REVIEW

Plant proteome analysis: A 2004–2006 update

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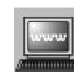
Since the appearance of the review entitled "Plant Proteome Analysis" in Proteomics in February 2004 (Cánovas, F. M., Dumas-Gaudot, E., Recorbert, G., Jorrín, J. *et al.*, *Proteomics* 2004, *4*, 285–298), about 200 original articles focusing on plant proteomics have been published. Although this represents less than 1% of the global proteomics output during this period, it nevertheless reflects an increase in activity over the period 1999–2004. These papers concern the proteome of at least 35 plant species but have concentrated mainly on thale cress (*Arabidopsis thaliana*) and rice (*Oryza sativa*). The scientific objectives have ranged from a proteomic analysis of organs, tissues, cell suspensions, or subcellular fractions to the study of plant development and response to various stresses. A number of contributions have covered PTMs and protein interactions. The dominant analytical platform has been 2-DE coupled to MS, but "second generation" techniques such as DIGE, multidimensional protein identification technology, isotope-coded affinity tags, and stable isotope labeling by amino acids in cell culture have begun to make an impact. This review aims to provide an update of the contribution of proteomics to plant biology during the period 2004–2006, and is divided into six sections: introduction, subcellular proteomes, plant development, responses to biotic and abiotic stresses, PTMs, and protein interactions. The conclusions summarize a view of the major pitfalls and challenges of plant proteomics.

Received: April 7, 2006
Revised: June 23, 2006
Accepted: June 23, 2006



Keywords:
Environmental proteomics / Plant proteomics / Post-translational modifications / Protein interactions / Subcellular proteomics

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Abbreviations: BN, blue-native; **CN**, colorless-native; **COMT**, caffeic acid/5-hydroxyferulic 3-*O*-methyltransferase; **DNP**, 2,4-dinitrophenylhydrazone; **GA**, gibberellin; **GPI**, glycosylphosphatidylinositol; **GPI-AP**, GPI-anchored protein; **GSH**, glutathione; **HSP**, heat-shock protein; **ICAT**, isotope-coded affinity tags; **iTRAQ**, isobaric tags for relative and absolute quantitation; **MudPIT**, multidimensional protein identification technology; **PCD**, programed cell death; **PLC**, phospholipase C; **PLD**, phospholipase D; **PR**, pathogenesis-related; **ROS**, reactive oxygen species; **RubisCO**, ribulose-1,5-bisphosphate carboxylase/oxygenase; **SA**, salicylic acid; **SILAC**, stable isotope labeling by amino acids in cell culture; **SOD**, superoxide dismutase; **SPR**, surface plasmon resonance; **SUMO**, small Ub-related modifier; **Trx**, thioredoxin; **Ub**, ubiquitin

1 Introduction

An "ISI Web of Knowledge" search covering the period February 2004–February 2006, using as a search string "proteomics or proteome", generated 6044 hits. A greatly reduced number was obtained by substituting the strings "plant and proteome" (125) or "plant and proteomics" (112). A similar relationship was obtained between global and plant-specific entries to the protein databases UniProtKB/Swiss-Prot (212 425 *vs.* 14 614) and TrEMBL (2 666 963 *vs.* 212 823). Nevertheless, activity within the area of plant proteomics, as measured by publication output, has increased significantly since the period 1999–2003, when only 100 hits were registered on ISI. A manual search of various biochemistry, proteomics, and plant science journals has identified in the region of 200 publications over this period, and 250 over the

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period 2004–2006. As many as 53 of the latter represent reviews, updates, editorials, or opinion articles (Supplementary Table 1) rather than original articles. This time trend can be interpreted as a measure of the rising potential of proteomics, but may also reflect more promise and expectations than hard reality.

The 200 or so original papers which have appeared since our first plant proteomics review [1], have described aspects of the proteome of at least 35 plant species (Table 1). Most focus either on the premier dicot model species thale cress (*Arabidopsis thaliana*) or on the model monocot rice (*Oryza sativa*). The complete genome sequence of both these species is in the public domain, and this simplifies the identification of proteins from MS data. As the rice proteome was extensively reviewed in 2006 [2], the present review will not dwell on this area. Second to the two major model species are the major cereal, legume and solanaceous crops, and the model legume barrel medic (*Medicago truncatula*). All of these species are associated with significant quantities of published genomic DNA and EST sequences. The literature covered by this review also includes proteomic studies of the lesser species (at least from a scientific point of view) holm oak (*Quercus ilex*) [3], Spanish broom (*Spartium junceum* L.) [4], and ginseng (*Panax ginseng*) [5]. In these cases, the success of protein identification is less certain, generally having to rely on *de novo* MS sequencing and similarity searches to provide clues to protein function.

The plant material involved includes a variety of genotypes, cultivars, transgenics and mutants, organs, cells, and subcellular fractions. The scientific objectives range from a global description of the proteome to the study of biological, physiological, or biochemical processes, most commonly exploiting a strategy of differential expression (see summary in Table 1). A number of studies have featured PTMs and protein interactions. Finally, as proteomics technology is becoming more practical [6–10], the term "industrial process proteomics" has recently been coined [11].

Protein extraction protocols for whole proteomic analysis have proliferated, with specialization to plant organ (leaf, roof, cell suspension), and with TCA-acetone precipitation and phenol extraction proving to be most generically useful [12]. While the latter allows for the simultaneous analysis of proteins and mRNAs [13], metabolites, proteins, and RNA have been sequentially extracted using a protocol [14] validated with leaf tissue from *Arabidopsis*. Precipitation protocols have been optimized to cope with small starting amounts of tissue, which is essential given that (1) source tissue is often limited, and (2) plant organs are composed of different cell types, each having its own proteome signature [15]. This is particularly relevant for the description of the response to pathogen attack, as only a restricted number of cells plays any active role in defense. Laser microdissection allows for the collection of homogeneous tissue- and cell-specific plant samples, but its use has only been reported once to date, in an experiment where vascular bundles were isolated from Arabidopsis [16]. Given the particular interest in the proteomes of cereal and legume seeds, special extraction protocols have been developed [17]. RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), the most abundant single protein on earth, accounts for up to 50% of the soluble protein in leaves and this complicates the analysis of minor proteins in green tissue. The problem is exacerbated under physiological (senescence, seed filling) or stressed conditions, where a significant number of proteolysis products of RubisCO, displaying variable M_r and p*I* values, has been identified [18]. Analogous to the treatment of albumins in blood serum samples, the development of rapid protocols to eliminate such majority proteins would be beneficial. PEG [19] and FPLC (fast performance LC) [20] have both been reported to have utility in this context. The definition of structural or functional subproteomes can provide valuable information inferred from the physical localization of the proteins. It would considerably simplify sample complexity and improve the quality of experimental data. Such studies of course require rigorous purification steps and will be described below later.

Although proteomic technology is advancing, it has not yet reached a level of sophistication sufficient to study plant proteins on a scale comparable with what can be achieved by genomics. Many proteins involved in the mechanisms of signal transduction (*e.g.*, transcription factors, protein kinases, and regulatory proteins) are present in low abundance, and thus are not readily detectable in crude extracts. Most plant proteomic studies still rely on 2-DE separations of crude cellular extracts, although the trend is toward the use of defined plant organs, isolated cellular compartments, and subproteomes to reduce sample complexity.

Before 2004, 2-DE (specifically IEF-SDS-PAGE) coupled to MS was the dominant separation platform, although multidimensional protein identification technology (MudPIT) has been reported in rice [21], and the bidimensional blue-native (BN-PAGE/SDS-PAGE) approach was appropriate for the study of mitochondrial and chloroplast protein complexes [22]. Over the last two years, second generation proteomic techniques have begun to make an impact. These include non-gel-based separation techniques such as LC, including MudPIT [23–25], which is particularly well suited for the identification of hydrophobic proteins [21, 26]. However, even simple techniques, such as 1-DE SDS-PAGE, can generate good quality data, especially from simple proteomes [27]. Both gel-based (such as DIGE (difference gel electrophoresis)), and LC-based (such as ICAT (isotope-coded affinity tags)) techniques have been exploited in a number of studies [15, 28–30]. Stable isotope labeling by amino acids in cell culture (SILAC) and cleavable stable ICAT have been successfully applied to measure relative protein abundance by MS in both yeast and mammalian cells. This technique has been recently extended to *Arabidopsis* cells [31]. The Jorrín group is optimizing iTRAQ (isobaric tags for relative and absolute quantitation) as a means toward comparative proteome analysis in leaf extracts from various species. Finally, *in silico* proteomics, although as yet

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Table 1. Studies, objectives, and contributions of the plant proteomics papers published from 2004 to February 2006 period

Plant	Proteomes (descriptive proteomics)	Biological processes (differential expression proteomics)	Practical aspects
Model systems	**Individuals**	**Development/differentiation**	Identify molecular markers
Arabidopsis thaliana (49)[a]	Genotypes	Seed germination	for assisting plant breeding
Medicago truncatula (7)	Mutants	Root formation (secondary, adventitious)	programs
	Transgenics	Pollen maturation	
Cereals		Somatic embryogenesis	Genotype characterization
Barley (7)	**Plant developmental stage**	Response to hormones (GA, ABA)[c]	(cataloguing) within species,
Maize (19)	Germinated seeds	Progammed cell death	populations
Rice (25)	Seedlings		
Wheat (14)	Mature plants (vegetative or flowering)	**Growth conditions**	Substantial equivalence
		Liquid medium composition (cell	analysis in genetically
Legumes	**Organ/Tissue/Cells**	suspensions)	modified plants
Alfalfa (1)	Suspension cells (cells and growth medium)	Nutrients (potassium, nitrogen)	
Lentil (1)	Seed (embryo, endosperm, gluten, kernel)	Light	Analysis of prepared
Lupinus (4)	Coleoptile		protein extracts
Pea (6)	Roots (tip, primary, secondary)	**Symbioses**	
Bean (*Phaseolus* spp.) (2)	Stem	Mycorrhiza	Food processing and
Soybean (6)	Leaves (different types in cereals, *i.e.*, leaf		trazeability
	sheath)	**Abiotic stresses and mechanisms of**	Allergen
Solanaceae	Petiol	**tolerance**	identification
Potato (2)	Xylem/Phloem (sap)	Drought	
Tobacco (7)	Trichomes	Osmotic (salt)	
Tomato (3)	Pollen	Temperature (cold, heat)	
	Pistil	Elevated CO_2	
Other crops[b]		Anoxia	
Carrot	**Subcellular fractions**	Nutritional deficiency	
Coffee	Cell wall (ionically or covalently bound proteins)	Metals (Ni, Cu, Zn), metalloids (As)	
Cucurbit	Apoplast	Herbicides (GSTs)[d]	
Grape	Membranes (plasma, tonoplast, organules)	Sludge	
Mustard	Cytosol (soluble fraction)		
Pepper	Plastids (etioplast)	**Biotic stresses and mechanisms of**	
Oilseed rape	Chloroplast (subfractions)	**resistance**	
Spinach	Mitochondria (subfractions)	Pathogens (virus, bacteria, fungi)	
	Microsomes	Herbivores	
Trees (fruit and forest trees)[b]		Parasitic plants	
Holm oak		Hypersensitive response	
Peach		Signaling (H_2O_2, SA)	
Peanut		Oxidative stress	
Poplar			
Rubber tree		**Biochemistry/metabolism**	
White spruce		Sulfur (sulfurtransferases)	
		Alkaloids	
Others[b]		Nitrogen mobilization	
Alyssum lesbiacum		Alternative oxidase	
Catharantus roseus		Protein import to chloroplasts	
Ginseng			
Grasses			
Rhododendron catawbiense			
Wild mustard			

a) Number in parentheses corresponds to papers reviewed.
b) 1–2 papers each.
c) Gibberellic and abscisic acids.
d) Glutathione-*S*-transferases.

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only applicable where full genomic sequence is available (*i.e.*, Arabidopsis and rice), is useful in both predicting and validating experimental data [32].

This review aims to update the contribution to plant biology of proteomics, covering the period 2004–February 2006. It has been divided into five sections, describing: subcellular proteomes, plant development, the responses to biotic and abiotic stress, PTMs, and protein interactions. Our concluding remarks summarize the pitfalls and challenges of plant proteomics. As in our previous review [1], methodological approaches and techniques are briefly discussed, making reference to relevant key original papers or reviews. We have attempted to minimize overlap with other recently published reviews [2, 33–35].

