REVIEW

Plant proteome analysis: A 2006 update

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This 2006 'Plant Proteomics Update' is a continuation of the two previously published in 'Proteomics' by 2004 (Canovas *et al.*, *Proteomics* 2004, *4*, 285–298) and 2006 (Rossignol *et al.*, *Proteomics* 2006, *6*, 5529–5548) and it aims to bring up-to-date the contribution of proteomics to plant biology on the basis of the original research papers published throughout 2006, with references to those appearing last year. According to the published papers and topics addressed, we can conclude that, as observed for the three previous years, there has been a quantitative, but not qualitative leap in plant proteomics. The full potential of proteomics is far from being exploited in plant biology research, especially if compared to other organisms, mainly yeast and humans, and a number of challenges, mainly technological, remain to be tackled. The original papers published last year numbered nearly 100 and deal with the proteome of at least 26 plant species, with a high percentage for *Arabidopsis thaliana* (28) and rice (11). Scientific objectives ranged from proteomic analysis of organs/tissues/cell suspensions (57) or subcellular fractions (29), to the study of plant development (12), the effect of hormones and signalling molecules (8) and response to symbionts (4) and stresses (27). A small number of contributions have covered PTMs (8) and protein interactions (4). 2-DE (specifically IEF-SDS-PAGE) coupled to MS still constitutes the almost unique platform utilized in plant proteome analysis. The application of gel-free protein separation methods and 'second generation' proteomic techniques such as multidimensional protein identification technology (MudPIT), and those for quantitative proteomics including DIGE, isotope-coded affinity tags (ICAT), iTRAQ and stable isotope labelling by amino acids in cell culture (SILAC) still remains anecdotal. This review is divided into seven sections: Introduction, Methodology, Subcellular proteomes, Development, Responses to biotic and abiotic stresses, PTMs and Protein interactions. Section 8 summarizes the major pitfalls and challenges of plant proteomics.

Keywords:
Plant and organ development / Plant proteomics / Plant stress responses / Post-translational modifications / Protein interactions

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Abbreviations: ICAT, isotope-coded affinity tags**; MudPIT,** multidimensional protein identification technology; **RuBisCO**, ribulose-1,5-bisphosphate carboxylase/oxygenase; **SILAC**, stable isotope labelling by amino acids in cell culture

1 Introduction

This 2006 'Plant Proteomics Update' is a continuation of the two previously published in 'Proteomics' by 2004 [1] and 2006 [2] and aims to update the contribution of proteomics to plant research during 2006 by reviewing the papers appearing on this field throughout this period. The number of

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reports found at the 'ISI Web of Knowledge' was 154, a slightly higher figure than that corresponding to the previous period [2] which indicates that proteomics is slowly gaining some confidence among plant biology researchers, but it still represents an insignificant percentage of the total proteomics papers published (4700 referenced at the ISI). In view of this data, we can conclude that the full potential of proteomics is far from being fully exploited in plant research, especially if it is compared to other organisms, mainly yeast and humans [3–7]. The great expectations generated by proteomics has surpassed the harsh reality, as illustrated, by the elevated number of reviews, 39, on different aspects of plant proteomics, methodology, or plant biology with an emphasis on proteomics [1, 2] (Supporting Information Table 1).

The nearly 100 original papers reviewed tackled the proteome of 26 plant species (Table 1). For most of them, only one, or two references at the most, have been found, whereas *Arabidopsis thaliana* and rice, with 28 and 11 papers published, respectively, keep monopolizing proteomic efforts, which is reflected by the publication of a number of reviews dealing specifically with these species. While still being aware of the general difficulties of doing proteomics with plant species, mainly due to methodological limitations, this fact accounts for the complete genome sequence for both species being publicly available, which simplifies the identification of proteins from MS data. For all the species with no significant amounts of published genomic DNA and EST sequences, success in protein identification efforts will be hampered, resulting in low confidence or poor percentage of the proteins identified. In this respect, it is important to point out that the number of plant-specific entries to the protein databases UniProtKB/Swiss-Prot and TrEMBL by February 2007, were of 376647 and 289134, respectively, which represent a significant increase with respect to figures reported in the previous update [2]. Consequently, the recent publication of the genome of *Populus trichocarpa* [8] is opening up new exciting possibilities for forest tree proteomics [9].

Concerning the plant material, a number of genotypes, cultivars, transgenic and mutants have been used for differential proteomic experiments, with the intention of identifying protein markers linked to, or that might account for the phenotypic differences observed [10–13].

Proteomics has proven to be a very valuable tool for assessing the substantial equivalence and the safety of food and feed derived from transgenic plants, this topic being addressed by Ruebelt *et al.* in three consecutive papers [14–16]. Allergen identification is another area of practical interest in which proteomics can make significant contributions [17, 18].

None of the revised papers published during the period reviewed here were aimed at the global description of organs, tissues or cell proteomes. Nevertheless, several proteomic activities have dealt with the proteome analysis of cells, calli, seeds, roots, stem, leaves, xylem/phloem sap, pollen or whole seedlings, in relation to different aspects of plant biology, from growth and development to stress responses (Table 1). In any case, information from these works would contribute to a global cataloguing of plant organ, tissue or cell proteomes, enlarging protein databases, as is happening for *Arabidopsis*, rice or *Medicago* [19–21] (http://expasy.org/ch2d/2d-index.html; http://proteomics.arabidopsis.info/; http://gene64.dna.affrc.go.jp/RPD/main_en.html). Plant responses to hormones or signalling molecules are being studied at the proteomic level, especially to gibberellins, with new reports on responses to ethylene, abscisic and jasmonic acids [22–24] (Table 1).

