

Proposal for a new COST Action

Plant Proteomics in Europe EUPP (European Plant Proteomics)

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Part I: Technical annex

A. Abstract

Plants, as all other living organisms depend on proteins to perform most of their vital functions. The name *protein* comes from the Greek *πρώτα* ("prota"), meaning "*of primary importance*". Proteins are the functional molecules that drive metabolic and regulatory pathways in a cell. Proteomics, i.e. the large-scale analysis of proteins in biological systems at a certain time point, aims to identify all proteins present and to characterize their qualitative and quantitative modifications, for example in response to environmental changes. Proteomics is a relatively recent technology currently undergoing fast development and growth, logically complementing the genomic and transcriptomic studies as well as the other emerging field of metabolomics. Although protocols have been developed to perform proteomic analysis in the human, animal and microbial domains of life, the plant kingdom still awaits a systematic approach for proteome analysis. This proposal aims to build up expertise in plant proteomics through an integrated network of European scientists. Tools for proteome analysis in fundamental and applied plant research areas will be developed and shared, to generate fundamental information about plant metabolism, investigate responses to environmental constraints and assess food quality. This proposal will also increase public understanding for new technologies, critical for further development by the industry.

Keywords

Mass spectrometry
Post-translational modifications
Protein-protein interactions
Plant proteome
Systems biology

B. Background

Plants provide a high diversification level on the biochemical, cellular and organism level to survive ecological threats. Production of metabolites, induction of defence mechanisms, and control of development are driven by proteins. As it addresses the true actors in the cell, proteome analysis is the level of choice to investigate and dissect mechanisms involved in plant responses to various effectors. Proteomics, i.e. the large-scale analysis of proteins in biological systems at a certain time point, is a scientific strategy undergoing rapid developments benefiting from the breakthroughs of mass spectrometry in addition to genomic and transcriptomic disciplines. Indeed, considerable progress has been made in the sequencing of plant genomes and availability of expressed sequence tags (ESTs). In 2000, the complete genome of *Arabidopsis thaliana* was published (The *Arabidopsis* Genome Initiative 2001); in 2004, a *Populus* EST resource was provided to the community (Sterky et al., 2004) and in 2005, the finished rice genome has been released (International Rice Genome Sequencing Project 2005). The sequencing of euchromatic regions of *Medicago truncatula* genome is close to completion (The *Medicago truncatula* Genome Initiative). Currently, different groups are working on the genome of maize, alfalfa, sorghum, wheat, tomato or soybean. However, beside this, biological systems have to be considered as complex networks, and therefore a large-scale study must bring more information. Until recently, plant proteomics was mainly aiming in simply cataloguing proteomes, as for example in different tissues of *Arabidopsis* (Giavalisco et al., 2005). As a logical complement of the post-genomic era, proteomics aims at profiling information by the characterization of proteins and their function, either by describing their differential synthesis, or their modifications observed during the study, their localization, the possible protein-protein interactions, and ultimately by unravelling how their synthesis is controlled within regulatory networks (Rossignol, 2001). For example, recent studies have been carried out on wood formation (Gion et al., 2005), evolution of cellular diversity (Ramsay and Glover 2005), on response to cold stress (Renaut et al., 2004; Amme et al., 2006), to water deficit (Riccardi et al., 2004), flower development (Theissen 2001) or on seed development (Hajduch et al., 2005; Hajduch et al., 2006).

At the transcriptome level, techniques like microarray analysis provide a wealth of information about genes. However, it must be taken into account that one gene does not equal one transcript, that one transcript is not equal to one protein (Peck 2005). So, the transcriptomic results must be interpreted with care since mRNA abundance and protein level are not clearly correlated (Gygi et al., 1999). Besides this systematic bias, discrepancy between mRNA, and protein abundance is naturally caused by translational regulation of gene expression that is even not uniformly distributed among plant tissues and individual cell types. For those reasons, transcriptomic studies do not provide reliable information about protein expression and abundance. Additionally, microarray studies cannot provide information about either the subcellular localization or the protein post-translational modifications (PTMs) that may be essential for its function, transport and activation (Gygi et al., 1999; Peck 2005). On the other hand, given the high heterogeneity of physicochemical properties, proteins are far more difficult to isolate, handle and

identify than mRNA, and there is no technique comparable to PCR to amplify low abundance proteins (Rose et al., 2004).

The rapid development of proteome research has led to many new applications and answers to a variety of biological questions. Two main approaches can be distinguished: either gel-based analysis, where the separation of proteins from a complex mixture, typically takes place through 2D electrophoresis, or gel-free analysis of the proteome that uses liquid chromatography devices. In both approaches, one or more mass spectrometers are involved in identification/characterization of proteins. Nonetheless, proteomics has still serious limitations: e.g. it does not allow the separation and visualization of the complete set of synthesized proteins (e.g. membrane proteins, very low abundant proteins, highly hydrophobic or basic proteins). On a 2D gel for example, it has been estimated that less than one-half of predicted proteins of *Arabidopsis* could be observed (Peck, 2005).

Moreover, proteomics techniques applied to plant tissues present the following specific constraints:

- The presence of multiple interfering substances during protein extraction. In addition to the classical interfering substances (e.g. lipids, nucleic acids, carbohydrates), efficient extraction of plant proteins also requires removal of proteases, polyphenols, tannins, pigments, lignin and waxes (Carpentier et al., 2005).
- Unlike animals, each plant cell is additionally surrounded by a cell wall, complicating the extraction.
- Plant cell organelles (mitochondria, chloroplasts, etc) have each, along with the cytoplasm, a distinct metabolic function and hence a unique proteome, requiring tissue pre-fractionation. Even in organelles such as chloroplasts, subfractions should be considered to analyze distinctly proteins from the stroma, the thylakoids and from the different membranes.
- In green tissues, the large abundance of ribulose 1,5-bisphosphate carboxylase/oxygenase, can disturb the 2D electrophoretic patterns. The abundance of such proteins raises significant dynamic range issues for the detection of low abundance proteins. In seeds, analogous problems exist with the high abundance of storage proteins.
- The identification of plant proteins is also more problematic than in human, microbial or animal field, as the number of sequenced genomes and therefore annotated plant proteins is relatively low, despite the fact that for certain plants (e.g. major crops) EST databases become now more and more publicly available.

