases (Hiks) are involved in the perception and transduction of signals in *Synechocystis*. To examine the potential role in thermotolerance of Hiks, we used microarray analysis to screen a Hik "knockout" library for mutations that affected the expression of genes for heat-shock proteins. Mutation of the *hik34* gene enhanced the levels of transcripts of a number of heat-shock genes, including *htpG* and *groESL1*. Overexpression of the *hik34* gene repressed the expression of these heat-shock genes. In addition, the cells with a mutant gene for Hik34 (?Hik34 cells) survived incubation at 48 °C for 3 h while wild-type cells and cells with mutations in other Hiks were killed. However, mutation of the *hik34* gene increased the expression of genes upon incubation of the mutant cells at 44 °C for 20 min. Quantitative two-dimensional gel electrophoresis revealed that levels of GroES and HspA were elevated in ?Hik34 cells after incubation of cells at 42 °C for 60 min. These findings indicated that levels of mRNAs and proteins were well correlated in the cells of *Synechocystis*. We overexpressed recombinant Hik34 protein in *Escherichia coli* and purified it. We found that the protein was autophosphorylated in vitro at physiological temperatures but not at elevated temperatures, such as 44 °C. These results suggest that Hik34 might negatively regulate the expression of certain heat-shock genes.
Keywords: Histidine kinase, Response regulator, *Synechocystis*, Heat shock, Thermotolerance

P4/E4.18
Proteomic analysis of stress response in plants

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Our research directions have been (1) the methodological establishment for isolation of proteomes, (2) proteomic analysis of the isolated proteomes to construct 2D reference maps and to identify stress-related proteins, (3) functional studies of the identified stress-responsive proteins, and (4) biotechnological application of the characterized target proteins for development of stress-resistant transgenic crops.
We have focused on three sub-proteomes, nuclear proteome, membrane proteome, and secretome. Nuclear proteome including transcription factors plays central roles in a broad range of cellular activities through regulating gene expression. Membrane proteome and secretome including receptors, channels, and secreted peptides are also of molecules that regulate cell–cell interactions of developmental processes and responses to the environment, and initiate and modulate diverse signaling pathways. Because of their active roles, nuclear proteome, membrane proteome, and secretome would be good resources of biotechnologically valuable molecules.
The proteomes were isolated and analyzed using 2D gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Approximately 400 protein spots were identified in reference 2D gels. We then analyzed the changes in the proteomes in response to stress. Of the 400 identified proteins, 70 were up- or down-regulated with a greater than 2-fold change in response to stress treatments. Among them, several proteins were initially selected for further characterization and biotechnological application. The details on functional studies will be presented at the seminar.
Keywords: Arabidopsis, Nuclear proteome, Membrane proteome, Secretome, Stress signaling

P4/E4.19
The search for validated biomarkers in the face of biosystems complexity

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The genomics and post-genomic sciences have delivered an excessive number of drug targets—few of which have been well validated. Indeed, apart from a few rare exceptions, genomics and many millions of dollars in expenditure have yet to greatly impact drug development. This situation has been further exacerbated by the 'hype' and over-selling of the biotechnology industry, much to the chagrin of the world's stock markets and investors.
Nonetheless, the genomic sciences have much to offer. For the first time in the history of the pharmaceutical industry, we possess a full parts-list for all the protein targets in many organisms, including humans. This information is invaluable and should allow us to move forward in a better-informed manner, particularly with respect to target selectivity analysis and prediction. The biomedical sciences must work better if biomarkers of high worth are to be delivered in the future. The search for biomarkers is laudable in that they allow for: discovery of new targets; early diagnosis of life-threatening disease; monitoring of adverse responses to one or more drugs; monitoring new treatments in clinical and pre-clinical trials; predicting disease and treatment outcomes; improved economic and practical efficiencies in healthcare delivery; and better targeting of high cost treatments to those patients most likely to benefit. For these reasons, much effort in genome mutation analysis, transcriptomics, proteomics, metabolomics and increasingly systems biology is being devoted to the search for biomarkers. Their relevance is equally valid to an improved understanding of plant systems.
Keywords: Biomarkers, Proteomics, Transcriptomics

P4/E4.20
Proteomic analysis of leaf plastids isolated from different cell types of *Arabidopsis thaliana*

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Using *Arabidopsis thaliana* lines with GFP labelled epidermal and mesophyll cells we have been able to isolate plastid populations from specific cell types. Plastids isolated from protoplasts and purified on a Percoll gradient were subjected to 2D-electrophoresis and proteomic analysis. The purification of these plastids followed by mass spectrometry analysis has led to information relating to the differential metabolic capacity between different plastids within a leaf.