As stated before, major advances in the field of plant proteomics correspond to the characterization of the proteome of different organella, with special interest in chloroplast and mitochondria. This topic has been extensively reviewed [25–33].

Finally, one area of great relevance in which proteomics will make important contributions towards the knowledge of living organisms is that of PTMs and interactomics. These aspects of plant biology need to be addressed by means of proteomic technology, but with the exception of the phosphoproteome, still remain unexploited in plants, with only a symbolic number of references appearing during 2006 (Table 1).

This review aims to update the contribution of proteomics to plant biology on the basis of the papers published during 2006 with references to our previous reviews [1, 2] and to those that appeared last year. It has been divided into six sections: methodology, subcellular proteomes, organ development, responses to symbionts, biotic and abiotic stresses, PTMs and interactomics. Section 8 summarizes the pitfalls and future challenges of plant proteomics. As in our previous reviews, 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, as it is the case of rice (Supporting Information Table 1).

2 Methodology

2-DE (specifically IEF-SDS-PAGE) coupled to MS still remains as almost the only platform utilized in plant proteome analysis. Application of gel-free protein separation methods [34–38] and 'second generation' proteomic techniques such as multidimensional protein identification technology (MudPIT) and those for quantitative proteomics including DIGE, isotope-coded affinity tags (ICAT), iTRAQ, stable isotope labelling by amino acids in cell culture (SILAC) [19, 39–43] still remains anecdotal. Other state-of-the-art platforms, successfully used in mammals for quantitative phosphoproteomics and simultaneous detection of several types of PTMs, remain unexploited in plant biology research [44–48].

The description of standard methodologies for plant proteomics, including a number of protein extraction methods optimized for different plant tissues and recalcitrant

Table 1. Studies, objectives and contributions of the plant proteomics papers published during 2006

Plant[a] (91)[b]	Proteomes (99)	Biological processes (differential expression proteomics) (47)	PTMs, interactomics (12)
Model systems	**Genotypes, mutants, transgenics (11)**	**Growth/development (12)[d]**	
Arabidopsis thaliana (28)		Pollen (1)	Phosphoproteome (6)
Medicago truncatula (5)	**Organ/tissue/cells[c] (57)**	Seeds (5)	Redox proteome (2)
	Cells, calli (11)	Roots (4)	Interactomics (4)
Cereals	Seeds (15)	Stems (1)	
Barley (*Hordeum vulgare*) (7)	Roots (12)	Pistils (pollination) (1)	
Maize (*Zea mays*) (7)	Stems (1)		
Rice (*Oryza sativa*) (11)	Leaves (6)	**Response to hormones and other signalling molecules (8)**	
Wheat (*Triticum aestivum*) (6)	Pistils (1)	Giberellin (4)	
	Xylem/phloem (sap) (2)	Auxin (1)	
Legumes	Pollen (7)	Abscisic acid (1)	
Pea (*Pisum sativum)* (4)	Whole seedlings (2)	Ethylene (1)	
Bean (*Phaseolus* spp.) (2)		Jasmonic acid (1)	
Soybean (*Glicyne max*) (1)	**Subcellular fractions (29)**		
	General (2)	**Symbioses (nitrogen fixing bacteria, cyanobacteria, mycorrhiza) (4)**	
Solanaceae	Cell wall, apoplast (2)		
Tobacco (*Nicotiana tabacum*) (1)	Membranes (plasma, tonoplast, organules) (6)	**Abiotic stresses (17)[e]**	
	Plastids (etioplast, amyloplasts) (3)	Various (3)	
Other crops	Chloroplast (subfractions) (12)	Osmotic (salt) (5)	
Buckwheat (*Fagopyrun esculentum*) (1)	Mitochondria (1)	Drought (2)	
Canola (*Brassica napus*) (2)	Nucleus (1)	Temperature (3)	
Cotton (*Gossypium hirsutum*) (1)	Vacuoles (2)	Heavy metals (1)	
Pepper (*Piper nigrum*) (1)		Nutritional deficiency (2)	
Sugar beet (*Beta vulgaris)* (1)	**Specific proteins (2)**	Herbicides (1)	
Sunflower (*Helianthus annuus*) (1)	ATP-dependent Clp complex		
	Chloroplastic ATP synthase	**Biotic stresses[f] and mechanisms of resistance to pathogens (virus, bacteria, fungi) (10)**	
Trees (fruit and forest trees)			
Apricot (*Prunus americana*) (1)			
Banana (*Musa*) (1)			
Holm oak (*Quercus ilex*) (1)			
Kiwi (1)			
Mandarin (1)			
Peanut (*Arachis hypogaea*) (3)			
Poplar (*Populus euphratica*) (1)			
Prunus campanulata (1)			
Others			
Amaranthus (1)			
Saueda aegyptiaca (1)			

a) With respect to the previous reviewed period, there has not been continuity in the proteome analysis of a number of species, including alfalfa, lentil, lupinus, potato, tomato, carrot, coffee, *Cucurbit*, grape, mustard, oilseed rape, spinach, peach, rubber tree, white spruce, *Alyssum lesbiacum*, *Catharantus roseus,* ginseng, grasses, *Rhododendron catawbiense,* wild mustard [2].
b) Number of papers.
c) Compared with the previous period [2] some organs are missed (coleoptile, petiol, trichomes).
d) Compared with the previous period [2] some processes are missed: somatyc embryogenesis, programmed cell death, effect of liquid medium composition (cell suspensions), and nutritional conditions (potassium, nitrogen) or light regime.
e) Compared with the previous period [2] some stresses are missed (elevated CO_2, anoxia).
f) Compared with the previous period [2] some studies are missed (herbivores, parasitic plants, hypersensitive response, H_2O_2, salicylic acid).