The priorities under the 7th EU framework will be plant genomics and biotechnology, as explained in the vision paper “Plants for the future”. In the ‘Stakeholder proposal for a strategic research agenda 2025’(see <http://www.epsoweb.org/catalog/TP/docs/SRA-I.PDF>), the technology platform intends to focus on a number of goals to meet the issues covered in this challenge, goal number one being a pertinent ‘vibrant basic research’ capability. Deliverables and research activities for this goal include research on proteomics. Indeed, in this document, one can read “Comprehensive functional genomics

programmes should investigate the various molecular levels: the RNA world, the protein world and the metabolome”. This illustrates that plant scientists start to realize that proteomics can make an essential contribution to their research. Beside the increasing scientific interest in proteomics, it is important to note the low proportion of scientific papers devoted to plant proteomics compared to all the papers published on proteomics (less than 10%, Fig. 1). Considering that plants form the basis of our food, environment and life, it is of outermost importance that plant proteomic research progresses at the same pace as human and yeast proteomics. Hence, the majority of papers dedicated to plant proteomics describes techniques or lists protein directories, while the pertinence of quantitative approaches linked with physiological and transcriptomic approaches seems to be the main challenge in the future. Proteomics research will involve identification of functions for proteins, including determining expression patterns for pathways or networks of proteins under specific environmental conditions or during developmental stages.

Number of publications including 'proteomics', 'proteomic' or 'proteome' (Source: PubMed, 2006.08.17)

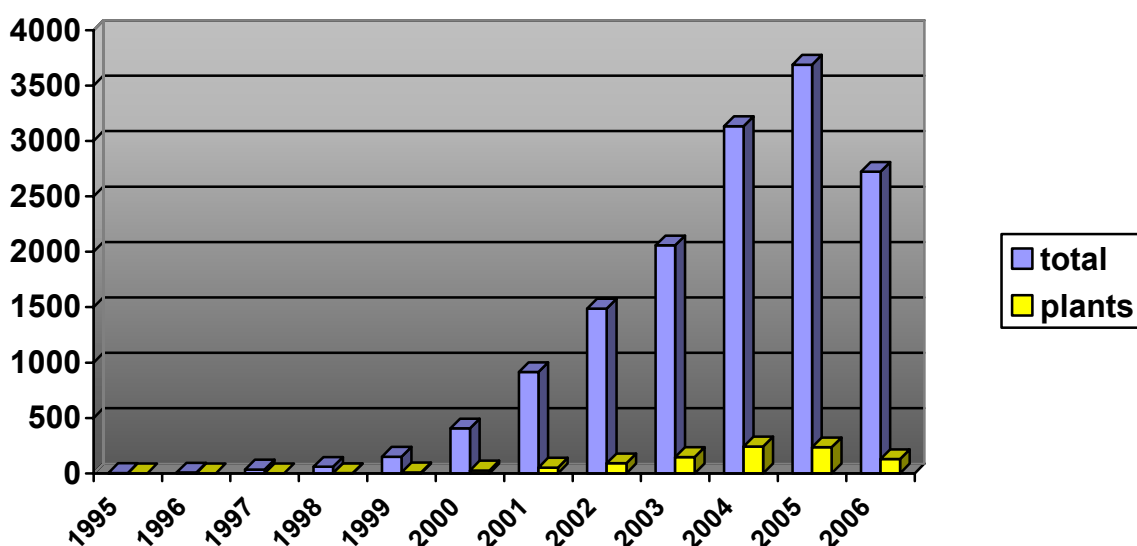


Figure 1: Number of publications in PubMed (2006.08.17) about proteomics and plant proteomics (including keywords proteomics, proteomic and proteome) since 1995.

Currently, many European laboratories that are involved in plant proteomics in model plants (i.e. *Arabidopsis thaliana*, *Medicago truncatula*), in crop species or in woody plants, are developing specific techniques for the extraction of proteins from different tissues or organelles. In other laboratories, efforts are put in the development of quantitative methods for the detection of variation in protein synthesis; some are working on more efficient methods of identification of proteins or PTMs; and finally some are focusing their research on deciphering plant physiological processes, involved in plants

relationship with their environment, or in plant bioproduct synthesis and accumulation for food, feed or wood quality. But it is the clinical and pharmaceutical research community, supported by important companies, that mainly drives the development of new techniques for proteome research. Launched in 2001, a global initiative was set up to study the human proteome (HUPO), ‘an international organization finally bringing discipline to the study of cells’ sets of proteins’ according to the journal Nature (n° 437, pp. 169-170) and regrouping proteomic societies dealing with human samples all around the world.

More recently, a European Proteomics Association (EuPA) was founded, regrouping national societies of proteomics in Europe, promising relations with FEBS and EMBO, and being ‘a network platform for HUPO’ as announced on their website (http://eupa.isb-sib.ch/images/stories/minutes/eupa_gc_20051205.pdf). The plant research community is called upon to generate appropriate networks and organize and coordinate initiatives in a manner similar to the HUPO consortium. This sort of initiative is both desirable and necessary to join and maximize efforts of the whole community, including laboratories at universities, public research centres, and companies worldwide. So far, there exists no such organization at a European level for plant proteomics. Only in France, a network called the “Green Proteome” (Protéome vert: <http://www.pierroton.inra.fr/genetics/2D/Proteomevert/>) was established. It gathers a large community of plant proteomists, who meet each year since 1999 to share their knowledge and skills. With respect to this proposal, more than 80 persons are currently registered denoting their interest in participating to a long-term structure where experiments on innovative techniques and applications can be discussed.

Experience in new and accurate technologies such as differential gel electrophoresis, multidimensional liquid chromatography, characterization of protein-protein interactions, protein identification, and bioinformatics will be extremely interesting to put together. Continued technological development is required to sustain a rapid evolution of plant proteomics.

In view of the significant expertise already present in different European laboratories and large amount of work needed to accelerate the production of knowledge in plant sciences, the aim of the present application is to develop a united European community in the field of plant proteomics, where information and skills can be shared and young scientists can get an effective training. A critical mass will be obtained only through a consortium of different institutes located in European countries bringing together their collective experience on (1) plant protein extraction techniques, (2) gel-based protein separation, (3) gel-free protein separation, (4) MS identification of proteins and (5) plant biology.