Keywords: Plastids, Proteomic analysis, 2-D electrophoresis

P4/E4.22
Identifying hydrogen peroxide sensitive proteins in *Arabidopsis thaliana*

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Hydrogen peroxide (H_2O_2) is now recognised as an important signalling molecule in both animals and plants. In plants, H_2O_2 is involved in gravitropic responses, gene expression, pathogen defence and regulating stomatal closure. Although several signalling components, such as kinases, have been shown to be activated by exogenous H_2O_2, little is known about the proteins that are direct targets of H_2O_2. Thiol groups on proteins are potential targets for oxidation by H_2O_2, resulting in alteration of protein structure and function. To identify thiol-modified proteins in *Arabidopsis*, we used a directed proteomics approach consisting of iodoacetamide-based fluorescence tagging of proteins in conjunction with mass spectrometric analysis. This approach identified cytosolic glyceraldehyde 3-phosphate dehydrogenase (cGAPDH) as a potential target of H_2O_2. cGAPDH activity is inhibited by H_2O_2 in a reversible manner, with the addition of either reduced glutathione (GSH) or the reductant dithiothreitol leading to restoration of activity. Recently, in animals and plants, cGAPDH has been found to have several cellular locations and roles not commensurate with its activity in glycolysis. Therefore, it is possible that cGAPDH in *Arabidopsis* might also have a role in mediating reactive oxygen species signalling.

Keywords: *Arabidopsis thaliana*, Glyceraldehyde 3-phosphate dehydrogenase, Hydrogen peroxide, Proteomics, Signalling

P4/E4.23
A proteomic approach to understanding herbicide safener mode of action

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Safeners are chemicals which selectively protect crops from herbicide injury. Although the mechanisms of safener action are generally poorly understood, these compounds are thought to work by increasing the ability of the crop to detoxify herbicides and a number of safener-induced genes have been identified. The aims of this project are to investigate the functions of a safener-induced wheat gene, TaGSTL1, and to use proteomic techniques to identify safener-induced changes in protein abundance and post-translational modification.

TaGSTL1 belongs to the lambda subclass of the glutathione-*S*-transferase (GST) superfamily. Since TaGSTL1 and a maize lambda class GST (1, 2) were identified as safener-induced genes, we hypothesised that lambda GSTs may play an important role in safener action. The active site of TaGSTL1, in common with other lambda class GSTs contains a cysteine in place of the conserved serine. Consequently, recombinant TaGSTL1 protein did not exhibit “classical” GST activity, but rather was shown to have thioltransferase activity, in common with Arabidopsis GSTLs (3). Potential roles of TaGSTL1 in protein (de)glutathionylation and oxidative stress tolerance are being investigated.

Conventional 2D and Difference Gel Electrophoresis (DiGE) have been used to identify soluble rice proteins which are differentially regulated in response to treatment with the herbicide, fenoxaprop and the safener, isoxadifen-ethyl. Recent data will be reported at the meeting.

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P4/E4.24
Analysis of the *Arabidopsis thaliana* guard cell proteome

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Guard cells are specialized cells mostly located on the undersurface of leaves. They form pores, so-called stomata, which play an important role in gas exchange and the regulation of water use efficiency. Guard cells sense and respond to a wide range of stimuli to open and close the stomatal pore, with an intricate web of signalling pathways being involved in regulating stomatal apertures. In order to investigate signalling pathways controlling these highly specialized cells from an additional perspective to physiological or molecular studies we are using a proteomic approach. To do so, a method by which guard cell enriched fragments are obtained from whole leaves has been adapted in our laboratory. The suitability of this procedure could be shown by DAPI and FDA staining in which fractions of 90–95% viable guard cell purity were obtained. Protein extracts from guard cells and whole leaves were then separated by 1D- and 2D-gel electrophoresis and proteins were identified by their peptide mass fingerprint (PMF) obtained using MALDI-TOF mass spectrometry. To date, several interesting proteins have been identified as being enhanced in guard cells. These proteins found represent starting points for exploring the complex network of guard cell signalling.