species, protocols for subfractionation and enrichment in proteins sharing certain characteristics, separation procedures, MS analysis and protein identification strategies, have been the subject of a number of recent monographs [49] journal technical issues (Nature Methods, Practical Proteomics) or reviews [34, 35, 39, 50].

The so called 'second generation' proteomic techniques developed firstly in yeast and mammals, including MudPIT, DIGE, ICAT, iTRAQ and SILAC, are now starting to be successfully applied to plants to conduct quantitative and large-scale proteomic studies (*i.e.* PTMs or interactomics). These analyses are resulting in documentation of low abundant proteins, PTMs, mostly phosphorylation events, modification site mapping, identification of enzyme substrates and protein complexes. The need for a functional validation of these data has become evident, and eventually, the results obtained from *in vitro* experiments must be analysed *in vivo* (*i.e.* by protein microarrays, site-directed mutagenesis) revealing their biological function [36, 51–55].

Another aspect to consider when working with complex organisms, as in the case of plants, is the fact that plant organs are composed of different cell types, each having its own proteome signature, or responding specifically or differently to certain effectors or environmental stresses, for example, pathogens [56]. The use of laser microdissection is particularly amenable in plant tissues [57] and its application to plant proteomic studies directed at identifying protein forms involved in specific functions, will help to prevent unwanted dilution effects that could mask cell-specific protein changes.

From the literature data (*i.e.* [58]) and our own lab experience, it has become clear that the protein extraction protocol to be used in each experiment needs be to chosen according to the plant material and objectives of the specific research carried out. Several extraction protocols have been optimized, precipitation or buffer solubilization methods being the choice for, respectively, whole organ analysis and subcellular proteome or PTMs studies.

Wang *et al.* [59] have reported a precipitation protocol which combines TCA/acetone and phenol extraction features. In our hands it has worked very well with root and leaf tissue from *Arabidopsis*, *Medicago truncatula* and sunflower, resulting in improved 2-DE gels compared to the former protocols, in terms of the number of spots resolved. The protein profile obtained by 2-D IEF/SDS-PAGE differs considerably from the ones obtained when using the TCA/acetone or phenol protocols (Supporting Information Fig. 1). Pointing towards the same direction, the comparison of a modified phenol-based protocol and a phenol-free protocol that involves hot SDS extraction followed by TCA precipitation resulted in considerably higher protein yields from apple and strawberry fruit in the former. 2-DE analysis of these protein extracts revealed spots only present in phenol gels, and other exclusive to SDS samples [60]. These results reinforce the idea that different extraction protocols are complementary and useful in characterizing the whole proteome.

Detection and analysis of low abundant proteins from complex mixtures which, very often, are involved in signalling events and represent targets of PTMs requires protein fractionation steps [61]. In this respect, the 'Protein Equalizer Technology' [62] would be very helpful. In other cases, the application of simple treatments to the protein extraction procedure has yielded good results, *i.e.* heat application to the protein extract has been successfully used to identify proteins involved in desiccation tolerance in *M. truncatula* seeds [63]. The presence of major proteins, *i.e.* ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), in photosynthetic tissues, jeopardizes the detection of less abundant proteins. Xi *et al.* [64] have reported the detection of a total of 5077 protein spots in RuBisCO-free fractions from *Arabidopsis* leaf tissue by using differential PEG precipitation. Among the analysed spots, *ca.* 80% corresponded to proteins nondetected previously using the TCA/acetone method, while the remaining spots exhibited a significant increase in their abundance. In our lab, the use of different solubilization conditions applied to the TCA-acetone pellet resulted in fractions with RuBisCO significantly under-represented (Supporting Information Fig. 2).

Nowadays 2-DE constitutes the dominant protein separation methodology, but several steps throughout the process, from protein extraction and separation to spots visualization can be optimized, resulting in improved resolution and increased reproducibility [65], and allowing the detection of low-molecular weight food allergens [18] or membrane proteins [66]. 1-D SDS-PAGE is still quite a valid technique especially when working with simple proteomes, enriched protein fractions or subproteomes [67]. The 2-D blue native/ SDS-PAGE has been successfully used for the analysis of thylakoid membranes and nuclear complexes [68–70]. By combining different staining methods (*i.e.* Coomassie, silver, Sypro) we have been able to increase the number of protein spots from *M. truncatula* roots which respond to broomrape infection (unpublished results).