The flexibility of the COST Actions allows the co-ordination of nationally funded research on a European level. COST is therefore the instrument of choice to bring together European plant proteomics specialists.

C. Objectives and benefits

Objectives

The main objective of the Action is improvement and exchange of scientific knowledge and technology in plant proteomics through the creation of a network between European proteomic scientists.

This Network aims to deepen cooperation in Europe, by regular meetings, scientific contribution to international conferences and collaboration with industries, in order to comply with the secondary objectives:

1: Develop **efficient protocols** to study plant proteomes. Currently, a lot of protocols are available and diversely used depending on the tissue, the species or the subcellular compartment. For example, a good separation of proteins through gel electrophoresis or liquid chromatography, is strongly depending on efficient extraction protocols. Therefore, an exchange and optimisation of efficient protocols to carry out these challenging steps is essential.

2: Promote the **use of proteomic tools** in a wide variety of research fields. These include:

- study of the genetic diversity,
- proteomics assisted breeding,
- field study for optimal use of arable lands (e.g. nutrient use efficiency, biotic and abiotic factor tolerance),
- study of symbiotic relationships between plants and microorganisms,
- detection of genetically modified organisms in field and food,
- metaproteomics (population proteomics).

3: Generation of fundamental knowledge of **protein-protein interactions**. Physical interactions between proteins in the same cell or variation of structure and/or composition in protein complexes in various biological conditions are of the utmost importance to unravel the plant regulatory networks and metabolic pathways.

4: Study the effect of **post-translational modifications** on metabolic pathways correlated to organogenesis, cell signaling and abiotic/biotic stress responses. Phosphorylations, ubiquitylations, thiolations, among others PTMs, are critical for the activation and function of most proteins. Many technical restrictions need to be overcome for an efficient identification of PTMs.

5: Integration of proteomic data in systems biology via **bioinformatic tools**. Bioinformatics is not only needed for the identification or the quantification of proteins, but it is also required to describe and validate the experimental data, implying the unification of data presentation and the standards parameters to allow published proteomic data to be readily comparable worldwide. After standardization, the data could

be integrated with transcriptomic and metabolomic results, and ultimately with physiological and morphological traits.

6: Increase of the information to **public and industries** for new technologies by popularization of plant proteomics. This implies the publication of articles on the technique and its applications in non-scientific journals targeting the general public.

Benefits

Benefits to the scientific community

The main benefit of this programme is to increase the competitiveness of the European plant scientific community through the promotion of useful interactions and the development of new techniques on plant proteomics. Exchange of published and non-published information, young researchers or material, as well as sharing proteomic facilities during short-term missions will contribute to enlarge the field of application of proteomics and to enhance progress in the understanding of the plants' life cycle. The creation of such a network that brings together the expertise from a wide variety of partners, will lead to the establishment of further EU research projects (for example FP7). Among the expected benefits for plant science are its importance in systems biology to evaluate crop plants, the use for safety assessment of novel foods, and plant-based production systems for valuable compounds, i.e. "molecular pharming".

Benefits to the society

Proteomics in general offers means to improve competitiveness of the European bioindustries. It can potentially enhance the efficiency and control of processes used by the biotechnology industry to produce bioactive compounds, to help in the discovery of biomarkers for multipurpose, and targets for drug development. Among the expected benefits of proteomics for plant science are its importance in systems biology to evaluate crop plants, the use for safety assessment of novel foods, and plant-based production systems for valuable compounds. New advances in protein separation, sample pre-fractionation and identification are expected to be made publicly available through this Action. Moreover, proteomics offers unique opportunities for improvement of public acceptance and understanding for new developments in science. This is necessary for further implementation by the industry.

Benefits to the consumers and environment

It is expected, that as part of this effort, proteomic methods will be applied for GMO safety evaluation and detection of GMO traces in the food chain. Thus consumers will benefit from up-to-date effort on GMO evaluation considering new scientific methodologies. Additionally, proteomic research of GMO and the surrounding plant populations is expected to be enhanced the understanding of the GMOs on the environment. Overall, the application of new post-genomics methodology such as proteomics will be beneficial for entire society. This will have consequences on food and feed quality, and importantly by decreasing the use of chemicals and pesticides. The development of plant varieties presenting higher tolerance characteristics to stress factors and the changing environment (e.g. biotic factors caused by pest and diseases as well as

abiotic factors like drought, frost, salinity, ...) using the proteomic approach would also help to decrease the quantity of chemical intrans and pesticides, by favoring sustainable agricultural practices and producing quality products.

Benefits for the employment

In long term, development of new proteomics methodologies along with exploration of new fields might lead into establishment of new “start-up” companies, or in enhancement of activities of existing biotechnology industries. European high-quality training will increase the skills of young scientists in several proteomic approaches widely used in pharmaceutical and medical industry and currently expanding in food quality (e.g. Monsanto or Dow AgroScience). Some companies have also expressed their interest in the activities of a putative network of European plant proteomists.

Benefits for EU research

The COST Action will strongly facilitate the diffusion of technical and scientific knowledge, making the EU consortium at the same level like some Asian or South Americas countries (for instance South Korea or Brazil), who have recently invested in important plant genomic platforms including proteomics.

This COST Action will also involve former Eastern European countries with the newest techniques on proteomics. Proposed COST Action will establish the basis for intensive collaboration between ‘old’ and ‘new’ EU countries, which is crucial for European scientific and technological development.

Benefits for reaching the millennium Developmental Goals (MDG)

Important challenges of the Millennium Development Goals are (i) to address hunger with respect to food availability and (ii) to ameliorate the incipient issues caused by unbalanced diets. Many of the objectives described in this project proposal will lead to the development of “superior” plants that can help in meeting the Millennium Development Goals. More specifically we think at the development of drought tolerant plants, a safe use of GMOs, nutritive quality of food ...

D. Scientific programme

To achieve the different objectives, the proposed scientific programme has been discussed with all scientists interested to take part in this COST Action.

The biological function of plants is determined by the action of extremely complex networks of interacting biomolecules. By linking gene expression to cell metabolism on one hand and to genetic maps on the other, proteomics is a pivotal tool for functional genomics, resulting in insights on evolution and on the regulation of gene expression. However given the enormous technical challenges and the need for information exchange, joined efforts are urgently needed to improve techniques and to increase the standardization.