2 Subcellular proteomes

The genome of Arabidopsis is well characterized, but the function of one-third of its encoded proteins remains unknown. Definition of their cell localization would doubtless furnish valuable functional information. In addition, the resolution of global proteomes is poor unless the genome is small, or the total cell proteome can be fractionated into its subcellular components. Since fractionation is inevitably imperfect, an assessment of the level of contamination is always necessary, although this is not technically straightforward. Over the past few years, organelle proteomics have reached a sufficient level of maturity to allow the identification of components in several subcellular compartments [1]. The past 2 years have seen the generation of a large amount of data, which have facilitated the construction of a number of organelle-specific proteome databases. Since the 2004 reviews [1, 36], two plastid (PPDB and plprot [37, 38]), one mitochondrion (AMPDB [39]) and one nucleolus (AtNoPDB [40]) databases have been created. In addition to these, information regarding organelle proteomes is present in global databases such as the Rice Proteome Database [41]. The extent of proteomic data is now sufficient to allow large-scale comparisons to be made between experimentally identified protein sets from enriched subcellular fractions and prediction sets based on *in silico* localization. This combined information has permitted the creation of a database delivering a genome-wide prediction of ultimate protein subcellular localization [39]. Much of this databasing effort has been reviewed elsewhere [42].

As in the previous review period, considerations of practicality have biassed organelle choice to plastids and mitochondria (see below, Sections 2.1 and 2.2). Nonetheless, significant efforts have been devoted to the membranes delimiting the cytoplasm, the plasma membrane and the tonoplast (terminal membranes; see Section 2.3), and to the cell wall and extracellular proteomes (see Section 2.4), although none of these have been assembled in a specific database. In addition, attention has been paid to the nuclear proteome (see Section 2.5) and innovative procedures have been trialled for subcellular compartments that cannot be efficiently purified, and so are generally sampled as mixtures (*e.g.*, the ER and the Golgi apparatus, see Section 2.6).

2.1 Plastids

Plastids, especially chloroplasts, have been the target of the most serious proteomic efforts since 2004, even though a large set of data was already available at the beginning of the period (reviewed in [43]). The initial focus concerned the cataloging of proteins localized in the envelopes and thylakoids of Arabidopsis and spinach, using either a classical 2-DE approach or other more specialized protein separation technologies [44]. This led to the characterization of certain protein complexes and PTMs. MS approaches have concentrated on systematic MS/MS. The utility of more precise configurations, such as FT-ICR-MS, although previously shown to have promise [45], remains anecdotal. A common feature has been the introduction of steps to improve protein or peptide resolution, thereby allowing a more in-depth analysis. Examples include multidimensional chromatography to resolve peptides [46] and three-phase partitioning to fractionate membrane proteomes [47], which, when combined, allowed for the identification of about 700 plastid proteins. After curation to eliminate redundancy due to the application of different gene models [47], approximately one third of these products remained of unknown function, a typical proportion in the light of the global functional annotation of *Arabidopsis* genes. This protein set includes many previously undetected membrane proteins, and the analytical approach can only provide provisional clues as to biological function. The identification of known function proteins has resulted in a better coverage of the chloroplast gene output, but has not led to a major evolution in the understanding of chloroplast function. In contrast, an analogous analysis of stromal proteome has led to some major advances [48]. For this research, colorless native (CN)-PAGE gels were used to resolve protein complexes and to quantify the relative abundance of proteins by image analysis after SYPRO Ruby staining. A number of oligomeric complexes were elucidated, emphasizing the importance of paralogues. The relative importance of the various functional classes or pathways in the stromal proteome was defined. Further successes include the identification of the targets of proteins involved in the redox poise (*e.g.*, thioredoxins (Trx)), described in more detail in Section 5; and the characterization of the plastoglobule [49, 50], a lipoproteic structure having an as yet unknown function. Detailed proteomic analysis has suggested that plastoglobules represent a functional metabolic link between the inner envelope and the thylakoid membranes. Collectively, prior data and these recent efforts have resulted in the characterization of approximately 1000 plastid proteins in Arabidopsis.

Arabidopsis, however, is not the only plant species to have benefited from proteomic study of the chloroplast. By coupling 2-DE with various data-clustering procedures, tem-

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poral changes in the proteome during the greening process were investigated in maize [51]. In barley, thylakoids were characterized by BN 2-DE without any intervening fractionation step [52], enabling the identification of the most abundant thylakoid protein complexes. In contrast, prior fractionation on density gradients was necessary to resolve the proteome of rice etioplasts by SDS-PAGE [53]. Thus, relatively comprehensive datasets for the chloroplasts of several species are now deposited in proteomic databases, including both dicots (Arabidopsis, tobacco) and monocots (barley, maize, and rice).

The preparation of very pure fractions of intact chloroplasts is technically feasible, and so, combined with the substantial volume of prior functional information, it is possible to accurately estimate the level of organelle contamination. This situation does not obtain for other subcellular compartments. For instance, in the case of the Arabidopsis stromal proteome, the contamination level was proposed to be under 2% [48]. In addition to the problem of contamination, both the statistical evaluation of changes in protein accumulation during a process and the robustness of derived protein complexes need to be considered. These features were specifically addressed in the study of chloroplast biogenesis using 2-DE-derived data [51].

2.2 Mitochondria

Mitochondria are the second organelle to have attracted significant proteomic analysis, since their purification is also relatively straightforward. However, in contrast to the plastids, the major focus has been to detect changes in the mitochondrial proteome induced by a range of treatments rather than attempting to construct a systematic catalog of all proteins recruited. The protein composition of mitochondria (reviewed in [54]) represents an approximately 20% coverage of the expected proteome, although this includes both individual proteins as well as protein complexes. Mitochondrial proteomics has not been restricted to model plants.

The only recent study of the mitochondrial proteome used a solvent extraction procedure initially developed for the chloroplast envelope [55]. Over 100 *Arabidopsis* proteins were identified, with a range of functions, as only 2% of these corresponded to known nonmitochondrial proteins, a low contamination level was claimed. Other studies have dealt with rice and pea. Using a dinitrophenylhydrazine tag, over 50 mitochondrial proteins susceptible to oxidation were detected in rice [25]. Also in rice, changes to the proteome induced by the relief from anoxic conditions were investigated, with a focus on protein complexes [56]. The effects of various abiotic stresses and herbicide treatments were characterized in pea [57]. In all cases, the mitochondria maintained their vital functions, but differential stress-dependent responses were observed, involving the degradation of key enzymes. Overall, the mitochondrial proteome remains rather poorly characterized, but is a favored target for physiological studies.

2.3 Terminal membranes

In recent years, a renewed interest in both the plasma membrane and tonoplast has led to significant and innovative attempts to characterize their proteomes including a large-scale analysis of PTMs. Solvent extraction procedures, previously developed for the purification of chloroplast envelope proteins, have been applied to isolate the hydrophobic *Arabidopsis* proteome [58]. This achieved the identification of almost 100 novel proteins, the functions of about 75% of which were classifiable. In addition, the plasma membrane localization of several was validated by the transient expression experiments using green fluorescent protein fusion proteins. The Arabidopsis plasma membrane was the first plant-based system to be submitted to systematic phosphoproteomic analysis [59, 60]. Over 300 phosphorylation sites were mapped, using inside-out plasma membrane vesicles and phosphopeptide selection and fractionation after trypsin release of cytoplasmic peptides. An *in silico* analysis was used to clarify the characteristics of these sites in the plant membrane. In addition to the phosphorylated proteins, 35 GPI-anchored plasma membrane proteins were also identified [61]. Outside of *Arabidopsis*, an MS approach allowed for the detection of two proteins in the plasmalemma of mycorrhizal roots (but not in noninoculated roots) of *M. truncatula*. These constitute the first potential markers for the peri-arbuscular membrane [62].

Two different approaches have been used to investigate the vacuolar membrane of Arabidopsis [24]. First, tonoplast proteins were resolved in micelles by 2-D LC followed by SDS-PAGE and MALDI-TOF MS [63]; and second, SDS-PAGE coupled to LC-MS/MS was combined with multidimensional peptide LC-MS/MS. Apart from the clear advantage of tandem MS in terms of the number of proteins identified, both approaches allowed for the characterization of largely hydrophobic protein sets with differences attributable, at least in part, to the source of sample (suspension cells *vs.* leaf). In rice, a 2-DE analysis of gibberellin (GA)-induced changes in the tonoplast peripheral proteome [64] suggested that both aldolase C-1 and V-ATPase are involved in the control of root growth.

2.4 Cell wall and extracellular proteomes

The purification of plant cell walls is hampered by a number of technical difficulties. Thus, the characterization of its proteome has long been difficult [65], and a combination of various treatments is required [66, 67]. MS analysis identified many proteins not previously believed to be extracellular. More recently, a multidimensional analysis of peptides has allowed for the characterization of additional subsets of proteins, resulting in the identification of over 250 *Arabidopsis* cell wall proteins, including some previously detected. The presence of numerous extracellular proteases was confirmed [23, 67]. Modifications in response to biotic and abiotic stresses have also been investigated. Both pathogen elicitors

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in maize [68] and salt stress in tobacco [69] induce significant changes in the extracellular proteome, including some changes in post-translational status.

2.5 Nuclear and nucleolus proteomes

The nucleus is a prominent target for proteomic analysis. However, the purification of intact nuclei (including both membrane and matrix) in sufficient quantity is difficult. The tight association between the nuclear membrane and the ER, and the similar density of nuclei and chloroplasts are at the heart of this challenge. By taking advantage of the presence of a detergent-resistant intranuclear skeleton (the lamina made of specific intermediate filament proteins, named lamins), a large preparation of stripped nuclei can now be purified relatively easily. The remaining problem is the minimization of protein loss from these leaky nuclei. Nevertheless, hundreds of nuclear or nucleolar proteins have already been described [70] and compared with human nucleolar proteins [71]. Some PTM approaches have also been initiated [72].

2.6 Mixtures of subcellular proteomes

The Golgi apparatus and the ER are not easily resolved from one another and copurify in overlapping fractions. The LOPIT (localization of organelle proteins by isotope tagging) strategy has been proposed for the characterization of these organelle mixtures [73]. LOPIT quantifies relative abundance by combining an assessment of membrane distribution (using known markers) with labeling by ICAT reagents of proteins in contrasting fractions. Proteins are then assigned to organelles according to their distribution and that of the markers in multiple pair-wise comparisons. This approach has proven to be suitable for Golgi and ER in the presence of contaminating plasma membrane, plastids, and mitochondria. Most recently, the use of iTRAQ labeling has allowed an expansion of this approach to distinguish genuine organelle residents from contaminating proteins [74].

3 Plant organ development

The proteomic analysis of whole organs and tissues is still a major task in the characterization of plant samples. Although limited in its separation capacity, 2-DE is still widely used to separate the components present in crude protein extracts. Large-scale analyses have been carried out in Arabidopsis [75], *M. truncatula* [76], and rice [2]. Some 6000 gel spots, resolved from extracts of *Arabidopsis* primary leaf, silique, and seedling, were purified from the gels and analyzed by MALDI-TOF MS, resulting in the identification of 2493 products encoded by 663 distinct genes. A Q-TOF MS analysis of an *M. truncatula* cell suspension culture has yielded an extensive protein reference map in which 1367 of the 1661 proteins resolved were identified (907 protein accession numbers). These comprised a complete tricarboxylic acid cycle; a nearly complete glycolytic pathway; a significant portion of the ubiquitin (Ub) pathway with the associated proteolytic and regulatory complexes; many enzymes involved in primary and secondary metabolism, and energy production; and proteins implicated in defense, protein destination/storage, protein synthesis, transcription, cell growth/division, and signal transduction.

A number of studies have demonstrated that proteomic analysis can generate information relevant for the elucidation of molecular mechanisms involved in developmental processes. Leaf developmental stages have been characterized in maize [77], revealing two isoforms of caffeic acid/5-hydroxyferulic 3-*O*-methyltransferase (COMT) among the differentially expressed gene products. Both COMT isoforms are involved in lignin biosynthesis and are maximally expressed 10–20 cm from the leaf point of insertion. The expression of several other enzymes of the lignin and flavonoid pathway, such as phenylalanine ammonia lyase and caffeoyl-3-*O*-methyltransferase, is correlated with the abundance of COMT isoforms. The lignification zone corresponds to the enzyme accumulation profiles, ensuring the reinforcement of cell walls as the elongation process tails off. In addition, a shift, in response to drought, in the maximum accumulation of these proteins was established, affecting the lignification process as a consequence of a reduced growth rate [77].

The consequences on the leaf proteome of a perturbation in polyamine metabolism have been studied in transgenic tobacco overexpressing *S*-adenosylmethionine decarboxylase. Groups of proteins, some with reduced and some with increased abundance were observed. Among the lower abundance proteins were the ribonucleoproteins, while those of higher abundance were isoforms of pathogenesis-related (PR) f-1 proteins. As the latter result was restricted to only one of the three independent transgenic lines, the molecular connection between altered polyamine levels and PR protein expression remains elusive [78].

Proteomic analysis of tree species still represents a major challenge. Protocol optimization for both leaf protein extraction and 2-DE was required for the holm oak. Values of 26% for the analytical variance and of over 58% for the biological variance between three independent trees were observed. The identification of proteins needed *de novo* sequencing and BLAST searches since only a limited number of database entries exist for *Quercus* [3]. Nevertheless, the feasibility of a proteomic analysis of tree tissues was established despite the considerable remaining technical challenges with respect to both 2-DE and MS-based protein identification.