Despite the existence of quite a number of different methods developed for protein extraction and separation, it is clear that all in all, it is not enough to allow for the analysis of entire proteomes (organelle, cell, tissue or organ). Some methodologies have proven to be more powerful and decisive than others, with regard to the number of proteins identified. This is the case of MudPIT, which allows the detection of a much larger number of proteins compared to gel-based methods, its drawback being the lack of quantitative data [41]. Fortunately, and surprisingly, it has been reported that a set of proteins can only be detected by a specific technology [43, 71, 72], which is in agreement with the idea that a combination of different methodologies is still needed to characterize entire proteomes.

While stable-isotope labelling (*e.g.* ICAT, O-18- or N-15-labeling, or AQUA) represents the technology currently used in MS-based proteomic quantification, increasing efforts are being directed towards label-free approaches, by means of LC-MS, using an IT or Fourier transform mass spectrometer

to analyse highly complex proteomes [59]. Its simplicity and cost effectiveness make its validation with plant extracts desirable.

In general terms, protein identification from mass spectra (PMF or *de novo* sequencing strategies), or occasionally by EDMAN sequencing [73] can be considered as being straightforward for plant spp. whose genome have been sequenced or with a considerable number of ESTs available in either general (UniProt, Swiss-Prot, NCBI) or plant specific databases [19, 74]. In parallel, the development of bioinformatic tools and specific algorithms permits data integration, modelling and prediction [75]. The opposite situation is encountered when dealing with proteomic analysis from nonmodel plants or with poorly characterized genomes [76, 77]. In such cases, sequence databases from closely related species are interrogated by *de novo* sequencing/BLAST similarity searching. For example, this approach has enabled the assessment of inherent genetic variability and differences during developmental stages and in response to drought in *Quercus ilex* [77]. BLAST results, however, are difficult to score and require a large amount of manual validation. Recent advances in the development of algorithms for database searching, offer the ability to account for genetic variability during first-pass database searches. Thus, the novel Paragon algorithm has been successfully utilized in the identification of a set of proteins from 2-DE gel spots of *Q. ilex* (Lenz and Jorrín, unpublished).

As stated for any high-throughput approach, proteomics experiments should be carefully designed, analysed and verified [78]. This is of special relevance for proteomics studies, considering the dynamic nature of the proteome and its higher degree of complexity as compared to the transcriptome or genome. Accurate, consistent and transparent data processing and analysis are integral and critical parts of proteomics workflows. Definition of common standards for data representation and analysis and the creation of data repositories are essential to compare, exchange and share data within the community [79] and some journals of the proteomics field published last year some guidelines defining these standards in order to improve the quality of published proteomic data [80]. A number of papers dealing with comparative (differential expression) proteomic studies lack a deep statistical analysis, which is mandatory, taking into account the amount of data handled and the necessity of validating differences [81], choosing arbitrary criteria for considering a difference as being biologically relevant (*i.e.* 2.0-fold increase or 0.5-fold decrease ratios). Also, a previous detailed analysis of both, biological and analytical variation for the system on study, is required in order to set up the basis for future comparative analyses. The CV has been determined for synchronized human cancer cells, ranging from 0.12 to 0.28 (1.3 to 2.1 ratios), depending on the protein [82]. This fact is of special relevance considering that tissue samples usually contain different cell types. 2-DE methodology, for example, is subjected to many sources of variability in protein spot pattern and intensity, some of them being associated with IEF separation [83], or with postelectrophoretic analysis of the 2-DE protein profiles [84]. Several papers have emphasized the pitfalls of 2-DE experiments, especially in relation to experimental design, poor statistical treatment of data and the high rate of 'false positive' results with regard to protein identification [85, 86].

Finally, it is important to mention that in any case, proteome studies are directed towards functional analysis. Hence, data coming from proteomic analysis need to be validated and compared with those obtained by using transcriptomics, cell biology and classical physiological and biochemical approaches. Only by combining the information coming from these different methodological approaches would it be possible to assess the biological function of a given protein form inferred from proteomic analysis [87]. Currently, this is the exception rather than the rule, and only a reduced number of plant proteome analyses published comply with these requirements [88, 89].

3 Subcellular proteomes

The major advances in the field of plant proteomics concern the characterization of organella proteomes, specifically those of chloroplast, mitochondria and membranes. The characterization of the subcellular proteomes has provided new insights not only into protein locations and function, but also valuable information on plant biological processes such as transport and metabolism [27, 68, 90, 91], the secretory pathway [92], protein degradation [93], stimuli perception [94], signal transduction, growth [95], seed germination [96], differentiation [97] and responses to stresses [22, 91, 98–101]. It is in this area of subcellular plant proteomics, that gel-free, second generation proteomic platforms, top-down MS analysis have been used, with *Arabidopsis* being the subject of study in most of the cases [67, 91, 94, 99, 102].

The study of membrane fractions remains as one of the most elusive and challenging tasks [26] due to intrinsic methodological difficulties, as it contains low abundant, and hydrophobic proteins, many of them displaying several transmembrane domains, hence they are recalcitrant to being purified by standard extraction protocols and solubilization by nonionic detergents. Due to their location, proteins contained in membranes are putative pivotal elements involved in signal perception and transduction between the environment and the plant cells, and could also mediate communication events between cells and/or subcellular organella. The aqueous two-phase partitioning protocol has proven to be an efficient protocol for obtaining highly pure membrane fractions, eliminating most soluble proteins [67, 102]. Proteins obtained from these preparations, are fractionated according to their differences in physicochemical properties [103] and further subjected to standard separation-MS techniques. By using this strategy, Hynek *et al.* [67] have identified 46 proteins associated with the barley aleurone plasma membrane, and Morel *et al.* [102] analysed 145 pro-

teins in membrane detergent-resistant fraction from tobacco. The use of gel-free platforms such as MudPIT facilitated the identification in *Arabidopsis* of an extra set of extracytosolic proteins (52) not previously reported by the classic 2-DE/LC-MS [19].