Two working groups (WGs) have been identified (Fig 2): a first WG will deal with the technical aspects inherent to plant proteomics. From the start, a large diversity of crops (not only the well known model plants such as *Arabidopsis*, *Medicago* or *Populus*) will be involved since for most such plant models many efficient techniques have already been developed. For more sophisticated techniques model plants will still be used. The second WG will focus more on the implementations. The latter includes proteomic tools in fundamental plant biology (e.g. tolerance to environmental changes), as well as in agronomy (e.g. characterizing and exploiting genetic diversity of crops or forestry species). Due to the complexity of this research, focus in this WG will initially be put more on model plants, since many techniques to analyse the proteome are already available. Moreover DNA sequences are known which makes identification of proteins (e.g. through PMF) much easier. In a later phase, results will be validated with non-model species.

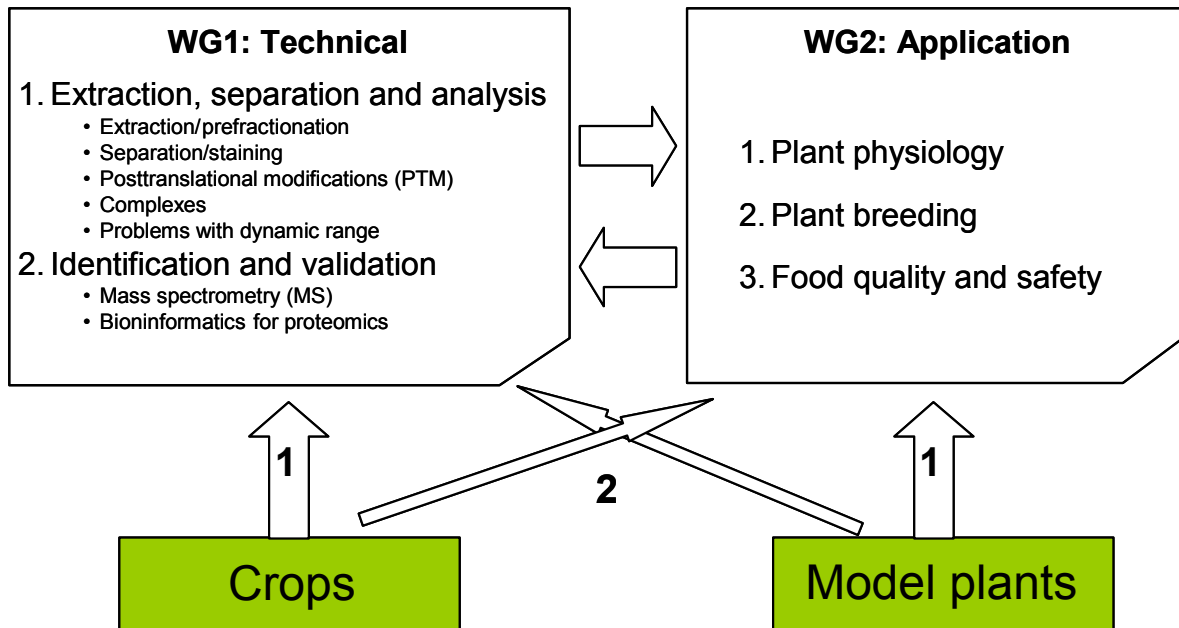


Figure 2. Interactions between WG1 and WG2

These working groups will be strongly linked together. Indeed, the development and/or improvement of efficient protocols will be beneficial for the application of the methods to the different fields covered in the second WG. In the same time, the results obtained in the second WG will also define new requirements for technical improvements through the expertise of the first WG.

Working group 1: Technical aspects of plant proteomics.

Techniques in plant proteomics are mainly focused on the separation and the identification of proteins. Different levels of complexity in the technical aspects of plant proteomics should be tackled. In first rank is the diversity of plant species used in this Action (e.g. *Arabidopsis thaliana*, *Brassica napus*, *Medicago truncatula*, *Pinus sp.*, *Pisum sativum*, *Populus sp.*, *Solanum tuberosum*, *Solanum lycopersicon*, *Triticum aestivum*,...). Then, the technical complexity decreases from the organs, cellular compartments, protein complexes, proteins, peptides and amino acids, common to all plants.

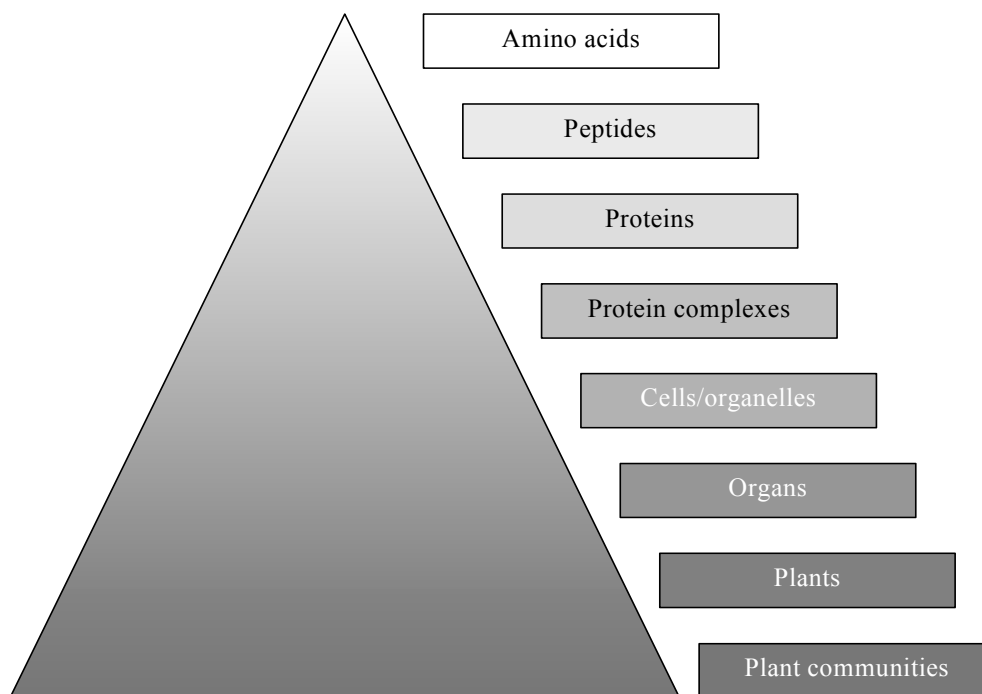


Figure 3: Levels of complexity involved in proteomics research

In this WG1, 2 main axes of research can be defined:

- Extraction, separation and analysis
- Identification and validation

1. Extraction, separation and analysis

This topic will mainly cover the issues of standardization of protocols aiming at a better separation of proteins. To address these issues, specific methods will be used to obtain the best resolution of targeted tissues, cells or subcellular compartments.