Proteins associated with adventitious root formation have been identified by comparing a number of *Arabidopsis* mutants [79]. The propagation of clonal populations of elite genotypes *via* tissue culture is a major goal in certain crop and many ornamental plants, and this is largely dependent on the ability to form adventitious roots, a character under polygenic control. Comparative 2-DE image analysis of gen-

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otypes contrasting in their capacity to form adventitious roots revealed 11 proteins positively or negatively correlated with endogenous root formation, the number of adventitious roots, or root primordia formed. Three of the positively correlated products were identified as auxin-inducible GH3-like proteins. Overall, the analysis revealed the regulatory pathways of adventitious root formation, and the proteins identified could be informative as novel markers to identify valuable genotypes. Interestingly, a microarray-based analysis of adventitious root formation in *Pinus contorta* identified similar candidate genes [80].

Root proteome analysis has also been performed on maize seedlings. Of all the proteins detected by 2-DE of extracts of 5-day-old plants, only 28% were still present at a later stage of development. These considerable alterations in the protein profile clearly reflect underlying cellular processes during primary root development, as well as processes associated with the formation of lateral roots at a later developmental stage [81].

Events associated with flower development have been tracked *via* a proteomic analysis of rose petals [82]. A series of 2-DE maps was obtained from closed buds, mature flowers, and flowers at anthesis, generating almost 1000 distinct products. Image analysis revealed that about 90 products are developmental stage specific. Only 6% of these products were constitutively present, whereas most showed a differential pattern of expression during flower development. MS identified 82 of the products which could be classified into various categories. Remarkably, stress-related proteins were strongly represented in the developing petals, suggesting that the protection of young floral tissue is an important component of flower development. Although secondary metabolism is a major sink for metabolite allocation during flower development, only one of the proteins identified (dihydroflavonol reductase) could be classified into this enzyme category.

A reference *Arabidopsis* 2-DE pollen map consisted of 135 identified proteins. In addition to those associated with basic cellular functions, such as energy generation, ten proteins were of unknown function and three were flower- or pollen-specific. Nine proteins, for which no equivalent mRNA had been found in a prior transcriptome experiment, were identified [83]. Pollen tube development in *Pinus strobus* was followed by comparing the proteomes of germinated and nongerminated pollen. Of almost 650 products resolved, 38 were pollen tube specific, and 19 showed increased expression in pollen tubes. As pollen tube development in gymnosperms differs considerably from that in angiosperms, a further in-depth analysis will be needed to define the cellular commonalities and specificities of this important process [84].

Over 350 distinct proteins could be recognized in extracts of tomato seeds at the onset of germination, for both embryo and endosperm. Nearly two-third of the 75 products purified from 2-DE gels could be identified by a combination of MS techniques. Proteins specific to both the embryo and the endosperm were identified and the major seed storage proteins were further characterized [85]. The measurement of protein carbonylation allowed the patterns of protein oxidation during germination of Arabidopsis seeds to be followed. In dry seeds, 12S cruciferins (legumin-type seed storage protein) were the major target of oxidation; but during imbibition, various other proteins were oxidized, although carbonylation was unevenly distributed. Protein oxidation is thought to be an adaptative mechanism to counteract oxidative conditions during germination [86]. The identification of 250 wheat starchy endosperm proteins permitted the definition of 13 metabolic processes such as ATP interconversion, and carbohydrate and lipid metabolism. Although less abundant proteins have yet to be identified in this system, changes in protein profiles during seed development were detectable [87].

Nitrogen mobilization from leaves during seed filling was analyzed in pea [18]. Once a reference proteome map had been established for leaves and stems, changes in protein profiles during N-mobilization could be tracked, revealing that 40% of products were significantly altered in abundance, including the loss of RubisCO and an increase in chloroplastic protease regulatory subunits. Many of the proteins present in higher amounts were related to protein folding, protein degradation, and defense mechanisms. For some, prior experiments had shown induction of higher levels of transcription. Also, among these were two 14-3-3 proteins which are members of a family thought to be involved in the regulation of C- and N-metabolism.

The capacity of the proteomic approach to investigate cellular processes occurring during somatic embryogenesis in spruce was demonstrated in a *Picea glauca* cell culture system [88]. The process is important as a tool for the clonal propagation of conifers, which is desirable to provide uniform seedling quality for forestry. Four stages of development were defined, and materials were analyzed by 2-DE, leading to the recognition of 48 differentially represented proteins. Identification of these was based largely on LC-MS/MS, using an EST database containing some 50 000 sequences obtained from three spruce species, along with ESTs from loblolly pine and poplar. An ontological classification of identified proteins revealed that the differentially expressed products included membrane and nuclear proteins as well as proteins involved in metabolism and energy production. Other proteins identified comprised a range of candidates newly associated with the embryogenesis process. The second most abundant protein was an enolase homolog which may be involved in the stress response. Although no experimental validation for this function was obtained, the protein was suggested as a marker for embryogenesis.

Somatic embryogenesis has also been studied in *M. truncatula* by comparing the 2-DE profiles of extracts of cell cultures from the wild-type Jemalong with those of line 2HA, which has a 500-fold higher capacity for regeneration [89]. Over 2000 spots could be visualized on individual gels, and image analysis identified 54 whose intensity

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changed over the 8 wk of cultivation. Of these, 60% were also polymorphic between the two test genotypes, and the protein identity of 16 has so far been identified. A remarkable expression pattern was displayed by 1-Cys peroxiredoxin – its abundance was low throughout the cultivation of Jemalong; but in 2HA, it increased in abundance between 5 and 8 wk. Peroxiredoxins are components of cell defense against oxidative stress, but the precise role of this isoform remains to be elucidated.

Cell-specific protein profiling has been achieved for the unicellular trichomes of Arabidopsis. Shotgun peptide sequencing led to the identification of 63 unique proteins, including a complete *S*-adenosylmethionine pathway cluster, two S-adenosylmethionine synthase forms, a glutathione (GSH) S-conjugate translocator, and other proteins involved in sulfur metabolism and detoxification [90]. Proteome analysis of a trichome preparation from tobacco revealed an abundance of defense-related proteins, including superoxide dismutase (SOD), GSH peroxidase, and PR proteins. Results of 2-DE were confirmed by Western blotting and enzyme activity staining for SOD. Unlike those in Arabidopsis, tobacco trichomes have a glandular function and are multicellular; thus, many differences in their protein complements are likely to be revealed in further proteome, transcript, and metabolic studies [91].

The targeted enrichment of whole tissue extracts has been successfully applied in a number of studies. Affinity purification of mannose-binding proteins was achieved from root, leaf, and seed of rice [92]. A total of 136 proteins were identified from these fractions following separation by 1-DE and HPLC-MS/MS. The proteins from separate rice tissues overlapped only partially indicating a high degree of organ specificity. A large fraction of the proteins identified was related to sugar metabolism, while a considerable one comprised proteins without an annotated function.

Gibberellin (GA)-regulated proteins have been identified in rice roots by comparison of the proteomes of a wild-type and a GA-deficient mutant. Among these was fructose-bisphosphate aldolase, suggesting that it plays a prominent role during GA-induced root growth [93]. Similarly, in an analysis of a cell suspension culture derived from a constitutive GA response rice mutant, methylmalonate-semialdehyde dehydrogenase was identified as the most up-regulated protein, relative to wild-type cells. In addition, this protein was present at higher amounts in the roots and leaf sheaths of the mutant, whereas in wild-type plants, its presence was stimulated by GA. Down-regulation of its expression using an antisense construct resulted in the production of thin seminal roots and a minor inhibition of leaf sheath elongation. The content of TCA cycle metabolites was reduced in these transgenic antisense plants. Overall, it appeared that methylmalonate-semialdehyde dehydrogenase plays a role in root development and its regulation forms part of the downstream response to GA signaling [94]. This study is a case example of the power of the proteomic approach for functional characterization since it achieves the initial identification of candidate genes and supplies the strategy for the further characterization of function in follow-up experiments.

Overall, the recent years have seen a rapid expansion in the proteomic analysis of whole plant tissues as an approach to the study of global gene expression, and hence various aspects of cellular biochemistry. A common experience is that many of the proteins identified belong to a small set of major housekeeping families. To obtain a detailed view of specific aspects in various plant tissues, a higher level of resolution is needed. Thus, the development of prefractionation techniques and the analysis of fractions by LC-MS or 2-DE will feature in future studies of whole plant tissues.

4 Responses to biotic and abiotic stresses

Plant survival, and hence crop productivity, depends greatly on the ability to adapt, respond, resist, and tolerate variable environmental conditions. Plants have evolved sophisticated mechanisms to cope with a variety of biotic (pathogens, herbivores, parasitic plants) and abiotic (drought, salinity, UV light, poor and polluted soils, *etc.*) stresses. How such environmental stimuli are perceived and trigger the complex defensive and adaptive signaling networks, leading to altered gene expression, protein and metabolic changes, and growth retardation, and how these events result in resistance/tolerance is of major practical interest. The study of the plant–environment interaction has been conventionally carried out using biochemical, genetic and, more recently, transcriptomic (qRT-PCR, micoarrays, SAGE) techniques. Proteomics has now begun to make a further contribution toward the unravelling of the elements involved in stress perception and transduction, and some reviews covering this area have already been published [95–97]. For such studies, the term “environmental proteomics”, if well-defined, can be used [2].

At least 52 papers reporting proteomic approaches toward studying alterations in protein levels in response to various stresses have appeared during the review period (Supplementary Table 2). The stresses take in both biotic (23 papers, covering viral, bacterial and fungal attack, resistance mechanisms and signaling, and response to herbivores and parasitic plants) and abiotic (25 papers, covering drought, salinity, high temperature, high light, nutritional deficiencies, elevated CO_2 levels, anoxia, heavy metal pollution, and herbicides) agents, along with a small number of miscellaneous topics (four papers). Most of these studies have been conducted using the roots or leaves of Arabidopsis, rice, or maize. Cell suspensions have also been exploited, especially for the dissection of signaling pathways and PTMs, since they are more suited for analytical techniques relying on amino acid radiolabeling (generally ^{35}S-Met). In particular, the transient suppression of *de novo* protein synthesis and protein degradation is a common phenomenon under stressed conditions, especially in susceptible genotypes [28].

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2-DE/MS analysis has been used to investigate genotypic variation in response to environmental stresses [98–100]. This variation typically takes the form of quantitative and qualitative differences in the protein profile between genotypes, and attempts have been made to correlate these with differential phenotype. Almost all environmental proteomics research uses comparisons of the 2-DE protein profiles among genotypes, mutants, or transgenics, where one line is resistant or tolerant and the other susceptible or nontolerant. Plants are typically grown both under optimal and suboptimal (stress) growth conditions, and differential spots once statistically validated, are extracted from the gel, purified, digested, and analyzed by MS for identification. Differential proteins, selected from contrasts between resistant/tolerant *versus* susceptible/nontolerant, or between optimal growth conditions *versus* stressed growth conditions, are taken as candidates involved in the stress response. The use of this approach acknowledges that only a minor fraction of the overall proteome can be analyzed (since 2-DE gels typically resolve less than 1000 spots), with the sample largely limited to the majority of the soluble proteins having a p*I* in the range of pH 4–8. For this group of proteins, there is a relatively good correlation among the level of mRNA accumulation, as assessed by microarray hybridization, quantitative real time reverse transcription-PCR and protein expression levels, and the level of translation product [29].

Irrespective of which stress is applied and what plant species is utilized, most of the differential proteins identified appear to be either constitutively present (preformed defenses) or are specifically induced in the resistant/tolerant plants [101]. A few are specific for the type of stress applied, for example, nitrogen-assimilating enzymes [100], heat-shock proteins (HSPs) [102], metal-chelating proteins [103], xenobiotics, detoxifying enzymes such as GST [104] and disruptors of the digestive processes of herbivorous insects [105]. For some differential proteins, multiple isoforms or specific PTMs could be detected, each responding differently according to the stress applied [104, 106].

4.1 Biotic stress

Descriptive proteomics is the topic of Sections 2 and 3. Nevertheless, the proteomic analysis of certain specific plant tissues and organs, such as the phloem [107] and xylem [108] sap, the trichomes [91], and subcellular fractions such as the cell wall [109] and apoplast [110], has revealed that defense and stress-related proteins are strongly represented even in the absence of stress, supporting the notion that they play important roles in many aspects of defense. Some of these proteins are present as multiple isoforms and have been shown to possess *in vitro* antifungal activity. In addition, novel proteins of unknown function, whose existence has been predicted from genomic analysis, have also been detected [111].