Dunkley *et al*. [92] have further developed the LOPIT (localization of organelle proteins by isotope tagging), a technique applying iTRAQ in conjunction with 2D-LC for mapping the organelle proteome of *Arabidopsis*. This study resulted in the determination of 689 protein density gradient distributions and the assignment of 527 proteins to multiple subcellular compartments, distinguishing between resident and contaminant or proteins that are in transit through the secretory pathway.

By using highly purified vacuoles from *Arabidopsis* protoplasts isolated by Ficoll density gradients, Jaquinod *et al*. [90] have reported the proteome of the membrane and soluble fractions of this organella. The protocol used permitted the identification of more than 650 proteins, 415 of them belonging to the membrane fraction (195 integral membrane, 110 transporters and related proteins), and, interestingly, only 20% of those proteins were previously known to be associated with vacuolar activities. The sub-cellular locations of some of these proteins were confirmed by transient expression of GFP-fusion constructs.

4 Plant and organ development

Most of the earlier plant proteomics studies were aimed at creating reference proteome maps of soluble proteins of plant organs at a certain developmental stage. Nevertheless in the last two years a number of papers have been published reporting protein changes associated with plant growth and development, in an attempt to identify key-related proteins involved in these events [1, 2, 29, 104]. During the 2006 period, at least 12 publications have reported differential expression proteomic studies associated with plant growth and developmental processes in vegetative (roots, stems) and reproductive (pistil, pollen, seeds) tissues (Table 1). The main features of the papers are summarized in Table 2.

Mooney *et al*. [105] have developed a quantitative comparative model of root and leaf metabolism on the basis of the differential protein profile between both organs in *Arabidopsis*. PMF analysis of the 288 most abundant 2-DE spots from each organ allowed 156 and 126 protein assignments for roots and leaves, respectively, 54 of which were commonly found in both tissues. Comparison of protein abundance with transcript abundance, using previously reported microarray data, yielded a correlation coefficient of approximately 0.6, indicating that it is inappropriate to make protein level or metabolic conclusions based solely upon data from transcript profiling. The model indicates elevated one-carbon and tricarboxylic acid metabolism in roots relative to leaves.

5 Responses to symbionts, biotic and abiotic stresses

Throughout 2006, at least 29 papers reporting proteomic studies on plant responses to symbionts (4), biotic (10) and abiotic stresses (17), including responses to stress-related hormones (8) (Table 1) have been published. The main features addressed are summarized in Table 3. Some of the above mentioned topics have been already covered by recent reviews [101, 106, 107] (Supporting Information Table 1).

Most of the studies have been conducted with *Arabidopsis*, rice, *M. truncatula* and wheat. By using a differential expression proteomics strategy, changes in proteomes as a consequence of the stress, inoculation or hormone-treatment have been analysed in wild, mutant or transgenic genotypes with different tolerance/susceptibility to stresses, showing compatible or incompatible symbiont/pathogen interactions or sensitive/insensitive response to hormones. Leaf tissue (seeds, roots, leaves) or cells/calli were sampled at different times post-treatment and used for extracting proteins or isolating organules or subfractions (chloroplast, mitochondria) for later proteomic analysis. While confirming changes in protein or genes previously reported by using transcriptomics, Western blot or classical biochemical approaches, proteomics is providing new valuable information. On a regular basis, a first functional group of proteins consisting of pathogenesis-, stress-related proteins and antioxidant enzymes are more represented or are present at higher amount in tolerant/resistant genotypes, while a decrease in enzymes of the photosynthesis and energetic metabolism is observed in the susceptible ones. A number of protein receptors, signal transducers and gene regulators have been shown to be involved in responses to stresses and have also been targeted by these studies. New changes, not previously reported, have been observed in response, for example, to jasmonic acid [108], *Glomus intraradices* [109], drought [110], *Rhizoctonia solani* [111] and virus [112].

6 PTMs

The reported post-translational studies in plants (previously reviewed in ref. [30, 52, 113–115] during 2006 are limited to the analysis of phosphoproteomes [116] in *Arabidopsis*, *M. truncatula*, barley, tobacco, and, to a lesser extent to the redox proteome [117]. Other PTMs, extensively studied in mammals and yeast, such as ubiquitination, and ubiquitination-like modifications, have started to be addressed in plants using global proteomic approaches [55, 118–123].