1) Extraction/prefractionation

Protein extraction can be considered as the most critical step for protein analysis. In plants, some tissues present a relatively low abundance of proteins, while they are rich in proteases and other components interfering with protein separation (Saravanan and Rose 2004). The **compounds** that **interfere** with protein extraction are as diverse as cell wall components, lipids, polysaccharides, phenolic compounds, lignin, waxes and other secondary metabolites that can lead to protein degradation or precipitation during the tissue disruption (Cánovas et al., 2004).

However, the optimal extraction procedure for a specific sample depends on the aim of and the techniques used in an experiment. In studies focused on the response of plants to biotic or abiotic stress, receptors present on/in **membranes** are the first sites to initiate stress signalling. While soluble proteins can readily be isolated, **hydrophobic proteins** (often membrane proteins) are poorly dissolved with a classical extraction protocol. In this case, preliminary enrichment of membrane proteins can be required. In some experiments, dissection of plant cells and pre-fractionation of **subcellular compartments** can be required (e.g. isolation of chloroplasts, mitochondria, nuclei).

2) Separation/staining

After their extraction and isolation, proteins will be solubilized and separated by **gel electrophoresis** or by **liquid chromatography**. An appropriate protocol needs to be determined to obtain an adequate and reproducible separation. Optimization of these protocols depends on the sample itself and on the technique used. Similarly for the staining, a broad range of protocols are available today (silver nitrate, Coomassie, fluorophores, ...)

The Action will be focused also on implementation of new electrophoretic techniques such as Fluorescence Difference Gel Electrophoresis (DIGE) (Unlu et al., 1997) or Pro-Q Diamond or Pro-Q Emerald fluorescence stains used for **phosphoprotein** and **glycoprotein** detection, respectively. The DIGE technology relies on protein labeling using CyDye fluorophores (Cy2, Cy3 and Cy5) and the separation of maximum 3 samples on the same gel. The advantage of this technology is the elimination of gel-to-gel variances and high sensitivity. Besides the presence/absence and the exact state of the proteins, cellular physiology is determined by the abundance and the ratio of proteins. Until relatively recently, 2-DE based proteomics quantification was reliable to quantify these ratios. Recently, an absolute quantification method using stable isotope labeling (Stemmann et al., 2001) was commercialized (AQUA, Thermo-Finningan). Additionally, stable isotope reagents such as ICAT or iTRAQ (Applied Biosystems) are available as well. The iTRAQ method uses four isobaric amine specific tags (Ross et al., 2004) for relative quantification. Most recently, implementations of comparative LC-MS algorithms enable highly detailed quantitative comparison of complex LC-MS datasets originating from a multitude of proteomics samples, also without the use of stable isotope labels (America et al., 2006). The proposed COST Action aiming also in implementation

of these new MS-based protein quantification methodologies to day routine of European plant proteomics laboratories.

3) Posttranslational modifications (PTM)

While transcriptomics allows determining which genes are activated, it offers little information on the actual state of gene products under specific growth conditions. In living organisms the cellular activity is governed by an intricate system of protein synthesis and degradation, modification, translocation and the formation of complexes. Many of these regulatory processes involve the formation or breaking of covalent bonds, so called posttranslational modifications (PTMs). Among the PTMs, processes such as removal of signal peptides, nitrosylation, glycosylation and phosphorylation are well studied; on the opposite, other PTMs are less ubiquitous or have not been studied because of the lack of appropriate techniques.

4) Complexes

Most physiological processes are not carried out by single proteins, but rather by protein assemblies (Alberts, 1998). The importance of protein complexes is clearly illustrated by their involvement in different physiological processes in plants like the cell cycle, cell proliferation, protein synthesis or respiratory system. Complex formation and activity is strongly regulated by parameters such as the participating components, cofactors, phosphorylation/dephosphorylation events and interaction with inhibitory or activating proteins. To date, protein-protein interactions are widely studied by techniques like tandem affinity purification (Rigaut et al., 1999), co-immunoprecipitation (Goodwin et al., 1993) and two hybrid screens (Fields and Song, 1989). These approaches revealed many protein-protein interactions, but a significant drawback exists in that they either depend on the foreknowledge of genomic data, on the availability of antibodies or are restricted to binary cytosolic interactions. A bio-analytical display method capable of presenting protein complexes in a context with other cellular complexes is highly desirable. An alternative is the BN-PAGE to study protein complexes at a proteome scale for most of the plant models with an unsequenced genome.

Blue Native gel electrophoresis (BN-PAGE) is a non-denaturing method used to separate protein complexes in their native form (Schagger and von Jagow, 1991). So far, BN PAGE has been mainly used to analyze pre-fractionated cellular extracts.

5) Problems with dynamic range

Because of the limited dynamic range of the different techniques currently used for protein separation, visualization and identification, the presence of a highly abundant protein often precludes the detection of low abundant proteins. The use of different staining procedures, both visible and fluorescent (Chevalier et al., 2006), can only partially resolve this problem. For plants, the elevated abundance of ribulose biphosphate decarboxylase/oxygenase (**Rubisco**) prevents an in-depth analysis of the proteome of green tissue in the same way as the abundance of albumin and immunoglobulin hinders the analysis of blood samples.

2. Identification and validation: the role of mass spectrometry and bioinformatics

This topic will focus on the protein identification techniques aiming at defining the best strategy in different experimental schemes with species whose genomes are sequenced and with non-model plant species. Special emphasis will be put on the support of bioinformatics.