The majority of the differential proteins present in resistant and susceptible genotypes (as a general rule, in increased amount in resistant types, and in decreased amount in susceptible ones) belongs to two major categories: (i) defense- or stress-related, and (ii) enzymes associated with C- and N-metabolism and secondary metabolism. Within the first group are the PRs (such as the glucanases, chitinases, proteases, protease inhibitors), antioxidants (catalases, SODs, peroxidases, enzymes of the ascorbate-glutathion cycle), late embryogenesis abundant (LEA) proteins, chaperones, and HSPs. Within the second group, a number of enzymes associated either with carbohydrate assimilation and metabolism (including photosynthesis, glycolysis, and the Krebs cycle), nitrogen assimilation, or secondary (phenolics) metabolism has been identified. Biochemical or transcriptomic approaches for the study of plant–pathogen interactions have emphasized group (i) much more than group (ii). The presence of greater amounts and/or activity of enzymes of the carbohydrate-energetic metabolism in resistant, as opposed to in susceptible genotypes may reflect, at least under limiting environmental conditions, a genetic advantage with respect to either photosynthesis and/or absorption of the photoassimilates needed to fuel biosynthetic pathways. There has long been evidence that constitutive expression of resistance leads to a reduction in plant growth and fitness as a consequence of "metabolic competition" directed toward the synthesis of defense elements [112]. Hence, the observed genotypic differences may balance the fitness cost of the resistance.

Plant carbohydrate and N-metabolism also plays an important role in the case of parasitic plants, flowering plant species that have lost their autotrophism during evolution by adapting themselves to obtain water and nutrients from other plants (about 3 500 species reported to date). Studies at the Jorrín lab using the legume/sunflower–*Orobanche* spp. interaction ([113] and unpublished results) support the existence of similar defense strategies against a broad range of pathogens – including bacteria, fungi, and parasitic plants – and have opened new possibilities for the deepening of our understanding of the redirection of host assimilates from host sinks to parasite.

Some published comparative proteomic studies have sought to characterize signaling pathway proteins which mediate changes in gene expression and protein modification in response to environmental cues, this representing one of the most innovative contributions of proteomics. Plant research has mainly focused on the unravelling of entire phosphorylation cascades involved in adaptation to different stress conditions, such as pathogen attack, as discussed in the following section.

The characterization of signaling events has been affected using proteomic or subproteomic comparisons in cell cultures treated with microbial elicitors, or exposed to key defense-related signaling molecules such as salicylic acid (SA), hydrogen peroxide (H_2O_2), and nitric oxide (NO). Treatment with some pathogen-derived elicitors induced changes in the extracellular matrix of maize cells [68], revealing some novel phenomena not previously reported, namely a change in the phosphorylation status of matrix

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proteins and the apparent recruitment of cytosolic proteins into the cell wall. In soybean cells, elicitor (syringolide) treatment induced an oxidative burst and protein phosphorylation [114]. Both phosphorylation and protein translocation events are important in signal modulation during induced defense responses.

Iodoacetamide-based fluorescence tagging was used in conjunction with MS analysis to identify a number of potential protein targets for H_2O_2 in the cytosolic fraction of Arabidopsis [115]. The most prominent protein identified was glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of glycolysis, suggesting a role in the mediation of reactive oxygen species (ROS) signaling in plants. Meanwhile, changes in the Arabidopsis "secretome" in response to SA led to the identification of GDSL LIPASE1, a lipase containing a GDSL-like motif [116]. This enzyme, in association with ethylene signaling, may be a critical component of the resistance against the fungal pathogen *Alternaria brassicicola*. In order to investigate the downstream signaling pathways of nitric oxide (NO) in plants, mung bean leaf tissue was treated with the NO donor sodium nitroprusside. The changes observed concern mainly photosynthetic proteins, suggesting that the chloroplast is a major subcellular target [117]. OsRac1, a rice homolog of a human small GTPase Rac, as deduced from 2-DE/MS analysis, is able to induce the expression of many gene products functioning in various signaling and metabolic pathways, and plays a dominant role in the defense response of cultured rice cells [118].

Programed cell death (PCD) in plants plays a central role in a wide range of processes including defense. In Arabidopsis cell suspensions, an extracellular glycoprotein has been identified that may serve to transmit a "death signal" from cell-to-cell [119]. In rice, proteomic comparisons of mutants displaying spontaneous cell death lesions and constitutive resistance features suggest that metabolic changes underlie the PCD phenotype [120].

Finally, and distinct from prokaryotes, unicellular eukaryotes and humans, little is known of the proteins in plants, involved in pathogen recognition. These processes are of crucial importance for plant survival, since sensing is the precursor of the early and co-ordinated activation of defense responses, both spatially and temporally, which culminate in resistance or tolerance. For host-plant resistance to pathogens governed by a gene-for-gene-interaction, it has been shown that the initial recognition event involves direct or indirect interactions of the gene products of the pathogen's avirulence genes and the corresponding resistance genes of the host plant [121], with some of them later identified recently by proteomic analysis [122]. A yeast-two hybrid analysis identified 12 proteins which interact with the *Arabidopsis RPS2* gene product [123], while a proteomic approach, based on membrane vesicles obtained from Arabidopsis and mustard cotyledons, succeeded in identifying 23 signaling proteins, among which were kinases, leucine-rich repeat (LRR)-receptor kinases, and G proteins [124]. Most recently, the induction of some of these LRR-receptor kinases has been reported in fungal-infected Arabidopsis roots [125].

4.2 Abiotic stresses

A number of unfavorable environmental factors can act singly or in concert to strongly impaired plant growth and productivity; these include the suboptimal availability of nutrients, excessive or inadequate light, lack of oxygen, lack of water, high or low temperature, wind, wound, and chemical pollution. As drought and salinity are the most commonplace environmental limitations to plant productivity, a number of important crop plants, in particular rice (reviewed in [2]), maize [77], and sugar beet [126] have been investigated using a proteomic approach.

2-DE profiling together with an analysis of secondary metabolism was applied to elucidate the impact of water deficiency on the lignification of maize leaves. The phenotypic response to water deficit was a reduction in the level of lignin and a shift of the lignification zone toward the leaf base. The expression profiles with respect to candidate proteins were consistent with these changes [77]. The response to drought stress of field-grown sugar beet has been described in [126]. Analysis of the 2-DE profiles of leaf extracts from two different cultivars identified 79 gene products whose intensity changed significantly as a consequence of water deficit; of these, 44 were down-regulated, 27 were up-regulated, and eight were not detected in the nondroughted controls. Some of these were also genetically variable. Selected spots were purified for protein identification, revealing proteins which have previously been shown to play a role in the stress response, including the small HSP, cytosolic Cu-Zn SOD, 2-cysteine peroxidase, cyclophilin, nucleoside-diphosphate kinase, a nascent polypeptide-associated complex α-chain, and the large subunit of RubisCO. These proteomic analyses based on a number of species have identified a significant degree of inter- and intraspecific genetic variation in the expression of these stress response genes. Some of these proteins clearly give a physiological advantage under stress conditions, and thus are simultaneously potential targets for marker-assisted selection and rational candidate genes for the identification of quantitative trait loci.

More recently, the effects of drought on the leaf proteome of the forest tree species holm oak have been described [127]. Differences at the level of the leaf proteome in response to drought stress variations related to different developmental stages and between provenances have also been observed in this study. The major physiological changes were the mobilization of storage proteins and carbohydrates and the inhibition of photosynthesis.

In a comparative analysis to define the cellular mechanisms operating in plant trichomes, the soluble proteins of whole leaves and isolated trichomes from tobacco were subjected to 2-DE [91]. Proteins related to stress defense responses (cytosolic Cu-Zn SOD, GSH peroxidase, PR-2, PR-Q, and

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an Arabidopsis stress-inducible protein) were strongly represented among the products specifically enriched in tobacco trichomes. The specificity of expression of these proteins in the trichome was confirmed by protein gel blotting and enzyme assays.

Changes induced in response to salinity stress have been described at the level of the soluble fraction of tobacco leaf apoplastic polypeptides. The expression of two chitinases and a germin-like protein increased significantly and two lipid transfer proteins were expressed *de novo* [69]. In a parallel study, salt stress-induced changes in the periplasmic fractions of *Synechocystis* were analyzed [128] and, more recently, a proteomic approach based on isolated plasma membranes was developed [129]. The largest defined group of proteins which consistently shows enhanced expression under salt stress contains the substrate-binding proteins of ABC transporters. Regrettably, a substantial number of proteins affected by salinity remain annotated as hypothetical proteins due to a lack of any sequence similarity to any proteins of known function. The effects of salinity and hyperosmotic stress on Arabidopsis cell suspension cultures have been investigated using DIGE technology [28]. Products displaying significant changes in abundance included H^+ transporting ATPases, signal transduction-related proteins, transcription/translation-related proteins, detoxifying enzymes, amino acid and purine biosynthesis-related proteins, proteolytic enzymes, HSPs, carbohydrate metabolism-associated proteins, and proteins of unknown function. In addition, the transient suppression of *de novo* protein synthesis was demonstrated.

A comprehensive study of the signaling events during salt stress in *Arabidopsis* analyzed salt-induced changes in the root microsomal proteome using 2-DE combined with protein gel blotting [130]. Ca^{2+}-dependent membrane-binding proteins, designated annexins, were identified as the major signaling components, a result validated by the observation that T-DNA insertion mutants of two isoforms of annexin displayed hypersensitivity to osmotic stress and abscisic acid (ABA) treatment during germination and early seedling growth.

In contrast to the intensive study of the influence of water and nutrient status on plant proteomes, studies on plant responses to light and temperature stress are rare. The response of Arabidopsis to excessive light was gauged from experiments with isolated chloroplasts [131]. Both the soluble and insoluble proteins were subjected to 2-DE, revealing 52 proteins which changed in abundance as a result of the imposed stress. Among these were a number involved in photosynthesis, as well as some known light stress-related proteins, such as HSP, dehydroascorbate reductase, and SOD. Low temperature has a detrimental effect on male reproductive development in rice, and 2-DE profiling of the rice anther proteome has been able to demonstrate the enhanced and induced selective partial degradation of proteins at the young microspore stage as a result of cold treatment [132]. The cold stress responses of Arabidopsis have been studied using DIGE analysis of the leaf proteome [133]. This identified the accumulation of a number of proteins, in particular dehydrins and low-temperature-induced protein 78.

A failing of much of the literature is the lack of supportive functional data from genetic and other approaches, and projects combining comprehensive protein expression mapping with transcript expression profiling are still uncommon. An example of this combined approach has been reported in reciprocal maize hybrids differing in their reaction to cold germination and desiccation tolerance [134]. Most of the differentially expressed traits were implicated in more than one structural, enzymatic, or regulatory function and could be localized in various subcellular fractions. In further studies, pleiotropic effects should be eliminated, leading to a set of traits closely involved in the control of cellular processes and resulting in the manifestation of differences in cold germination and desiccation tolerance phenotypes.

5 PTMs

Proteomics provides the best available tools for the study of PTMs, which are indicative of the final players in most biological responses. The ongoing development of methods for the identification of PTMs, involving combinations of extraction protocols and affinity-based purification with multidimensional separation methods and MS, is making large-scale analysis of PTMs a reality [135]. Nevertheless, global analyses present some intrinsic difficulties. First, the number of potential protein modifications is large (about 300, including phosphorylation, glycosylation, ubiquitination, acetylation, and various forms of oxidation that can occur simultaneously in a single protein [136]). Second, many PTMs, at least those relevant to regulatory processes, are reversible. In addition, low abundance proteins or proteins located in very specific subcellular compartments, or present at certain stages of plant development, may only be detected if specific enriched protein subpopulations are analyzed.

At present, PTM research in plants has been mostly limited to phosphorylation in Arabidopsis, and this has been extensively and recently reviewed [33, 137]. The present update will therefore focus mainly on advances in defining the plant redox proteome, featuring protein S-nitrosylation and S-glutathionylation. Phosphorylation will be considered in relation to methodological aspects developed to unravel phosphorylation events – “Phosphoproteomics Technology” – that could be extrapolated for the study of other PTMs. Other PTMs of critical importance in the regulation of protein function, protein interactions, and protein turnover, such as acetylation, methylation, glycosylation, ubiquitination, and Ub-like modifications have been the object of proteomic research in human, yeast, and prokaryotic systems. Despite their interest, plant proteomic studies devoted to

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these PTMs are either scarce (glycosylation) or absent in the literature. At the time of writing, an update of PTM methodology has appeared [138], summarizing sample preparation strategies for the study of phosphorylation, ubiquitination, and glycosylphosphatidylinositol (GPI) modification.

5.1 Phosphoproteomics and phosphoproteomic technology

The enormous attention paid to phosphorylation has driven the development of specific methods for global analysis and has led to the emergence of the novel research field "phosphoproteomics". The current MS-based phosphoproteomics technology has established itself as an invaluable tool for the large-scale identification of novel phosphorylation sites, and protein kinases, and to uncover entire phosphoproteomes and their dynamics during stress and signaling (reviewed in [97, 139–141]).