P4/E4.25
The C-terminal domain of histone H1 and protamine bind preferentially to scaffold-associated regions (SAR) DNA

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Histone H1 preferentially binds and aggregates scaffold-associated regions (SARs) via the numerous homopolymeric oligo(dA).oligo(dT) tracts present within these sequences. Here we show that the mammalian somatic subtypes H1a,b,c,d,e and H1° and the male germ line-specific subtype H1t, all preferentially bind to the *Drosophila* histone SAR. Experiments with the isolated domains show that whilst the C-terminal domain maintains strong and preferential binding, the N-terminal and globular domains show weak binding and poor specificity for the SAR. The preferential binding of SAR by the H1 molecule thus appears to be determined by its highly basic C-terminal domain. Salmine, a typical fish protamine, which could have its evolutionary origin in histone H1, also shows preferential binding to the SAR. The interaction of distamycin, a minor groove binder with high affinity for homopolymeric oligo(dA).oligo(dT) tracts, abolishes preferential binding of the C-terminal domain of histone H1 and protamine to the SAR, suggesting the involvement of the DNA minor groove in the interaction.

Keywords: Histone H1, Carboxi-terminal domain, Scaffold-associated regions, Protamine

P4/E4.26
Self-incompatibility in *Coffea canephora* and *Petunia hybrida*

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Coffee is a key global cash crop. Selection and breeding are therefore important in meeting consumer demand. However, gametophytic self-incompatibility, which operates in *Coffea canephora* (Lashermes et al., 1996), disrupts breeding activities. Identifying self-incompatibility (*S*) genotypes in current cultivation will lead to improved breeding strategies.

In *Petunia hybrida* (and other members of the Solanaceae), a ribonuclease-based mechanism of self-incompatibility has been identified. A ribonuclease activity assay has revealed polymorphisms between stylar extracts of different lines of *C. canephora* and *P. hybrida*. These polymorphisms in stylar ribonucleases with high pI values suggest that the same mechanism of self-incompatibility may exist in *Coffea* and *Petunia*.

Polymorphisms in stylar protein molecular weights have also been identified between four experimental *P. hybrida* lines that possess different *S*-genotypes using SDS-PAGE. These differences have been shown to occur at a low molecular weight typical to known *S*-RNases (25 kDa–28 kDa). Mass spectrometry analysis performed on the polymorphic proteins has confirmed that these are *S*-RNases. Similar work is to be performed on *C. canephora*.

Microsatellites have also been used to reveal differences between the genetic background of *C. canephora* accessions. The microsatellites will offer a powerful tool to be used in conjunction with *S*-RNase polymorphisms to facilitate coffee breeding.

Keywords: Coffee, Petunia, *S*-RNases, Polymorphisms

1. Lashermes, P., Coutron, E., Moreau, N., Paillard, M. and Louarn, J. (1996) Inheritance and genetic mapping of self-incompatibility in *Coffea canephora* Pierre. *Theor. Appl.Genet.* 93 458–462.

P4/E4.28
Proteomic analysis of the differential responses to biotrophic and necrotrophic pathogens in pea

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Peas comprise the largest planted area of field vegetables in the UK, and downy mildew, caused by the oomycete pathogen *Peronospora viciae* is the most common foliar disease of this crop with up to 55% losses in yield observed where plant resistance is ineffective. Control of downy mildew is achieved through the use of resistant pea cultivars and treatment with fungicides. Resistance to these fungicides is now a problem, so optimal application is required to prolong their effectiveness in controlling downy mildew outbreaks. This DEFRA-funded work uses proteomics and mass spectrometry to identify protein biomarkers that increase or decrease in abundance during infection of pea by *P. viciae*. The specificity of these proteins to the downy mildew infection is being assessed by examining proteins differentially regulated during infection of pea by other pathogens including the biotrophic fungus *Erysiphe pisi* and the necrotrophic fungus *Botrytis cinerea*. Fundamental information on proteins specifically involved in these plant–pathogen interactions is being generated, which may be employed in novel control strategies for downy mildew and other fungal diseases. Biosensor devices will be developed to detect specific biomarkers and deployed for early diagnosis of infection, allowing fungicides to be applied more efficiently and effectively.

Keywords: Downy mildew, Peronospora, DIGE

P4/E4.29
Changes in protein expression in *Lolium perenne* roots on switching from an inorganic to organic N supply

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The degree to which plants take up organic nitrogen as intact molecules under field conditions is still subject to controversy. The transporters and enzymes used in the acquisition and initial metabolism of an amino acid such as glycine differ from those used for the uptake and metabolism of nitrate and ammonium. A plant's protein complement will therefore depend on the forms of nitrogen it utilises. Potentially this difference in protein complement can provide a means of assessing the utilisation of amino acids by field grown plants. We investigated the change in protein expression in roots of *Lolium perenne* after switching the form of nitrogen supplied to the plant from ammonium nitrate to glycine.