1) Mass spectrometry (MS)

MS, an analytical technique used to measure the mass-to-charge ratio of molecules, has emerged as a key platform technology in proteomics. Recently the impact of the development of MS-techniques was honoured by attributing the 2002 Nobel Prize in Chemistry to Fenn and Tanaka, respectively for their contribution to the development of matrix-assisted-laser-desorption/ionization MS (**MALDI**) and electrospray ionization MS (**ESI**).

MALDI MS utilises laser pulses to ionise and volatise peptides that have been embedded in a dry, crystalline, organic matrix. The high resolution and throughput attained by MALDI-TOF made it the most important instrument in the identification of proteins with the **peptide-mass-fingerprint** (PMF) method. In this method the masses of peptides resulting from the digestion of a protein with sequence-specific proteases are submitted in database searches. Several software packages that compare theoretical peptide mass fingerprints from entries in public protein sequence databases with the experimentally determined fingerprint are available. These algorithms can also be used for identification using translated genomic and expressed sequence tag (EST) databases. The disadvantages of PMF are that the sequence of the protein must be known and that any mass change of a specific protein (e.g. PTM) may hinder protein identification.

When identification by PMF is inconclusive, structural information from peptides may provide sufficient information to allow identification. This can be obtained by fragmentation in **tandem mass spectrometry** (MS/MS). In MS/MS, an ion with a specific m/z value is selected, collided with inert gas molecules and the fragment ions resulting from these collisions are detected after passing through a second mass-analyzer. This process produces fragment ions that are representative for the sequence of the peptide, allowing the determination of a portion of the peptide sequence used for database searching.

During **ESI/MS**, ions are formed from a liquid phase, allowing easy coupling to liquid phase separations. While MALDI predominately results in the formation of singly-charged ions, the ions formed during ESI are most often multiply charged. Because of the better fragmentation properties of ions containing more than one charge, ESI was most used in protein identification, a dominance now alleviated by the development of MALDI-instruments with real MS/MS capabilities.

The genomes of most of the plants used in this COST Action have not been fully sequenced yet. Because protein **identification** relies on matches with sequence databases, high-throughput proteomics is currently restricted to those species for which comprehensive sequence databases are available. For this reason, *Arabidopsis thaliana* is considered as the ideal model plant to start analyzing the response of proteomic networks to genetic and/or environmental perturbations (Weckwerth et al., 2004; Wienkoop et al.,

2004). This allows the use of 'gel-free' **shotgun proteomics**, fully automated two-dimensional nano-liquid chromatography coupled to an ion trap MS or MALDI MS and database-searching algorithms, to rapidly generate a global profile of the proteins present in the sample (Peng et al., 2003).

However, working with unsequenced genomes, as most crops or tree species, implies the identification of proteins by either manual or automated **de novo peptide sequence determinations** and the use of the generated sequences for cross-species protein identification.

The importance of **PTMs** for the description of the biological function of proteins, and thus for the determination of the physiology of a tissue, was already mentioned. Because PTMs results in a characteristic mass shift of the modified protein/peptide, MS is the most common way to characterize PTMs.

2) Bioinformatics for proteomics

The field of proteomics yields a vast and large amount of highly heterogeneous data. Various informatic and bioinformatic resources are required to store, process, analyse and share proteome data (Kremer et al., 2005). Typical workflows in proteomics laboratories involve the use of different software applications and databases that are operated in a sequential way. This can be automated by devising pipelines. Manufacturers often provide analysis pipelines specific to their instruments which are most of time not compliant with software from competitive companies. Furthermore, proteomics labs generally use instruments from different vendors, complexifying the design of pipelines. The community also offer increasing number of alternative software and pipelines to help user to process data (Kiebel et al., 2006; Rauch et al., 2006). Despite the significant contributions, no standard data processing protocol have emerged yet and laboratories face the tedious problem of choosing the appropriate software and then to implement them in the laboratory.

Other problems that require considerable efforts from bioinformatics: the mining of mass spectra to reveal protein modification, the validation of novel identifications (Shadforth et al., 2005), the improvement of de novo sequencing algorithms, the description of experiments and associated data using standardized terminology and format (Pedrioli et al., 2004; Taylor et al., 2006). To resolve this, it will be crucial to combine datasets, share data and integrate them with gene expression, metabolic, phenotypic and genotypic data.

Ultimately, with the holistic approach, the goal of integrating metabolite and protein data and applying multivariate statistics at a systems biology level (Weckwerth et al., 2004; Cassman 2005) will be reached. The integration of the data will lead to an improved interpretation of the experiments, and to biomarker identification (Morgenthal et al., 2005).

Objectives of WGI:

- Develop efficient extraction protocols for a wide variety of plant species/tissues with special emphasis on membranes, hydrophobic proteins and subcellular compartments,
- Evaluate the advantages of the different staining methods and propose standardized methods,

- Develop user-friendly methods for a rapid characterisation of PTMs and quantification of the different isoforms in the same sample and in different samples,
- Develop and improve protocols that can determine interactions between proteins or the structure of protein complexes,
- Improve the dynamic range to detect less abundant proteins,
- List and evaluate the existing tools for bioinformatics,
- Recommend a set of evaluated software or/and propose new developments when no satisfying solutions were found,
- Develop tools for the database-independent identification of proteins from MS data, and de novo sequencing.
- Compare different methods of MS-based protein quantification (e.g. ICAT, SILAC, iTRAQ, AQUA, direct methods of quantification without the aid of stable isotopes) and define their different domains of application,
- Define the best strategy for protein identification in species whose genomes were not extensively sequenced.

Working group 2: Applications of plant proteomic tools

This working group will mainly focus on the fundamental and applied research fields in which proteomics is included as a part of the system biology approach.

In this working group, emphasis is put on the study of plant developmental processes in relation with environmental key constraints together with a special interest for plant products in terms of quality and safety. An understanding of the mechanisms by which plants develop and resist infection or mount a defensive response, or increase tolerance ability is vital from an agricultural standpoint. Thanks to spectacular advances in the techniques for identifying proteins separated by two-dimensional electrophoresis and in methods for large-scale analysis of proteome variations, proteomics is becoming an essential methodology in various fields of plant biology. In the study of pleiotropic effects of mutants and in the analysis of responses to hormones and to environmental changes, the identification of involved metabolic pathways can be deduced from the function of affected proteins. In molecular quantitative genetics, proteomics can be used to map translated genes and loci controlling their expression, which can be used to identify proteins accounting for the variation of complex phenotypic traits. Linking gene expression to cell metabolism on the one hand and to genetic maps on the other, proteomics is a central tool for functional genomics.