The problem of the low abundance of phosphopeptides within the proteome is solved by the application of several fractionation and enrichment steps prior to MS analysis. The combination of these methods with radioactive and fluorescent labeling techniques allows a quantitative analysis of phosphorylation between samples. One of the most significant technological advances in phosphoproteomics has been immobilized metal-affinity chromatography (IMAC)-enrichment of phosphopeptides produced by tryptic digestion, followed by tandem MS [142]. The prepurification of single organelles or prefractionation of complex mixtures by, for example, SAX/SCX (strong cation/anion exchange chromatography), or the esterification of carboxylic acids groups prior to IMAC purification of peptides, increases the specificity and coverage of the phosphoproteome in each experiment. The power of the combined use of IMAC and MS for site-phosphorylation mapping in plants has been demonstrated by the large scale analysis of Arabidopsis phosphopeptides from plasma membranes [60], thylakoid membranes [143], and cytosolic and nuclear proteins [59, 140, 144, 145]. Recently, the phosphoproteome of the Arabidopsis seed has been characterized using a strategy combining the thermostability of many LEA proteins with the use of phosphoaffinity chromatography followed by phosphoprotein identification by MS [146]. Seven previously unknown phosphorylation sites were identified in the spinach stroma membrane by taking advantage of titanium dioxide microcolumns for phosphopeptide enrichment, coupled to MS analysis [147]. A large-scale phosphoproteome analysis of rice, in particular tracking changes in response to stress and hormone treatment [148], represents one of the few plant studies carried out in species other than Arabidopsis.

5.2 Redox proteome

Oxidative/nitrosative stress, an imbalance between the presence of ROS and reactive nitrogen species (RNS), and the antioxidant defense capacity of the organism is associated with the stress phenomenon (both abiotic and biotic) in many biological systems. The interaction between ROS/RNS and cellular components can result in a large variety of protein modifications. The formation of carbonyl groups is considered to be irreversible and is generally associated with a permanent loss of biological activity; in contrast, the reversible redox cycling of cysteines and methionine residues is associated with the activation/inactivation of protein function or the modulation of protein–protein interactions [149]. Few comprehensive analyses of cellular processes regulated by redox modifications of proteins (redox proteome) have been conducted in plants. Most of those directed toward the systematic identification of proteins containing redox-regulated cysteine residues have focused on the isolation and identification of subsets of proteins, such as targets for Trxs, and candidates for glutathionylation, nitrosylation, and carbonylation.

5.2.1 Carbonylation of proteins

Protein carbonylation is an oxidation process which leads to a permanent loss of function, and thus represents a valuable marker for oxidative stress. Its study has been simplified by the development of an immunochemical assay, which relies on conjugation with 2,4-dinitrophenylhydrazone (DNP) and immunodetection of the resulting DNP-tagged protein by a specific antibody. Two recent studies have described protein carbonylation in plants. In the first [25], the impact of oxidative stress on mitochondria was elucidated by use of immunoprecipitation of DNP-tagged proteins followed by 2-D-LC MS/MS of tryptic peptides to identify the oxidized proteins of the soluble matrix. An *in vitro* oxidation assay was then exploited to further identify a number of potentially oxidation-prone proteins. In the second [86], a combination of 2-DE and DNP immunoassay was used to research the impact of increased levels of ROS during the early stages of *Arabidopsis* seed germination, revealing some selective carbonylation targets, including glycolytic, mitochondrial and chloroplastic enzymes which very probably have implications for the success of the germination process.

5.2.2 Disulfide proteome

The oxidation/reduction of cysteine residues is increasingly recognized as an important modulator of protein activity and signal transduction in various physiologically important reactions in mammals, yeast, bacterial, and plant systems [149]. In plants, this dynamic PTM has long been known as a regulating mechanism for photosynthetic enzymes, and there is considerable interest in defining all the possible protein candidates for such regulation in the plant cell, *i.e.*, to define the "disulfide proteome" [150]. Thus, a large-scale proteomic approach was developed to investigate the total redox-responsive disulfide proteome of Arabidopsis [27]. Sulfhydryl groups were fully blocked by alkylation, and any disulfide cysteines remaining unmodified from the first step

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were converted to sulfhydryl groups by reduction. In the next step, thiol proteins were purified by affinity chromatography, separated by SDS-PAGE, and identified by MS. The use of affinity chromatography to enrich target proteins permits the detection of relatively low-abundance proteins: in all, 65 soluble, membrane and secreted putative disulfide proteins, of which 20 had not been previously described as being regulated by the redox state.

5.2.3 Targets of Trxs

Accumulating evidence suggests that Trxs, a ubiquitous family of small (12 kDa) oxidoreductases, is one of the major protein disulfide-reducing systems present in all cells. Plants contain several forms of Trx, which differ from one another in their subcellular localization, and thus, in the proteins with which they interact. Initially discovered as regulators of light-dependent malate biosynthesis in the chloroplast, plant Trxs are now implicated in a large spectrum of reactions related to metabolism, defense, and development. The development of new proteomic approaches designed for the characterization of Trx targets has greatly extended the putative functions of Trxs, and generated evidence for crosstalk between Trxs and glutaredoxins, the second major family of thiol disulfide reductases [151]. The identification of Trx targets in the soluble and membrane fractions of organelles known to contain Trxs, *i.e.*, plastids, mitochondria, nuclei, and plasma membrane is an active area of investigation which should lead to the elucidation of Trx-linked regulatory mechanisms. Two complementary strategies have been used.

5.2.3.1 Capture *via* protein–protein interactions using an affinity chromatography column grafted with a monocysteinic Trx

The monocysteinic Trx forms a disulfide bond with the target protein, but, unlike the wild-type molecule, the disulfide intermediate cannot be resolved because of the absence of a reactive thiol at the active site [152]. This method has been successfully applied to trap Trx targets from various plant organelles [150] and to search for putative Trx targets in the leaves of Arabidopsis [153]. After enzymatic reduction of the trapped targets, followed by radioactive labeling of unmasked thiols and 2-DE separation, 40 proteins were identified. These were involved in a variety of processes, including primary metabolism, folding, defense, and protein synthesis, and some were not previously known to be targets of Trxs [153]. A similar approach was employed to identify target proteins of cytosolic Trx in Arabidopsis [154].

5.2.3.2 The mBBr/2-DE strategy

This method begins with the chemical blocking of all free thiols, followed by the labeling of the newly formed-protein sulfhydryl groups with a specific fluorescent thiol labeling probe, monobromobimane (mBBr), before and after incubation with Trx. This is followed by a 2-DE protein separation and the detection of targets under UV light. This approach has been applied in a number of plant studies to identify Trx targets, for example in peanut seeds [155], and, most recently, in rice, aiming at the characterization of the role of Trxs during seed germination [156].

A combination of both strategies has been applied to investigate the role of Trx in wheat endosperm [157] and pea, spinach, and potato mitochondria [158]. In the former case, a unique set of proteins characteristic of each developmental stage was revealed, and in the latter, 50 potential Trx-linked proteins functioning in diverse processes were identified, supporting the view that Trxs act as sensors and enable mitochondria to adjust to key reactions in accordance with the prevailing redox state. The research was extended to study Trx regulation in wheat endosperm amyloplasts, where 42 potential Trx targets were identified, and a mechanism was hypothesized in which thiol and sugar have a signaling function which coordinates photosynthesis with amyloplast reactions [159].

The methods have been applied both to total extracts, and to cytosolic, plastid, and mitochondrial fractions. Currently, over 200 proteins have been associated with Trx in this way. In some cases, the same protein was detected with each approach providing an independent validation of the methods. Additional approaches are still needed to reveal the *in vivo* function of a given Trx and to evaluate the physiological consequences of reduction of the target. The feasibility of this challenging task has been recently demonstrated by the immunoprecipitation of *in vivo* target complexes from leaf extracts containing CDSP32, an environmental stress-induced chloroplastic Trx [160]. Comparing extracts from plants overexpressing Wt-CDSP32 with the active site mutant-CDSP32, two peroxiredoxins were identified, confirming that the interaction occurred *in vivo*. As peroxidase activity was dependent on CDSP32-interaction, it was concluded that CDSP32 was specifically involved in the plastidic response to oxidative stress.

5.2.4 *S*-Glutathionylation

The ability of proteins to form mixed protein disulfides with the redox buffer GSH in a process known as *S*-glutathionylation, is now recognized as a common reversible PTM that can act as a redox-driven regulator of signal transduction cascades and metabolic pathways. Various studies have identified proteins that have undergone glutathionylation as part of their regulation. The recently reported Trx f, a chloroplastic Trx from Arabidopsis, was among the first plant proteins found to undergo this PTM, indicating the existence of crosstalk between the Trx and GSH systems [161]. At the time of writing, we are only aware of one publication which has attempted the large-scale identification of proteins than can be reversibly *S*-glutathionylated in plants [162]. Using Arabidopsis cell suspension cultures and a combination of

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in vivo and *in vitro* GSH-biotin labeling methods, followed by 2-DE and MS analysis, 79 proteins undergoing glutathionylation in response to oxidizing conditions were isolated and identified. Some of these were analyzed further to identify mechanisms of thiolation and the effect of this modification on biological activity.

5.2.5 *S*-Nitrosylation

Nitric oxide has been identified as a key signaling molecule in plants over the past few years, and is an important determinant of the response to biotic and abiotic stress, iron homeostasis, and regulation of respiration and photosynthesis [163–166]. A growing body of evidence suggests that protein S-nitrosylation, the covalent attachment of NO to the thiol group of a cysteine residue, is a redox-based regulation mechanism that plays a pivotal role in plants in a similar manner as occurs in animal systems. Recently, the first detailed characterization of S-nitrosylation in Arabidopsis has been described, which involved the inhibition of the enzyme methionine adenosyltransferase [167]

Except for an *in silico* search for *S*-nitrosylation in Arabidopsis, which resulted in more than 100 hits [137], only one experimental attempt to identify *S*-nitrosylated plant proteins on a global scale has been reported [168]. This research used a recently developed, highly specific biotin switch method for the purification of S-nitrosylated proteins in animals to identify S-nitrosylated proteins in *Arabidopsis* plants and cell suspensions in response to nitrosative stress. In combination with nano-LC and MS analysis, a number of *S*-nitrosylation candidates were identified, including stress-related, redox-related, signaling and cytoeskeletal proteins, and metabolic enzymes. About 60% of these proteins had already been described in various animal systems in the context of *S*-nitrosylation and *S*-glutathionylation, validating the specificity of the method and further indicating that NO-regulation in plants and animals share common features.

In vitro and *in vivo* experiments frequently identify different polypeptides [162, 168]. Nevertheless, *in vitro* experiments do allow a reasonable picture to be formed of the range of proteins that can undergo this sort of modification. The priority now, as stated in [162], will be to develop protocols which allow a reliable monitoring of *in vivo* reactions while avoiding nonspecific side effects.

5.3 GPI-anchoring and peptide-protein modifications

The GPI anchor is a special form of glycosylation, and is a common modification at the C-terminus of some proteins, attaching them to the outer cell membrane. The unique functional and structural properties of GPI-anchored proteins (GPI-APs) conferred by this tag explain their well-recognized importance in signaling during developmental and differentiation processes in various systems, including in plants. One key feature of this group of proteins is their dynamic release into the extracellular space *via* a specific phospholipase cleavage of the GPI moiety. The only two plant-based investigations devoted to high scale characterization of GPI-APs in plants have taken advantage of the enzymatic release of these proteins after treatment of plasma membrane fractions with either phospholipase C (PLC) [169] or phospholipase D (PLD) [61]. The use of PLD proved to be more versatile than PLC for the investigation of GPI-APs in a variety of cells, tissues, and organisms. PLD treatment-release of GPI-APs followed by capillary LC and MS/MS led to the identification of 35 Arabidopsis GPI-APs. Considering that GPI anchors are site-specific, the integration of both proteomic experimental approaches and computational prediction tools should improve the efficiency, specificity, and sensitivity for characterizing this subgroup of proteins [61].

All aspects of plant life are controlled by the regulated synthesis of new polypeptides and the precise degradation of preexisting ones. Consistent with the importance of protein turnover for cell control, the Ub/26S and Ub-related proteasome pathways play a pivotal role in housekeeping and cell regulation processes. Thus, these polypeptide-based PTMs are involved in every aspect of plant physiology: cell division, response to hormones, light, and biotic and abiotic stresses. In the Ub/26S proteasome pathway, the dominant proteolytic system in plants, Ub polymers are covalently bound to protein targets using a three-step (E1-E2-E3) conjugation cascade that detects specific Ub signals. The resulting peptide-tagged proteins are then degraded by deubiquitinating enzymes in the 26S proteasome, an ATP-dependent proteolytic complex, with the concomitant release of the reusable Ub residues. In addition to Ub, the number of identified plant Ub-related modifiers is expanding continuously consistent with the idea that these peptide tags are key players in plant cell regulation. Among these are RUB1 (related to Ub-1), SUMO (small Ub-related modifier), and HUB-1 (homologous to Ub-1), which, although they apparently have a much more limited set of targets and perform more specialized functions, use analogous mechanisms to modify and regulate target proteins (reviewed in [170]).