In tobacco cells, quantitative and qualitative changes in the phosphoproteome in response to lipopolysaccharides were studied, providing new information on the signal perception and transduction mechanisms mediating induced innate immunity. Changes in the phosphoproteome occurred very early during the response and affected, among others, a G-protein, a Ca2+/calmodulin-dependent, a W-ATPase,

Table 2. Plant proteome analyses related to plant developmental processes published in 2006

Organ	Plant spp.	Developmental process	Reference	Main feature
Pollen	Rice (*O. sativa*)	Germination	[130]	2300 spots detected in 2-DE, 186 differentially expressed in mature and germinated, 66 specific to developmental stages. 160 proteins identified, matching 120 protein species (mostly involved in metabolism)
Seed	Cotton (*G. hirsutum*)	Fibre development	[58]	Optimization of an extraction protocol. 1700 spots resolved in 2-DE Coomassie stained gels, 43 spots showing significant changes, 10 identified
	P. campanulata	Break dormancy	[131]	2-DE of cotyledons, embryo and testae protein extracts. From 320 to 490 spots resolved 71 dramatic changes after break dormancy, 4 protein spots identified (dehydrin and pruning 1 and 2)
	Barley (*H. vulgare*)	Grain-filling and maturation	[10]	450 spots on 2-DE, 105 identified Differential proteins are grouped according to their appearance kinetics and function. Isoforms may show different kinetics (*i.e.* cys-peroxiredoxin, β-1,3-glucanase)
Root	Maize (*Z. mays*)	Embryonally preformed primary root	[11]	350 spots detected in 2-DE. 14 differentially accumulated between wild and mutant genotypes coded by 12 different genes (lignin biosynthesis, defence and Krebs cycle)
		Postembryonically formed shoot-borne roots (crown root)	[132]	146–203 spots detected in 2-DE, 19 differentially accumulated between wild and mutant genotypes, all identified. The expression of some of them (*i.e.* G-protein and putative auxin binding) was studied by transcriptomics
		Primary root elongation	[95]	Cell wall fraction (vacuum infiltraction technique). Proteome of the cell wall I and II types. New proteins, acting on the major polysaccharides are reported
Stem	Rice (*O. sativa*)	Uppermost internodes (milky stage)	[133]	762 spots detected in 2-DE, 98 proteins identified (80 gene products), most belonging to the energetic metabolism and stress related
Pistil	Apricot (*P. armeniaca*)	Pistil (self- and crosspollinated)	[134]	10 qualitative and 133 quantitative differences in the 2-DE protein profile, 10 identified

thioredoxin and 14-3-3 proteins [121]. In a different study conducted in barley [122], an in-depth analysis of the phosphorylation pattern of the plastid ATP-synthase beta subunit isoforms revealed different grades of phosphorylation, with Ser and Thr phosphorilation sites identified. Jones *et al.* [124] have reported quantitative changes in the *Arabidopsis* phosphoproteome in response to *Pseudomonas syringae*, by using iTRAQ. They identified five proteins potentially phosphorylated as part of the plant basal defence response, including a putative p23 cochaperone, heat shock protein 81 and a plastid-associated protein)/fibrillin and the large subunit of RuBisCO.

Analysis of redox modifications in plant systems during this period is limited to only two works. The first analysis reports redox changes in membrane proteins of the barley aleurone layer after gibberellic acid treatment, which induced the reduced form in at least 17 proteins, and the oxidized form in another 5 [94]. In a second work, analysis of rice bran proteins led to the identification of fragments of embryo specific proteins and a dienelactone hydrolase as putative targets for thioredoxins, suggesting that thioredoxins controls the life time of specific proteins [125].

7 Interactomics

The dissection of protein–protein interactions constitutes the most challenging task in plant proteomics. Nevertheless, the number of published works covering this topic remains extremely small, compared to those for yeast and mammals, with just four references appearing at the ISI during 2006.

By using tandem affinity purification of protein complexes coupled to MS-based proteomic analysis Rohila *et al.* [126] reported the identification of a number of rice proteins interacting with kinases. 41 cDNAs encoding protein kinases were fused to a tandem affinity purification (TAP)-tag and expressed in transgenic rice plants. The TAP-tagged kinases and their corresponding interacting proteins were then pu-

Table 3. Plant proteome analyses related to plant symbiosis, biotic and abiotic stresses published in 2006