Plant proteomics should culminate in knowledge and tools/assays that can be practically applied in diverse research fields with direct impact on agriculture, breeding, biotechnology and /or consumer quality issues.

One can distinguish applications in the following fields:

1. Plant physiology

Several physiological processes involved in different phases of plant development of various plant species (crops, legumes, cereals, trees etc) are being studied at a proteome level. This includes:

- Plant reproduction: fertility/sterility, seed setting, seed quality, germination,
- Growth regulation: hormone response, fruit setting, ripening, photosynthesis,
- Stress response to abiotic stresses: temperature, salinity, drought, light, pollution, soil mineral depletion,
- Stress response to biotic stresses: aerial and soil-borne microbial pathogens, insects, nematodes, beneficial microorganisms such as arbuscular mycorrhizal fungi, Plant Growth Promoting Rhizobacteria, nitrogen fixing bacteria.

These researches aim towards the comprehension of the biochemical and molecular events underlying all the physiological processes briefly listed above together with mechanisms involved in plant responses to various environments. Identification of protein markers also opens perspectives for plant breeders to select agronomical important traits and to develop crops for low input systems.

2. Plant breeding

The very helpful property of proteomics for distinguishing expressed genotypes, even in closely related backgrounds, will be used to investigate the genetic variation in relation to the above-mentioned physiological processes. In this respect, development of all types of quantitative proteomics will be of importance in order to help searching “candidate proteins” i.e. proteins whose quantitative variation can be responsible for the variation for phenotypic traits. These different aspects of applications aim to determine proteomic markers linking genotype to phenotype, and furthermore to help breeders to select crops presenting a better adaptation to a changing environment.

3. Food quality and safety

Food quality is a major socio-economic concern. In this area, plant proteomists are giving an active contribution by identifying proteins controlling interesting traits. Proteomics applied to plant product quality is based on the characterization of protein/enzymes involved in metabolic pathways leading to production of molecules of interest together with the assessment of harmful plant compounds as well as GMOs detection.

Plant proteomics for food quality mainly investigate proteins involved in genetic variation of:

- Organoleptic quality (aroma, content in sugars, weight and texture, pigments),
- Health promoting compounds (anti-oxidants, unsaturated fatty acids, vitamins, essential amino acids),
- Health affecting compounds like allergens, glutenins and toxins,
- Factors affecting quality during storage,
- Prediction of ripening timing to market chain,
- Yield and quality of animal fodder.

The assessment of plant allergenicity with particular attention to glycosylation, plant toxicity and GMO detection is also an important part of plant proteomics, covered by several groups that will strongly benefit from the framework of this COST action.

In total, the fields of applications of proteomics in plant research are quite diverse. The aims of this WG2 are to bring together these different fields of applications in order to improve knowledge transfer and stimulate new research initiatives bringing proteomic technology into practical applications for improvement of plant growth, plant/food quality and food safety.

Objectives of the WG2

- Communicate and disseminate knowledge of proteomics applications in diverse fields of plant research,
- Integrate of knowledge at several levels from physiology, biochemistry, genomics, microbiology, breeding, growing and (industrial) processing,
- From black numbers to green practice: bidirectional communication of plant proteomists with commercial partners, like breeders, growers, food processors, etc. Identifying needs and opportunities for future collaborations. (this may be organised in dedicated workshops),
- Investigate possibilities for standardisation and linking of multiple datasets generated within the community (as far as publicly available) enabling meta-level datamining and data integration.

**

These working groups will lead to scientific publications in international peer-reviewed journals and to a book dedicated to plant proteomics, protocols and applications. A public forum website will allow discussion about technical aspects of analyses. Articles published in non-scientific periodicals will target general public.

In both working groups, special emphasis will be put on organization of training for young researchers and practical courses on new techniques. This action is expected to lead to a long-term networking at the European level by creating the basis of a plant proteome association.

E. Organisation

The Action will be coordinated by the **Management Committee (MC)** that is preceded by a chair and vice-chair. For organisational purposes, two **Working Groups (WGs)** will be established; WG1: technical aspects of plant proteomics and WG2: applications of plant proteomic tools.

Each of the **WGs** will be managed by a WG co-ordinator. These WG co-ordinators will have as main tasks:

- Participate in the plenary and restricted meeting of the MC
- Plan the appropriate scientific meetings
- Co-ordinate the activities within their WG in order to meet the objectives that are defined in the scientific programme
- Promote the set-up of joint research (e.g. making use of short-term scientific mission)
- Promote the writing of common publications
- Report the WG progress to the Action chair and MC

Meetings of the WGs will be organized on a 10 monthly basis at different partner locations. These frequent gatherings are planned in order to have an optimal exchange of ideas. It is planned to hold two to three days meetings for each WG. The first one to two days would be devoted to specific WG activities. This would allow the exchange of information and ideas, encourage the collaboration between scientists and institutes, stimulate the planning of joint experimental work and will address WG specific topics. The last day of the meeting would be combined with the other WG. This would greatly enhance integration of activities from the different fields, and promote interface between WGs. Indeed, the results obtained in the first working group on the standardization and optimisation of plant proteomic protocols will be extremely helpful during the application of these protocols by the partners involved in working group 2. Feedback from the partners of WG2 will also be essential to define new challenges and technical requirements for the working group 1.

The **MC** that coordinates the Action will have as main responsibilities:

- Appointment of chair, vice-chair(s) and WG co-ordinators
- Planning and coordination of the different meetings: MC meetings, scientific meetings as well as workshops
- Assessment of the different activities (such as meetings, short-term scientific missions, publications, training schools, etc.) in order to meet the general objectives defined for this Action
- Report of the progress made by the different WG to meet their respective objectives in the framework of the Action
- Promotion of collaboration and of exchange of knowledge (and data) between the partners from the different WGs
- Promotion and approval of short-term scientific missions according to the recommendations of an established ad-hoc committee

- Creation and regular updating of a website in order to enhance communication between partners and to disseminate the results generated in the different WGs
- Coordination and facilitation of all efforts that can lead to the preparation of research project related to plant proteomics. Our aim is to establish at least one EU Seventh framework project.
- Preparation of annual reports
- Dealing with matters related to IPR and the possibilities for exploitation and dissemination of project results.
- Organization of contacts and common workshops with appropriate ongoing COST Actions and other research frameworks such as ESF, EUREKA or the EU Framework Programmes to address problems of common interest. Cost Actions of interest are e.g. Phytotechnologies to promote sustainable land use management and improve food chain safety and Cryopreservation of crop species in Europe)

Meetings of the MC will take place once a year, preferably linked with the WG meetings or with workshops. This will insure efficient coordination of the activities and discuss about the objectives and critical points of the programme.