The application of emerging technologies is making proteomic studies of protein modification by Ub- and Ub-like proteins feasible. Many recent proteomic studies in mammals and yeast have targeted the ubiquitination and Ub-like modification of proteins, with a view to identify the proteolytic machinery and target proteins, and to elucidate the modification sites in the target proteins [171, 172]. The methodological approaches include, in most cases, a multistep procedure consisting of affinity purification with an epitope-tagged Ub/Ub-like protein, followed by proteolytic digestion of the covalent bond between the modifying peptide and the substrate and the use of chemical probes, coupled to various MS techniques. A combination of various C-terminal SUMO mutants with different protease

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digestion strategies has enabled an efficient identification of SUMO attachment sites in *Saccharomyces cerevisiae* [173]. Proteomic approaches used for animals and yeasts could be easily adapted to study the proteolytic regulatory pathways in plants, and thus lead to the identification of the substrates, interacting partners, enzymatic components, and effector enzymes of these systems. As with the entire field of MS-based proteomics, a critical future goal will be to perform quantitative experiments to study the dynamic behavior of such modifications under various physiological conditions. These experiments have already been initiated to decipher both SUMOylation and ubiquitination pathways in animals using ICAT and SILAC procedures.

6 Interactomics

The dissection of protein–protein interactions constitutes a challenging task. In the plant area, in addition to well-established methods with a truly proteomic dimension (see below), several complementary approaches have been attempted over the review period. These include yeast two-hybrid technology [174, 175] and its split-Ub membrane-based version [176], as well as less generic techniques such as bimolecular fluorescence complementation [177, 178] and far Western blotting [179]. *In silico* analysis is increasingly being used to predict interactions and evaluate the relevance of experimental data in the light of archival information [180, 181]. Most proteomic experiments have relied on either native gel electrophoresis (see Section 6.1) or affinity methods (see Section 6.2).

6.1 Native electrophoresis

Blue-Native (BN) and CN gels were introduced in the late 1980s to study protein–protein interactions with membrane proteins. In these methods, protein complexes are gently delipidized with nonionic or zwitterionic detergents and then electrophoresed through nondenaturing polyacrylamide gels, which discriminate on the basis of both molecular mass and net charge. The introduction of CBB G250 (BN-PAGE), a negatively charged molecule able to bind to various amino acids, reduces the effect of charge on the migration of the protein complexes, leaving them to be resolved mainly on the basis of their mass. The BN/CN-PAGE method has been used to investigate complexes in the tomato respiratory chain [182], and was recently extended to both mixtures of membrane and membrane-associated proteins [52] and to soluble complexes [48] in plastids (see Section 2.2.1). BN/CN-PAGE allows a simple global overview of stable complexes and is somewhat equivalent of a 2-DE but does not introduce all the biases linked to 2-DE (*e.g.*, bias against high MW proteins). The resolving power of this technique is at least as good as gel filtration, but is still limited, while the prolonged electrophoresis necessaryily restricts the analysis to stable complexes.

6.2 Tandem affinity purification of tagged proteins (TAP-tag), affinity chromatography, and surface plasmon resonance (SPR)

In the TAP-tag system, a fusion protein is created by the insertion of two tags (in tandem), usually at the N- or the C-terminus of the protein. The ideal tag needs to be small (to minimize interference with the biological function of the target) and must facilitate a rapid and flexible purification from a complex mixture. In addition, the affinity chromatography of the tagged protein should ideally be achieved under native conditions so that the interacting molecules can be recovered along with the protein. This approach, with careful inspection for plausible interactions, has led to the identification of partners for more than 20 protein kinases in rice [183]. Direct affinity chromatography can be also used to trap target proteins in a different fashion, as exemplified by the grafting of a modified Trx to an affinity column ([152], see Section 5.2.3).

SPR technology represents a sophisticated version of affinity chromatography in which the kinetics of binding is recorded in real time. SPR involves label-free binding interactions between an analyte in solution and an immobilized ligand. The interactions are monitored directly by changes in the refractive index at a biosensor surface, where the response is directly proportional to the mass of the bound analyte. As the nature of the analyte and ligand can be very different, this versatile method can analyze a broad spectrum of interactions and was recently used to characterize chitopentose–lectin interactions in wheat [184]. However, this technology has yet to be used on any large scale in plants.

Compared to the situation in 2004, the last 2 years have seen both an increase in the use of classical approaches and the introduction of novel strategies for the systematic investigation of certain protein complexes. Perhaps more importantly, it is becoming clear that, even when addressing a clearly defined subproteome, additional information can be extracted by deploying procedures that do not destroy complexes before MS/MS characterization. This simple improvement is likely to be even more beneficial to functional approaches as the abundance and the stoichiometry of the partners can be determined. To this end, the introduction of absolute quantification procedures would be highly desirable.

7 Conclusions

The early part of the review period was preoccupied with the completion of protein directories (protein identification, corrections of false genome annotations, improvement and validation of *in silico* targeting predictions). Major advances have involved an increase in the number of subcellular territories investigated on a large scale and the implementation of organelle proteomics in physiological studies. On the other hand, despite some significant pioneering efforts, pro-

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gress in the characterization of complexes and of post-translational status has been limited. Modern quantitative tools have been little used to date, thus restricting relevance, particularly in physiological studies. Obviously, the generalization of this level of investigation will represent the major challenge for the coming period.

Despite significant advances gained in the understanding of plant physiology, partly as a consequence of the deployment of genomics and proteomics, significantly less effort has been devoted to plant studies as a whole, compared to parallel work in other biological systems, particularly human and yeast. The same is true for plant proteomics. Even though this field has undergone exponential growth over the last few years, showing as yet no sign of tapering off, proteomic research in plants has not advanced at the same pace as have human and yeast proteomics. Comprehensive quantitative comparative studies of dynamic protein profiling during developmental or stress responses, and functional analysis and characterization of regulatory processes are now needed to understand plant physiology and how plants interact with and adapt to the environment. This will have a substantial impact on agriculture and through this, on human health. Plants have particular advantages for biological study, as they are easily handled and enjoy an enormous breadth of genetic diversity, both within and between species.

While the state-of-the art technology (including bioinformatic tools) appropriate for proteomic analysis is at hand, high cost and dependence on human expertise remain an impediment. Additional challenges for the plant research community flow from the fact that only experiments conducted on Arabidopsis and rice can take full advantage of the technological cutting edge, as has been underlined throughout this review. For example, the need for a fully sequenced genome for the positive identification of PTMs is the major obstacle preventing extension of this field beyond the genomic model species. It is clear that plant proteomics will progress in harness with genomic progress, and the expectation is that the coming years will see an increasing number of fully sequenced and annotated plant genomes. The vast amount of empirical information derived from proteomics studies should, in turn, assist in generating plant-specific databases (organelle-, tissue-, stress response-specific) and efficient and accurate prediction programs to infer localization, PTMs, and functional and interacting domains. Thus, the forthcoming challenge lies in the development of bioinformatic tools and robust prediction programs based on plant sequences in order to facilitate data organization, to generate testable hypotheses, and to model holistic biological networks. These tools will inform a wide range of plant processes in Arabidopsis and other model plants. Although much of this model knowledge will be transferable to other plant systems, it will remain necessary to directly explore plants of agricultural importance, since many production traits are crop-specific. This will only become possible as genomic information is obtained for these species.

A further important challenge concerns the development of methodology, in relation to the establishment of easy, reliable, and efficient protocols for sample preparation, reducing the amount of starting material needed, and allowing high-throughput studies compatible with the technology available. Plant sample preparation has to be optimized for each sample type, always with an eye kept on the purpose behind each experiment. A standardization of protocols for tissue fractionation, protein extraction, and solubilization made available to the whole plant research community will accelerate the use of leading edge proteomic technologies such as MudPIT, DIGE, iTRAQ, and SILAC in combination with prefractionation and enrichment protocols (such as IMAC) for specific subgroups of proteins. This will enable quantitative studies with an unprecedented level of sensitivity, covering entire sub-proteomes and the investigation of their dynamic behavior throughout specific processes such as stress, development, *etc.*

Toward this end, the plant research community is called upon to generate appropriate networks and organize and coordinate initiatives in a similar manner to the existing HUPO. This sort of initiative is both desirable and necessary to unite and maximize the efforts of the whole community, including laboratories at universities, public research centers, and companies worldwide.

Proteomics is a relatively new field which is growing at an unprecedented rate. Thanks to its potential to unravel biological questions not readily accessible by other technologies, it has been enthusiastically applied to many biological systems, creating large volumes of raw data for databases, and succeeding in assigning function to numerous proteins. Comparative proteome analysis still relies heavily on 2-DE profiling, but the evaluation of experimental data remains dependent on the judgements of the individual researcher. A rule-of-thumb applied to the design of proteomics experiments is that at least three independent replicates must be used for the assessment of the biological variance, and at least three independent gels run to establish sample reproducibility. However, although most of the authors cited follow this level of control in their experiments, a comprehensive statistical evaluation of the quality of experimental data, as described in [126], is still an exception. Thus it will be important to define the guidelines for the analysis and documentation of proteomic data for routine future use [185].

New plant proteomics studies will be more likely directed toward functional analysis than simple mapping. Therefore, request of documentation will change. In addition to descriptions of experimental data, data validation, protein inference problems, and particularly protein quantification will grow in importance. The implementation and management of heterogeneous instrument platforms for gel imaging, MS, and database searches are a priority. Unifying modes of data presentation and establishing standard parameters to ensure the integrity of protein assignments will help to assure the credibility of scientific records, and allow published proteomic data to be readily comparable to experiments conducted in laboratories worldwide [185].

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The ultimate goal of proteomic technology is to define protein function. Although high-throughput experiments typically generate multiple candidates as components of a biological response, the critical demonstration as to which, if any, of these do indeed participate in the process under investigation is usually lacking. Localization, modification, and interactions can only provide supportive evidence. Function can, in the end, only be proven through a combination of biochemical and genetic experiments. Several robotic platforms have endeavored to obtain protein profiling automation based on direct enzyme activity measurement [186] or protein arrays, but these platforms are still being considered more of a promise than a reality.

The expectation is that integration of proteomic and genomic data will deliver much of the raw information necessary to predict which protein forms, PTMs, and protein complexes are present at a specific moment in a given tissue. Quantitative comparisons during development or in response to stress, robust predictions of the role(s) of particular proteins in metabolic and signal pathways, and large-scale functional testing of candidate proteins, will serve to fill the gaps in genetically defined pathways. Progress in these directions will lead to the modeling of entire metabolic pathways in the coming years, and thus usher in an era of predictive biology. This will represent a giant step for biotechnology, allowing it to contribute significantly to the design of genetic solutions to the ever-present threats of biotic and abiotic stress.

The authors thank www.smartenglish.co.uk for linguistic advice in the preparation of this manuscript.

8 References

[1] Cánovas, F. M., Dumas-Gaudot, E., Recorbert, G., Jorrín, J. *et al.*, *Proteomics* 2004, *4*, 285–298.

[2] Agrawal, G. K., Rakwal, R., *Mass Spectrom. Rev.* 2006, *25*, 1–53.

[3] Jorge, I., Navarro, R. M., Lenz, M., Ariza, D. *et al.*, *Proteomics* 2005, *5*, 222–234.

[4] Di Michele, M., Chiatante, D., Plomion, C., Scippa, G. S., *Plant Sci.* 2006, *170*, 926–935.

[5] Nam, M. H., Kim, S. I., Liu, J. R., Yang, D. C. *et al.*, *J. Chromatogr. B* 2005, *815*, 147–155.

[6] Perrocheau, L., Rogniaux, H., Boivin, P., Marion, D., *Proteomics* 2005, *5*, 2849–2858.

[7] Carbonaro, M., *Food Sci. Technol.* 2004, *15*, 209–216.

[8] Cellini, F., Chesson, A., Colquhoun, I., Constable, A. *et al.*, *Food Chem. Toxicol.* 2004, *42*, 1089–1125.

[9] Wait, R., Gianazza, E., Brambilla, D., Eberini, I. *et al.*, *J. Agric. Food Chem.* 2005, *53*, 4599–4606.

[10] Lehesranta, S. J., Davies, H. V., Shepherd, L. V., Nunan, N. *et al.*, *Plant Physiol.* 2005, *138*, 1690–1699.

[11] Incamps, A, Hely-Joly, F., Chagvardieff, P., Rambourg, J. C. *et al.*, *Biotechnol. Bioeng.*, 2005, *91*, 447–459.

[12] Carpentier, S. C., Witters, E., Laukens, K., Deckers, P. *et al.*, *Proteomics*, 2005, *5*, 2497–2507.

[13] Dumas-Gaudot, E., Amiour, N., Weidmann, S., Bestel-Corre, G. *et al.*, *Proteomics* 2004, *4*, 451–453.

[14] Weckwerth, W., Wenzel, K., Fiehn, O., *Proteomics* 2004, *4*, 78–83.

[15] Majeran, W., Cai, Y., van Wijk, K. J., *Plant Cell* 2005, *17*, 3111–3140.