Symbiont/abiotic stress/pathogen	Plant spp.	Plant material	Reference	Main features[a,b]
Symbiosis				
Azoarcus	Rice (*O. sativa*)	Compatible and incompatible genotypes	[108]	A number of protein spots were increase by JA[b] and bacteria in compatible (2) or incompatible plants (7; PRs and receptor-like kinases). A new JA-induced protein (RAN) is reported
G. intraradices	*M. truncatula*	Wild-type, mycorrhiza-defective and autoregulation-defective genotypes	[109]	Except for a chalcone reductase, none of the differentially displayed proteins that could be identified previously was known as appressorium responsive
S. meliloti	*M. truncatula*	Wild and *skl* mutant (ethylene-insensitive/hypernodulating) genotypes	[25]	Mutant *vs.* wild root proteome: pprg-2, Kunitz proteinase inhibitor and ACC oxidase isoforms were down-regulated in skl roots, while were up-regulated trypsin inhibitor, albumin 2 and CPRD49 were up-regulated. During early nodule development, the plant induces ethylene-mediated stress responses to limit nodule numbers
Stresses				
Cold/pathogen	Rice (*O. sativa*)	Suspension cells of the *dwarf-1* mutant (gibberellin-insensitive) and wild genotype	[135]	16 proteins were increased and 14 were decreased in *gid1* mutant compared with its wild type. Among the proteins hyperaccumulated in gid1 were osmotin, triosephosphate isomerase, probenazole inducible protein (PBZ1) and pathogenesis-related protein 10
Cold/salt	*A. thaliana*	Transgenic plants expressing PR10 (ABR17) cDNA	[136]	Several proteins were observed to be significantly altered in the transgenic line, including some with a role(s) in photosynthesis, stress tolerance and the regulation of gene expression
Abiotic stresses				
Salt	Wheat (*T. aestivum*)	Salt-tolerant and -resistant genotypes	[137]	The expression of more than 50% proteins was changed, but the difference between the genotypes in various categories of protein change (up-regulated, down-regulated, disappeared and new-appeared) was only 1–8%.
	S. aegyptiaca (halophyte)	Plants grown under different salt concentrations	[138]	102 spots showed significant response to salt treatment. 27 protein spots were identified including proteins involved in oxidative stress tolerance, glycinebetain synthesis, cytoskeleton remodelling, photosynthesis, ATP production, protein degradation, cyanide detoxification and chaperone activities
	Peanut (*A. hypogaea*)	Salinity-tolerant/susceptible callus cell lines	[139]	PR 10 proteins mediate stress response; the can be phosphorylated
	Rice (*O. sativa*)	Leaf lamina	[65]	Analytical and biological variance analysed in detail, 32 spots showed significant changes in response to salinity (11 identified).
Drought		Seedlings	[110]	In response to drought conditions 10 proteins increased in abundance and the level of 2 proteins decreased. An actin depolymerizing factor is one of the target proteins induced by drought stress. In addition to drought stress, accumulations of protein were analysed under several different stress conditions
	M. truncatula	Desiccation tolerant and nontolerant seed stages	[63]	In heat stable fraction of radicles, the abundance of 15 polypeptides was linked with DT, out of which 11 were identified as late embryogenesis abundant proteins from different groups

Table 3. Continued

Symbiont/abiotic stress/pathogen	Plant spp.	Plant material	Reference	Main features[a,b)]
Cold	*A. thaliana*	Chloroplasts of plants subjected to low temperature.	[99]	DIGE. 43 differentially displayed proteins that participate in photosynthesis, other plastid metabolic functions, hormone biosynthesis and stress sensing and signal transduction
	Wheat (*T. aestivum*)	Subzero cold-acclimated plants	[140]	DNA arrays, RT-PCR and proteomic (2-DE) analysis. Changes resulting from subzero acclimated plants often appeared to be a loss of rather than an appearance of new proteins
Light		Wild and ascorbate-deficient mutant genotypes	[100]	Changes in the thylakoid proteome of 45 protein changes as a consequence of the genotype, light treatment or both. Data confirmed by Western blot. Changes affected plastid metabolism and oxidative stress defence
	A. thaliana Pepper (*C. annuum*)	Plastoglobules	[91]	Differences were determined by differential stable-isotope labelling using formaldehyde. Differences in four enzymes of carotenoid biosynthesis
Heavy metals	*A. thaliana*	Roots	[88]	In response to Cd2+ 42 spots showed significant changes, 17 proteins (25 spots) identified. Selective enrichment of GST, confirmed by Western blot.
Iron deficiencies	Sugar beet (*B. vulgaris*)	Thylakoids from Iron deficient plants	[98]	SDPAGE/BN-2DE. The relative amounts of electron transfer protein complexes were reduced, whereas those of proteins participating in leaf carbon fixation-linked reactions were increased
Herbicides	*M. truncatula*	ALS-inhibitor treated plants	[141]	Changes in meristematic and nonmeristematic tissue (81 spots, increased accumulation of cell division and redox-mediating proteins) and herbicide-treated and nontreated plants (51, increased accumulation of PRs and decreased of metabolic enzymes)
Responses to stress-related hormones				
ABA	Rice (*O. sativa*)	Root tips	[22]	ABA-induced or -repressed proteins, including actin depolymerization factor (ADF), late embryo abundant protein (LEA), putative steroid membrane-binding protein, ferredoxin thionine reductase and calcium-binding protein
Biotic stresses[c)]				
P. syringae	*A. thaliana*	Inoculated and noninoculated plants with wild and mutant *P. syringae* strains	[124]	Transcriptomic and proteomic profiles are compared. Changes in the soluble, chloroplast and mitochondria proteomes. 57 (73 spots) differential soluble (36, 51 spots), chloroplast (8, 9 spots), mitochondria (13, 13 spots) proteins. Differences between basal disease resistance and hypersensitive response are discussed. Important processes related to resistance are communication between organelles and regulation of primary metabolism through redox mediated signalling
			[42]	iTRAQ. Early changes in the phosphoproteome are reported. RuBisCO large subunit show significant difference between basal and hypersensitive response