Initially, plants were grown in sterile solution culture with all nitrogen supplied to the plants as 1 mol m^{-3} ammonium nitrate. After 26 days, half the plants continued to receive 1 mol m^{-3}

ammonium nitrate whilst the remaining plants received 2 mol m^{-3} glycine. Plants were harvested after a further 1 and 3 days, the root tissue was weighed, frozen in liquid nitrogen and stored at −80 °C. Proteins were extracted from the roots using a mixture of trichloroacetic acid, deoxycholic acid and acetone then separated by 2D-gel electrophoresis. Principal component analysis (PCA) demonstrated that protein expression discriminated clearly with treatments (2 N-forms and 2 harvest dates). The key proteins contributing to the specificity of the respective protein complements will be identified by peptide mass mapping and potentially utilised as indicator-proteins of root function.
Keywords: Protein, Inorganic, Organic, Nitrogen, Ryegrass

P4/E4.30
Global analysis of stress responses: the pea proteome following biotic and abiotic stresses

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Plants have evolved a complex variety of responses to stresses from the environment. Many different stress conditions produce common responses, some of which are mediated by abscisic acid. However, specific responses are also induced by particular stress conditions. We are examining the proteome of pea leaves following a variety of stress treatments including fungal, oomycete and bacterial infections as well as drought and wounding. Protein extracts are separated using two-dimensional difference gel electrophoresis and identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry or peptide sequencing by Q-TOF mass spectrometry. We have identified proteins that increase or decrease in abundance following stress treatment. Some of the proteins we have identified show increased abundance under all of the stress conditions examined, while others show altered abundance in response to only one or a few of the conditions. Some of the proteins we have identified have previously been reported to be involved in responses to stress and/or abscisic acid, whereas others have not previously been associated with stress responses or are unknown.
Keywords: DIGE, Drought, Wounding, Plant pathogens

P4/E4.31
Proteomic-based analysis of AtMPK6 signaling in *Arabidopsis thaliana*

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In *Arabidopsis thaliana*, ROS-induced signaling has been shown to utilize a protein kinase-based, multicomponent phosphotransfer system involving the mitogen-activated protein kinase (MAPK) AtMPK6. We have previously shown (Miles et al., 2005) that RNAi-mediated silencing of AtMPK6 renders the plant sensitive to ozone as determined by visible leaf damage and elevated levels of leaf-localized hydrogen peroxide. AtMPK6 thus appears to be central to oxidant-induced signaling making it a prime candidate for further analysis. To this end, we investigated the role(s) of AtMPK6 in response to ozone-induced oxidative stress, via isotope-coded affinity tagging (ICAT) technology to both identify and quantify alterations in protein expressions between WT and AtMPK6-RNAi genotypes. This quantitative proteomic approach allowed the detection and positive identification of 81 proteins in the 0 h-air and 151 proteins in the 8 h-ozone samples. The majority of the 0 h-air and 8 h-O_3 proteins fell in the GO categories of “other metabolic processes”, “electron transport”, “energy pathways” or “antioxidant-related” with an assortment of other categories covering the rest of the protein entries.
Keywords: Ozone, AtMPK6, Isotope-coded affinity tag, *Arabidopsis thaliana*
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P4/E4.32
Plant membrane trafficking and proteomics

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Although lipid rafts are well studied in the mammalian field, the existence and consequently function of lipid microdomains in plants is unknown. Many experiments in mammalian cells suggest that Glycosylphosphatidylinositol- (GPI-) anchored proteins have a high affinity for lipid rafts. By developing raft preparation methods and applying these to plant cell cultures, we found conditions that gave a remarkable enrichment of a GPI-anchored reporter protein in detergent resistant membranes (DRMs). Two-dimensional fluorescence difference gel-electrophoresis, as well as separation of proteins by 1D PAGE (GelC-MS) and a quantitative proteomics technique of isotopic labelling (ICAT) coupled with tandem mass spectrometry were used to characterise the DRM composition in comparison to the control membranes. DRMs were highly enriched in a defined sub-set of plasma-membrane proteins and largely depleted of intracellular proteins. Several of the proteins enriched in the DRMs were GPI-anchored proteins, supporting the hypothesis that some GPI-anchored proteins are present in rafts. A comprehensive proteomic analysis (GelC-MS and ICAT) of the plasma membrane was used to assess the contribution of PM and non-PM (e.g., Golgi, endosome or vacuolar proteins) in the detergent resistant membranes. PM proteins not present in the DRMs in the conditions tested were identified as well. This will be important for comparative localisation and trafficking studies of DRM and non-DRM proteins. Furthermore it could be shown that the DRMs were enriched in several phytosterols relative to mixed organelle membranes, and also in sphingolipids.
Keywords: Lipid rafts, GPI-anchored proteins, DIGE, ICAT, GelC-MS