[16] Schad, M., Lipton, M. S., Giavalisco, P., Smith, R. D., Kehr, J., *Electrophoresis* 2005, *26*, 2729–2738.

[17] Natarajan, S., Xu, C., Caperna, T. J., Garret, W. M., *Anal. Biochem.* 2005, *342*, 214–220.

[18] Schiltz, S., Gallardo, K., Huart, M., Negroni *et al.*, *Plant Physiol.* 2004, *135*, 2241–2260.

[19] Kim, S. T., Cho, K. S., Jang, Y. S., Kang, K. Y., *Electrophoresis* 2001, *22*, 2103–2109.

[20] Wienkoop, S., Glinski, M., Tanaka, N., Tolstikov, V. *et al.*, *Rapid Commun. Mass Spectrom.* 2004, *18*, 643–650.

[21] Koller, A., Washburn, M. P., Lange, B. M., Andon, N. L. *et al.*, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 11969–11974.

[22] Heinemeyer, J., Eubel, H., Wehmhoner, D., Jansch, L., Braun, H.-P., *Phytochemistry* 2004, *65*, 1683–1692.

[23] Bayer, E. M., Bottrill, A. R., Walshaw, J., Vigoroux, M. *et al.*, *Proteomics* 2006, *6*, 301–311.

[24] Carter, C., Pans, S., Zouhar, J., Avila, E. L. *et al.*, *Plant Cell* 2004, *16*, 3285–3303.

[25] Kristensen, B. K., Askerlund, P., Bykova, N. V., Egsgaard, H., Moller, I. M., *Phytochemistry* 2004, *65*, 1839–1851.

[26] Borner, G. H., Sherrier, D. J., Weimar, T., Michaelson *et al.*, *Plant Physiol.* 2005, *137*, 104–116.

[27] Lee, K., Lee, J., Kim, Y., Bae, D. *et al.*, *Electrophoresis* 2004, *25*, 532–541.

[28] Ndimba, B. K., Chivasa, S., Simon, W. J., Slabas, A. R., *Proteomics* 2005, *16*, 4185–4196.

[29] Casati, P., Zhang, X., Burlingame, A. L., Walbot, V., *Mol. Cell. Proteomics* 2005, *4*, 1673–1685.

[30] Dunkley, T. P., Dupree, P., Watson, R. B., Lilley, K. S., *Biochem. Soc. Trans.* 2004, *32*, 520–523.

[31] Gruhler, A., Schulze, W. X., Mathiessen, R., Mann, M., Jensen, O. N., *Mol. Cell. Proteomics* 2005, *4*, 1697–1709.

[32] Heazlewood, J. L., Tonti-Filippini, J., Verboom, R. E., Millar, A. H., *Plant Physiol.* 2004, *139*, 598–609.

[33] Peck, S. C., *Plant Physiol.* 2005, *138*, 591–599.

[34] Hirano, H., Islam, N., Kawasaki, H., *Phytochemistry* 2004, *65*, 1487–1498.

[35] Rose, J. K. C., Bashir, S., Giovannoni, J. J., Jahn, M. M., Saravanan, R. S., *Plant J.* 2004, *39*, 715–733.

[36] Schwacke, R., Flügge, U. I., Kunze, R., *Plant Physiol. Biochem.* 2004, *42*, 1023–1034.

[37] Friso, G., Giacomelli, L., Ytterberg, A. J., Peltier *et al.*, *Plant Cell* 2004, *16*, 478–499.

[38] Kleffmann, T., Hirsch-Hoffmann, M., Gruissem, W., Baginsky, S., *Plant Cell Physiol.* 2006, *47*, 432–436.

[39] Heazlewood, J. L., Millar, A. H., *Nucleic Acids Res.* 2005, *33*, 605–610.

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[40] Brown, J. W. S., Shaw, P. J., Shaw, P., Marshall, D. F., *Nucleic Acids Res.* 2005, *33*, 633–636.

[41] Komatsu, S., *Plant Mol. Biol.* 2005, *59*, 179–190.

[42] Pan, S., Carter, C. J., Raikhel, N. V., *Expert Rev. Proteom.* 2005, *2*, 781–792.

[43] van Wijk, K. J., *Plant Physiol. Biochem.* 2004, *42*, 963–977.

[44] Rolland, N., Ferro, M., Seigneurin-Berny, D., Garin, J. *et al.*, *Photosynth. Res.* 2003, *78*, 205–230.

[45] Zabrouskov, V., Giacomelli, L., van Wijk, K. J., McLafferty, F. W., *Mol. Cell. Proteomics* 2003, *2*, 1253–1260.

[46] Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W. *et al.*, *Curr. Biol.* 2004, *14*, 354–362.

[47] Peltier, J. B., Yttergberg, A. J., Sun, Q., van Wijk, K. J., *J. Biol. Chem.* 2004, *279*, 49367–49383.

[48] Peltier, J. B., Cai, Y., Sun, Q., Zabrouskov *et al.*, *Mol. Cell. Proteomics* 2006, *5*, 114–133.

[49] Ytterberg, A. J., Peltier, J. B., van Wijk, K. J., *Plant Physiol.* 2006, *140*, 984–997.

[50] Vidi P. A., Kanwischer M., Baginsky S., Austin J. R. *et al.*, *J. Biol. Chem.* 2006, *281*, 11225–11234.

[51] Lonosky, P. M., Zhang, X., Honavar, V. G., Dobbs, D. L. *et al.*, *Plant Physiol.* 2004, *134*, 560–574.

[52] Ciambella, C., Roepstorff, P., Aro, E. M., Zolla, L., *Proteomics* 2005, *5*, 746–757.

[53] von Zychlinski, A., Kleffmann, T., Krishnamurthy, N., Sjölander, K. *et al.*, *Mol. Cell. Proteomics* 2005, *4*, 1072–1084.

[54] Millar, A. H., Heazlewood, J. L., Kristensen, B. K., Braun, H. P., Møller, I. M., *Trends Plant Sci.* 2005, *10*, 36–43.

[55] Bruguière, S., Kowalski, S., Ferro, M., Seigneurin-Berny, D. *et al.*, *Phytochemistry* 2004, *65*, 1693–1707.

[56] Millar, A. H., Trend, A. E., Heazlewood, J. L., *J. Biol. Chem.* 2004, *279*, 39471–39478.

[57] Taylor, N. L., Heazlewood, J. L., Day, D. A., Millar, A. H., *Mol. Cell. Proteomics* 2005, *4*, 1122–1133.

[58] Marmagne, A., Rouet, M. A., Ferro, M., Rolland, N. *et al.*, *Mol. Cell. Proteomics* 2004, *3*, 675–691.

[59] Nühse, T. S., Stensballe, A., Jensen, O. N., Peck, S. C., *Mol. Cell. Proteomics* 2003, *2*, 1234–1243.

[60] Nühse, T. S., Stensballe, A., Jensen, O. N., Peck, S. C., *Plant Cell* 2004, *16*, 2394–2405.

[61] Elortza, F., Mohammed, S., Bunkenborg, J., Foster, L. J. *et al.*, *Proteome Res.* 2006, *5*, 935–943.

[62] Valot, B., Negroni, L., Zivy, M., Gianinazzi, S., Dumas-Gaudot, E., *Proteomics* 2006, *6*, suppl. 1, S145–155.

[63] Szponarski, W., Sommerer, N., Boyer, J. C., Rossignol, M., Gibrat, R., *Proteomics* 2004, *4*, 397–406.

[64] Konishi, H., Maeshima, M., Komatsu, S., *J. Proteome Res.* 2005, *4*, 1775–1780.

[65] Lee, S. J., Saravanan, R. S., Damasceno, C. M., Yamane, H. *et al.*, *Plant Physiol. Biochem.* 2004, *42*, 979–988.

[66] Watson, B. S., Lei, Z., Dixon, R. A., Sumner, L. W., *Phytochemistry* 2004, *65*, 1709–1720.

[67] Boudart, G., Jamet, E., Rossignol, M., Lafitte, C. *et al.*, *Proteomics* 2005, *5*, 212–221.

[68] Chivasa, S., Simon, W. J., Yu, X. L., Yalpani, N., Slabas, A. R., *Proteomics* 2005, *5*, 4894–4904.

[69] Dani, V., Simon, W. J., Duranti, M., Croy, R. R., *Proteomics* 2005, *5*, 737–745.

[70] Khan, M. M., Komatsu, S., *Phytochemistry* 2004, *65*, 1671–1681.

[71] Pendle, A. F., Clark, G. P., Boon, R., Lewandowska, D. *et al.*, *Mol. Biol. Cell.* 2005, *16*, 260–269.

[72] Gonzalez-Camacho, F., Medina, F. J., *Proteomics* 2004, *4*, 407–417.

[73] Dunkley, T. P. J., Watson, R., Griffin, J. L., Dupree, P., Lilley, K. S., *Mol. Cell. Proteomics* 2004, *3*, 1128–1134.

[74] Dunkley, T. P. J., Hester, S., Shadforth, I. P., Runions, J. *et al.*, *Proc. Natl. Acad. Sci. USA* 2006, *103*, 6518–6523.

[75] Giavalisco, P., Nordhoff, E., Kreitler, T, Klöppel, K. D., *Proteomics* 2005, *5*, 1902–1913.

[76] Lei, Z., Elmer, A. M., Watson, B. S., Dixon, R. A. *et al.*, *Mol. Cell. Proteomics* 2005, *4*, 1812–1825.

[77] Vincent, D., Lapierre, C., Pollet, B., Cornic, G. *et al.*, *Plant Physiol.* 2005, *137*, 949–960.

[78] Franceschetti, M, Perry, B., Thompson, B., Hanfrey, C., Michael, A. J., *FEBS Lett.* 2004, *576*, 477–480.

[79] Soria, C., Negroni, L., Balliau, T., Corti, H. *et al.*, *Plant Physiol.* 2006, *140*, 349–364.

[80] Brinker, M., van Zyl, L., Liu, W., Craig, D. *et al.*, *Plant Physiol.* 2004, *135*, 1526–1539.

[81] Hochholdinger, F., Woll, K., Guo, L., Schnable, P. S., *Proteomics* 2005, *5*, 4885–4893.

[82] Dafny-Yelin, M., Guterman, I., Menda, N., Ovadis, M. *et al.*, *Planta* 2005, *222*, 37–46.

[83] Holmes-Davis, R., Tanaka, C. K., Vensel, W. H., Hurkman, W. J., McCormick, S., *Proteomics* 2005, *5*, 4864–4884.

[84] Fernando, D. D., *Proteomics* 2005, *5*, 4917–4926.

[85] Sheoran, I. S., Olson, D. J., Ross, A. R., Sawhney, V. K., *Proteomics* 2005, *5*, 3752–3764.

[86] Job, C., Rajjou, L., Lovigny, Y., Belghazi, M., Job, D., *Plant Physiol.* 2005, *138*, 790–802.

[87] Vensel, W. H., Tanaka, C. K., Cai, N., Wong, J. H. *et al.*, *Proteomics* 2005, *5*, 1594–1611.

[88] Lippert, D., Zhuang, J., Ralph, S., Ellis, D. E. *et al.*, *Proteomics* 2005, *5*, 461–473.

[89] Imin, N., Nizamidin, M., Daniher, D., Nolan, K. E. *et al.*, *Plant Physiol.* 2005, *137*, 1250–1260.

[90] Wienkoop, S., Zoeller, D., Ebert, B., Simon-Rosin, U. *et al.*, *Phystochemistry* 2004, *65*, 1641–1649.

[91] Amme, S., Rutten, T., Melzer, M., Sonsmann, G. *et al.*, *Proteomics* 2005, *5*, 2508–2518.

[92] Andon, N. L., Eckert, D., Yates, J. R. III, Hayness, P. A., *Proteomics* 2003, *3*, 1270–1278.

[93] Konishi, H., Kitano, H., Komatsu, S., *Plant Cell Env.* 2005, *28*, 328–339.

[94] Tanaka, N., Takahashi, H., Kitano, H., Matsuoka, M. *et al.*, *J. Proteome Res.*2005, *4*, 1575–1582.

[95] Jorrín, J., Rubiales, D., Dumas-Gaudot, E., Recorbert, G. *et al.*, *Euphytica* 2006, *147*, 37–47.

[96] Renaut, J., Hausman, J. F., Wisniewski, M. E., *Physiol. Plant.* 2006, *126*, 97–109.

[97] Thurston, G., Regan, S., Rampitsch, C., Xing, T., *Physiol. Mol. Plant Pathol.* 2005, *66*, 3–11.

[98] Riccardi, F., Gazeau, P., Jacquemot, M. P., Vincent, D., Zivy, M., *Plant Physiol. Biochem.* 2004, *42*, 1003–1011.

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[99] Chevalier, F., Martin, O., Rofidal, Devauchelle, A. D. *et al.*, *Proteomics* 2004, *4*, 1372–1381.

[100] Bahrman, N., Gouy, A., Devienne-Barret, F., Hirel, B. *et al.*, *Plant Sci.* 2005, *168*, 81–87.