Table 3. Continued

Symbiont/abiotic stress/pathogen	Plant spp.	Plant material	Reference	Main features[a,b)]
R. solani	Rice (*O. sativa*)	Inoculated and noninoculated R and S plants	[111]	6 protein induced in S and R, 11 only in R (antifungal proteins and metabolic enzymes). A defensive role is suggested, for the first time, for 3-β-hydroxysteroid dehydrogenase/isomerase
Yellow mottle virus		Inoculated plant extracts and *in vitro* virus–plant extracts mixtures	[112]	Plant protein–virus complexes purified by LC have been characterized
P. triticina	Wheat (*T. aestivum*)	Inoculated and noninoculated R and S plants	[142]	32 proteins spots increased in infected plants, 7 host, 22 fungal and 3 unknown
Black point disease		Black point-affected and nonaffected grains	[143]	Changes in affected grains: 252 in germ extracts (67 identified) and 317 in endosperm-bran extracts (86 identified) differential spots. The major group corresponds to stress-related proteins
F. graminearum		Inoculated and noninoculated, compatible and incompatible plants (spikelets)	[144]	41 proteins differentially present in inoculated and noninoculated plants, with some increased (signalling, PRs, nitrogen metabolism) and other decreased (phtosynthesis). 8 fungal proteins were identified
	Tobacco (*N. tabacum*)	Lipopolysaccharide treated cells	[121]	Changes in the phosphoproteome
B. cinerea *R. solani*	Bean (*P. vulgaris)*	Double (plant and pathogen) or triple interaction (plant, pathogen and *Trichoderma*)	[145]	A number of proteins associated to the multiple player interactions have been identified: PR and disease-related factors in plant, virulence factors in the pathogen
E. pisi	Pea (*P. sativum*)	Inoculated and noninoculated R and S plants	[146]	Existence of quantitative and qualitative differences between extracts from: (i) noninoculated leaves of both genotypes (77 spots); (ii) inoculated and noninoculated S plant leaves (19 spots); and (iii) inoculated and noninoculated R plant leaves (12 spots). Some of the differential spots have been identified as proteins belonging to several functional categories, including photosynthesis and carbon metabolism, energy production, stress and defence, protein synthesis and degradation and signal transduction

a) If not indicated, 2-DE-MS has been used.
b) Abbreviations: ABA, abscisic acid; ABR, abscisic acid-responsive; JA, jasmonic acid; PR, pathogenesis related.
c) R, resistant; S, susceptible.

rified and identified by MS. The validation of the method is based on previously known orthologous identifications, and its advantages, drawbacks and improvements are discussed in detail.

A work of great interest and with possible practical implications for plant biotechnology is that reported by Brizard *et al.* [112]. The complexes formed by plant virus (rice yellow mottle) and host proteins (rice) were extracted from virus-infected plants or from *in vitro* binding experiments and then purified by LC. The virus-containing fractions were subsequently recovered (the presence of virus particles being confirmed by electron microscopy), the proteins separated by SDS-PAGE and bands were finally analysed by MS. The presence of virus coat protein was further confirmed by Western blot analysis. This study resulted in the identification of 223 host-proteins, and further validated the specificity of the host recruited proteins.

By using coimmunoprecipitation followed by proteomic analysis, seven and nine proteins interacting with phytochrome A and B, respectively were identified in *Arabidopsis*; the validation of the involvement of the identified proteins in phytochrome-related functions comes from physiological data showing that knockout mutants in two of these proteins had impaired light-signalling phenotypes [127].

By using cupper-immobilized metal affinity chromatography (Cu-IMAC) 35 unique Cu-interacting proteins from *Arabidopsis* root extracts were identified, allowing potential copper-interacting motifs to be predicted [128].

8 Conclusion

In view of the number of papers on plant proteomics published during 2006 (around 100 original papers and nearly 40 reviews) we can affirm that there has been a slight quantitative leap from the previous period reviewed, and that proteomics is slowly gaining confidence within the plant scientist community. However, with regard to the papers reviewed, we cannot claim any qualitative improvement, and, compared to other systems, mainly yeast and humans, plant proteomics, with the exception of the organular proteome (chloroplast, mitochondria and membranes), is in a very early stage, and its full potential is far from being fully exploited. This conclusion is justified taking into account the technical platforms used, in a very high percentage of the work published, limited to the 2-DE/MS workflow. The utilization of gel-free separation methods and those of 'second generation' quantitative proteomics remains anecdotal. Despite the large number of plant species studied at the molecular level by using gene-expression profiling methods, cellular biology or physiological/biochemical classical approaches, only five of them have been the subject of more than three proteomics publications during 2006 (*Arabidopsis*, *M. truncatula*, and the cereal crops, rice, maize and wheat). Apart from describing subcellular proteomes, most of the studies reviewed dealt with developmental changes and response to stresses. PTMs, and interactomics, the real thermometer of the proteomics status in a field, still remains a major challenge. Even so, proteomics is providing new information, validating, complementing or correcting that information already provided by different approaches, and, as a consequence, giving us a deeper knowledge of plant biology. A number of critical issues need to be re-evaluated in works already published and those considered for future publications in order to improve the quality of the proteomic data presented, such as a good experimental design and a proper statistical treatment. The comparative statistical analysis of hundreds of spots in 2-DE gels with a reduced number of replicates (usually three) requires the use of multivariate, hypothesis-generating, methods such as principal component analysis and partial least squares regression [129]. Only after the detailed analysis of expression data, involving data normalization, appropriate transformation, determination of the inherent variance and the use of suitable uni- and multivariate statistical tests, we can conclude about treatment-specific spots [80]. After protein identification and while biologically interpreting such differences, special care must be taken with excessive extrapolations, nonfunctionally validated, from protein data to phenotypes. Data standards for experimental design, sampling, protocols, data handing and analysis are needed. A number of the problems encountered are intrinsic to the databases used for protein identification, such as redundancy, incorrect annotations and the use of different identifiers for the identical protein sequence. These and other challenges also emphasized in our last update [2] should be considered when approaching plant biology studies through proteomics.

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