P4/E4.33
Comprehensive sizing of Arabidopsis membrane protein complexes using isotope encoded quantitation of polypeptides

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We present a technique for the identification of soluble and membrane bound proteins while simultaneously estimating the size of their respective protein complexes. Current techniques for the separation of protein complexes include rate zonal centrifugation in sucrose/glycerol gradients and 2D Blue Native (BN)/SDS-PAGE gels. However, the measurement of protein distribution in such experiments is generally performed on a protein-by-protein fashion using western blotting. Additionally, in the case of 2D BN-PAGE gels, unknown spots can be identified by individual MS analyses for each protein. Our technique utilizes isotope coded affinity tag (ICAT) technology for the quantification of protein abundance across rate zonal gradients to produce sedimentation data for large numbers of identified proteins simultaneously.
Consistent with published work on plant mitochondrial protein complexes and rate zonal centrifugation, subunits of the same protein complex were shown to have a similar distribution. The migration of different protein complexes was also proportional to their molecular weight. To our knowledge, this is the first time that a rate zonal analysis has been performed for a large group of proteins simultaneously by obtaining quantitation and identification in parallel. Additionally, to compare our technique with those currently in use we ran the same plant mitochondrial protein samples on a 2D BN-PAGE gel with subsequent MS based identification of spots. The isotope tag based technique was able to produce similar sizing data for complexes yet identify more proteins.
Keywords: Proteomics, Stable isotope quantitation, Nanoscale chromatography, Membrane proteins

P4/E4.34
Isolation and purification of isopentenyl adenine binding proteins during the cell cycle of synchronized tobacco BY-2 cell line

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Plant hormones are known to be involved in the control of plant growth and development. The presence of both cytokinins and auxins is required to maintain cell proliferation during in vitro culture. Cytokinins are implicated in the regulation of cell cycle, and are N6-substituted adenines. The N6 side chain can be either an aromatic moiety or an isoprenoid derived chain such as isopentenyl adenosine. We aimed to identify the isopentenyl binding protein(s) using synchronised tobacco BY-2 cells as model system. The coupling of isopentenyl adenine to Epoxy activated Sepharose 6B was optimized in order to purify isopentenyl adenine binding proteins by affinity chromatography and afterwards to identify them by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Several isopentenyl adenine binding proteins isolated are phosphorylated by Pro-Q Diamond dye but not with SYPRO Ruby; indicating that these proteins are low abundant but highly phosphorylated. The isopentenyl adenine binding adenosine kinase (ADK) is the most abundant protein isolated by this method and it proved to be phosphorylated. ADK is believed to play an important role in cell cycle. In animal system the ADK is mostly involved in the regulation of adenosine levels, while in plant it might have an additional regulatory function in order to convert cytokinin ribosides to nucleotides, which are believed to be inactive forms.
Keywords: Cytokinins, Cell cycle, BY-2, MALDI-TOF, Isopentenyl adenine

P4/E4.35
The effect of plant hormones on the protein composition of the nuclei in the *Arabidopsis thaliana* cell suspension culture

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Plant hormone auxin plays an essential role in the communication between cells during the development of plant organs. The efforts to identify the mechanism of auxin action have identified a complex signaling network, which involves two major processes. One of them is a polar transport system that is responsible for a discrete distribution of auxin within the plant (1). The second one is the proteasome-dependent degradation of the nuclear AUX/IAA repressor proteins in the presence of auxin, which leads to a specific regulation of transcription (2). This leads to the recognition of the importance of the nuclear proteome map as tool in the study of the primary targets of the plant hormone action. We use the auxin-dependent MMD-2 *Arabidopsis thaliana* cell suspension culture as a model system in our effort to develop a reliable and reproducible method for a comparative 2-D analysis of the nuclear protein content, which allows us to detect protein modifications which are caused by the hormone treatment, such as phosphorylation, polyubiquitination and degradation.
Keywords: Nuclear extracts, 2-D electrophoresis, Auxin, Ubiquitin
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