[101] Bahrman, N., Gouy, A., Devienne-Barret, F., Hirel, B. *et al.*, *Plant Sci.* 2005, *168*, 81–87.

[102] Sule, A., Vanrobaeys, F., Hajos, G., Van Beeumen, J., Devreese, B., *Phytochemistry* 2004, *65*, 1853–1863.

[103] Ingle, R. A., Smith, J. A., Sweetlove, L. J., *Biometals* 2005, *18*, 627–641.

[104] Smith, A. P., DeRidder, B. P., Guo, W.-J., Seeley, E. H. *et al.*, *J. Biol. Chem.* 2004, *279*, 26098–26104.

[105] Chen, H., Wilkerson, C. G., Kuchar, J. A., Phinney, B. S., Howe, G. A., *Proc. Natl. Acad. Sci. USA* 2005, *102*, 19237–19242.

[106] Jones, A. M., Thomas, V., Truman, B., Lilley, K. *et al.*, *Phytochemistry* 2004, *65*, 1805–1816.

[107] Walz, C., Giavalisco, P., Schad, M., Juenger, M. *et al.*, *Phytochemistry* 2004, *65*, 1795–1804.

[108] Alvarez, S., Goodger, J. Q. D., Marsh, E. L., Chen, S. *et al.*, *J. Proteome Res.* 2006, DOI: 10.1021/pr050471.

[109] Jamet, E., Canut, H., Boudart, G., Pont-Lezica, R. F., *Trends Plant Sci.* 2006, *11*, 33–39.

[110] Haslam, R. P., Downie, A. L., Raveton, M., Gallardo, K., *Ann. Appl. Biol.* 2003, *143*, 81–89.

[111] Yoo, B. C., Lee, J. Y., Lucas, W. J., *J. Biol. Chem.* 2002, *277*, 15325–15332.

[112] Heil, M., Balwin, I. T., *Trends Plant Sci.* 2002, *7*, 61–67.

[113] Castillejo, M. A., Amiour, N., Dumas-Gaudot, E., Rubiales, D., Jorrín, J. V., *Phytochemistry* 2004, *65*, 1817–1828.

[114] Slaymaker, D. H., Keen, N. T., *Plant Sci.* 2004, *166*, 387–396.

[115] Hancock, J. T., Henson, D., Nyirenda, M., Desikan, R. *et al.*, *Plant Physiol. Biochem.* 2005, *43*, 828–835.

[116] Oh, I. S., Park, A. R., Bae, M. S., Kwon, S. J. *et al.*, *Plant Cell* 2005, *17*, 2832–2847.

[117] Lum, H.-K., Lee, C. H., Butt, Y. K.-C., Lo, S. C.-L., *Nitric Oxide* 2005, *12*, 220–230.

[118] Fujiwara, M., Umemura, K., Kawasaki, T., Shimamoto, K., *Plant Physiol.* 2006, *140*, 734–745.

[119] Swidzinski, J. A., Leaver, C. J., Sweetlove, L. J., *Phytochemistry* 2004, *65*, 1829–1838.

[120] Tsunezuka, H., Masayuki, F., Tsumotu, K., Shimamoto, K., *Mol. Plant-Microbe Interact.* 2005, *18*, 52–59.

[121] Hammersmidt, R., *Physiol. Mol. Plant. Pathol.* 2004, *65*, 223–224.

[122] Rep, M., van der Does, H. C., Meijer, M., van Wijk, R. *et al.*, *Mol. Microbiol.* 2004, *53*, 1373–1383.

[123] Quirino, B. F., Genger, R., Ham, J. H., Zabala, G., Bent, A. F., *Physiol. Mol. Plant. Pathol.* 2004, *65*, 257–267.

[124] Shahollari, B., Peskan-Berghofer, T., Oelmuller, R., *Physiol. Plant.* 2004, *122*, 397–403.

[125] Shahollari, B., Varma, A., Oelmuller, R., *J. Plant. Physiol.* 2005, *162*, 945–958.

[126] Hajheidari, M., Abdollahian-Noghabi, M., Askari, H., Heidari, M. *et al.*, *Proteomics* 2005, *5*, 950–960.

[127] Jorge, I., Navarro, R. M., Lenz, C., Ariza, D., Jorrín, J., *Proteomics* 2006, *6*, S207–214.

[128] Fulda, S., Huang, F., Nilson, F., Hagemann, M., Norling, B., *Eur. J. Biochem.* 2000, *267*, 5900–5907.

[129] Huang, F., Fulda, S., Hagemann, M., Norling, B., *Proteomics* 2006, *6*, 910–920.

[130] Lee, S., Lee, E. J., Yang, E. J., Lee, J. E. *et al.*, *Plant Cell* 2004, *16*, 1378–1391.

[131] Phee, B. K., Cho, J. H., Park, K. S., Jung, J. H. *et al.*, *Proteomics* 2004, *4*, 3560–3568.

[132] Imin, N., Kerim, T., Rolfe, B. G., Weinman, J. J., *Proteomics* 2004, *4*, 1873–1882.

[133] Amme, S., Matros, A., Schlesier, B., Mock, H. P., *J. Exp. Bot.* 2006, *57*, 1537–1546.

[134] Kollipara, K. P., Saab, I. N., Wych, R. D., Lauer, M. J., Singletary, G. W., *Plant Physiol.* 2002, *129*, 974–992.

[135] Jensen, O. N., *Curr. Opin. Chem. Biol.* 2004, *8*, 33–41.

[136] Aebersold, R., Goodlett, D. R., *Chem. Rev.* 2001, *101*, 209–295.

[137] Huber, S. C., Hardin, S. C., *Curr. Opin. Plant Biol.* 2004, *7*, 318–322.

[138] Kwon, S. J., Choi, E. Y., Choi, Y. J., Ahn, J. H., Park, O. K., *J. Exp. Bot.* 2006, *57*, 1547–1551.

[139] Laugesen, S., Bergoin, A., Rossignol, M., *Plant Physiol. Biochem.* 2004, *42*, 929–936.

[140] de la Fuente van Bentem, S., Roitinger, E., Anrather, D., Csaszar, E., Hirt, H., *Physiol. Plant.* 2006, *126*, 110–119.

[141] Peck, S. C., *J. Exp. Bot.* 2006, *57*, 1523–1527.

[142] Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J. *et al.*, *Nat. Biotechnol.* 2002, *20*, 301–305.

[143] Hasson, M., Verner, A. V., *Mol. Cell. Proteomics* 2003, *2*, 550–559.

[144] Nühse, T. S., Boller, T., Peck, S. C., *J. Biol. Chem.* 2003, *278*, 45248–45254.

[145] Glinkski, M., Weckwerth, W., *Mol. Cell. Proteomics* 2005, *4*, 1614–1625.

[146] Irar, S., Oliveira, E., Pagès, M., Godoy, A., *Proteomics* 2006, *6*, DOI: 10.1002/pmic.200500387.

[147] Rinalducci, S., Larsen, M. R., Mohammed, S, Zolla, L., *J. Proteome Res.* 2006, *5*, 973–982.

[148] Khan, M., Takasaki, H., and Komatsu, S., *J. Proteome Res.* 2005, *4*, 1592–1599.

[149] Ghezzi, P., Bonetto, V., *Proteomics* 2003, *3*, 1145–1153.

[150] Yano, H., Kuroda, S., Buchanan, B. B., *Proteomics* 2002, *2*, 1090–1096.

[151] Meyer, Y., Reichhld, J. P., Vignols, F., *Photosynth. Res.* 2005, *86*, 419–433.

[152] Motohashi, K., Kondoh, A., Stumpp, M. T., Hisabori, T., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 11224–11229.

[153] Marchand, C., Le Marechal, P., Meyer, Y., Miginiac-Maslow, M. *et al.*, *Proteomics* 2004, *4*, 2696–2706.

[154] Yamazaki, D., Motohashi, K., Kasama, T., Hara, Y., Hisabori, T., *Plant Cell Physiol.* 2004, *45*, 18–27.

[155] Yano, H., Wong, J. H., Lee, Y. M., Cho, M. J., Buchanan, B. B., *Proc. Natl. Acad. Sci. USA* 2001, *98*, 4794–4799.

[156] Yano, H., Kuroda, M., *Proteomics* 2006, *6*, 294–300.

[157] Wong, J. H., Cai, N., Balmer, Y., Tanaka, C. K. *et al.*, *Phytochemistry* 2004, *65*, 1629–1640.

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[158] Balmer, Y., Vensel, W. H., Tanaka, C. K., Hurkman, W. J. *et al.*, *Proc. Natl. Acad. Sci. USA* 2004, *101*, 2642–2647.

[159] Balmer, Y., Vensel, W. H., Cai, N., Manieri, W. *et al.*, *Proc. Natl. Acad. Sci. USA* 2006, *103*, 2988–2993.

[160] Rey, P., Cuine, S., Eymery, F., Garin, J. *et al.*, *Plant J.* 2005, *41*, 31–42.

[161] Michelet, L., Zaffagnini, M., Marchand, C., Collin, V. *et al.*, *Proc. Natl. Acad. Sci USA* 2005, *102*, 16478–16483.

[162] Dixon, D. P., Skipsey, M., Grundy, N. M., Edwards, R., *Plant Physiol.* 2005, *138*, 2233–2244.

[163] Delledone, M., Xia, Y., Dixon, R. A., Lamb, C., *Nature* 1998, *394*, 585–588.

[164] Durner, J., Wendehenne, D., Klessing, D. F., *Proc. Natl. Acad. Sci. USA* 1998, *95*, 10328–10333.

[165] Wendehenne, D., Durner, J., Klessing, D. F., *Curr. Opin. Plant Biol.* 2004, *7*, 449–455.

[166] Feechan, A., Eunjung, K., Yung, B.-W., Wang, Y. *et al.*, *Proc. Natl. Acad. Sci. USA* 2005, *102*, 8054–8059.

[167] Lindermayr, C., Saalbach, G., Bahnweg, G., Durner, J., *J. Biol. Chem.* 2006, *281*, 4285–4291.

[168] Lyndermayr, C., Saalbach, G., Durner, J., *Plant Physiol.* 2005, *137*, 921–930.

[169] Elortza, F., Nüshe, T. S., Foster, L. J., Stansballe, A. *et al.*, *Mol. Cell. Proteomics* 2003, *2*, 1261–1270.

[170] Downes, B., Viestra, R. D., *Biochem. Soc. Trans.* 2005, *33*, 393–399.

[171] Kirkpatrick, D. S., Denison, C., Gygi, S. P., *Nat. Cell. Biol.* 2005, *7*, 750–757.

[172] Denison, C., Kirkptrick, D. S., Gygi, S. P., *Curr. Opin. Chem. Biol.* 2005, *9*, 69–75.

[173] James, A., Wohlschlegel, J. A., Johnson, E. S., Reed, S. I., Yates, J. R. III, *J. Proteome Res*. 2006, *5*, 761–770.

[174] Yang, Y., Jack, T., *Plant Mol. Biol.* 2004, *55*, 45–59.

[175] Burkle, L., Meyer, S., Dortay, H., Lehrach, H., Heyl, A., *Funct. Integr. Genomics* 2005, *5*, 175–183.

[176] Pasch, J. C., Nickelsen, J., Schunemann, D., *Appl. Microbiol. Biotechnol.* 2005, *69*, 440–447.

[177] Walter, M., Chaban, C., Schutze, K., Batistic, O. *et al.*, *Plant J.* 2004, *40*, 428–438.

[178] Stolpe, T., Susslin, C., Marrocco, K., Nick, P. *et al.*, *Protoplasma* 2005, *226*, 137–146.

[179] Krab, I. M., Caldwell, C., Gallie, D. R., Bol, J. F., *J. Gen. Virol.* 2005, *86*, 1841–1849.

[180] Kelley, B. P., Yuan, B., Lewitter, F., Sharan, R. *et al.*, *Nucleic Acids Res.* 2004, *32*, W83–W88.

[181] Zhou, H., Zhang, C., Liu, S., Zhou, Y., *Nucleic Acids Res.* 2005, *33*, W193–W197.

[182] Navet, R., Jarmuszkiewicz, W., Douette, P., Sluse-Goffart, C. M., Sluse, F. E., *J. Bioenerg. Biomembr.* 2004, *36*, 471–479.

[183] Rohila, J. S., Chen, M., Chen, S., Chen, J. *et al.*, *Plant J.* 2006, *46*, 1–13.

[184] Vila-Perelo, M., Gutiérrez Gallego, R., Andreu, D., *Chembiochem.* 2005, *6*, 1831–1838.

[185] Wilkins, M. R., Appel, R. D., Van Eyk, J. E., Chung, M. C. M. *et al.*, *Proteomics* 2006, *6*, 4–8.

[186] Gibon, Y., Blaesing, O. E., Hannemenn, J., Carillo, P. *et al.*, *Plant Cell* 2004, *16*, 3304–3325.

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