Short-term scientific missions will enhance exchange of knowledge (technology transfer) and moreover strengthen the collaborations between partners. An ad-hoc short-term scientific mission evaluation committee will be appointed by the management committee with one co-ordinator and one representative of each working group. This committee will favour the mobility and training of **young researchers**, with a special accent put on the less-developed EU regions (below 70% of EU'GDP average) and EU accession countries (Bulgaria, Romania, Croatia and Turkey).

Training schools and summer courses have already been randomly organised in some laboratories that showed their interest in this Action. A training school will be organised more systematically in the framework of this COST Action. A practical proteomics course will be organised as an intensive one-week training available to anyone from participating cost countries who wants to acquire hands-on experience of modern proteomic techniques. The aim of training schools will be to build up on good proteomic practices. Training schools will demonstrate excellent practices across the range of training activities.

The 80 researchers (scientists and engineers from more than 45 institutions) willing to take part in this COST Action are representing a unique panel of experts in plant proteomics, plant development, plant stress physiology, mass spectrometry, sustainable agriculture and food safety assessment. This consortium of both academic researchers and institutional partners has the necessary expertise, updated technical infrastructure and technological know-how to successfully address all scientific aspects of this proposal.

F. Timetable

- The proposed duration for this Action is **4 years**.
- The **Kick-off meeting** will start the Action and during this meeting, the WG coordinators will be selected.
- The **homepage** will be created soon after this kick-off meeting and will be updated on regular basis (4 times per year).
- **Each WG** will hold a **meeting every 10 months**. Since the two WG meetings will have a one day overlap, specific combined meetings will not be organised.
- **Training schools** of the two WG will be organised every year.
- **Plenary management committee meeting** will take place once a year. It will be linked to the meeting of the working groups or during a workshop.
- **Short-term scientific missions** can be requested anytime after the first WG meeting.
- **Inter-COST Meetings** will be held to address problems at the interface of WGs and ongoing COST Actions, and will allow for the cross-fertilisation of outputs and ideas.
- The Action will be closed with a **final conference**, combining a meeting where all the partners involved will present their results.

Table 1. Timetable

	Year 1				Year 2				Year 3				Year 4			
Coordination	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Kick-off meeting	■															
Homepage	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Reporting				■				■				■				■
MC meeting	■					■				■						■
WG1 meeting			■			■				■				■		
WG2 meeting			■			■				■				■		
Inter-Cost*																
Training schools							■				■				■	

STSMs				■	■	■	■	■	■	■	■	■	■	■	■	■
Final Conference																■

MC meeting: Management committee meeting

WG meeting: Working group meeting

STSMs: Short-term scientific missions

Inter-Cost*: Timing of the Inter-COST Workshops will be defined in agreement with the Management committee of that specific Action.

G. Economic dimension

The following 21 COST countries have actively participated in the preparation of the Action or otherwise indicated their interest: AT, BE, BG, CH, CZ, DE, DK, ES, FI, FR, GR, IT, LU, NL, NO, PL, PT, SE, SI, SK, UK. It is expected that more participants will join this Action. For example, shortly before the submission of this proposal, contacts have been established with Turkish laboratories.

On the basis of national estimates provided by representatives of these countries, the economic dimension of the activities to be carried out under the Action has been estimated at roughly EUR 64 Millions for the total duration of the Action.

This estimate is valid under the assumption that all the countries mentioned above, but no other countries, will participate in the Action. Any departure from this will change the total cost accordingly.

H. Dissemination plan

Press releases

The first press release will be organised on the occasion of the kick-off MC meeting in all the participating countries.

Publications

- a. Scientific results of the project will be disseminated through **refereed scientific journals** provided that publication does not impair IPR. The MC will promote co-publications as much as possible.
- b. The publication of **Common review articles and book chapters** will help to disseminate results to a broader, less specialized public.
- c. **Technical guidelines** focussing on one or more technical aspects related to plant proteomics, and that mainly linked to the activities of WG1 will be produced.
- d. At the end of the Action it is foreseen to publish at least one **scientific book** that gives an overview of the most important results. Again the MC will take the lead in this initiative.

Website

A public website will be made available to provide all kinds of information to the international scientific community but also to facilitate communication flow between the partners of this project. This website will be kept active by one designated partner in this Action. Part of the website will be available to everyone while other information (for example new non published protocols) will be on webpages that are protected with a password.

Content of the website

- General information about COST and this Action (activities, meetings, ...)
- Publications and contact information for Action participants
- On-line courses, proceedings of meetings, talks and posters from meetings
- STSM Calls and Reports
- Teaching tools (e.g. slides, course notes, protocols)

- Links to the websites of the participating institutions and websites related to proteomics
- Job announcements

Workshops

The consortium will organize workshops to inform interested scientists, regulatory bodies and policy makers about the results of the project and about new technologies developed throughout the project. These workshops will provide hands-on practical training as well as theoretical information.

WG meetings

WG meetings will be combined as much as possible with MC meetings and will be organised in different geographic regions to allow participants from all regions to come into contact with the European technical know-how.

STMS

Short-Term Scientific Missions (STSMs) will be offered to young scientists and scientists from developing regions. STSMs will facilitate technology transfer within the Action and training in new techniques or shared use of critical equipment or field sites.

International Conferences

Knowledge and data resulting from the COST Action activities will be integrated and presented at International Conferences. This will promote the European know-how and increase the international collaboration.

Teaching activities

Teaching activities in Universities at undergraduate and post-graduate level will also take advantage of the knowledge and experience acquired during this COST Action. Young scientists and engineers will thus be trained and informed on the latest developments in proteomics

Part II: Additional information

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C. Selected publications of participating experts

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