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Review

System, trends and perspectives of proteomics in dicot plants Part II: Proteomes of the complex developmental stages

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Abstract

This review is devoted to the proteomes of the complex developmental stages of dicotyledoneous (dicot) plant materials. The two core technologies, two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS), independently or in combination with each other, are propelling dicot plant proteomics to new discoveries and functions, with the establishment of tissue-specific and organelle proteomes, mostly in *Arabidopsis thaliana* and *Medicago truncatula*, revealing their complexity and specificity. These experimental proteomes have provided a good start towards the establishment of high-density 2-DGE reference maps and peptide mass fingerprint databases, for not only the model dicot plants, *A. thaliana* and *M. truncatula*, but also other important dicot plants, which will serve as a basis for proteomes of many other dicot plants and plant materials.

Keywords: Dicotyledoneous plants; Development; Proteomes; 2-DGE; MS

Contents

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1. Introduction

Developmental proteomics can be defined "as a set of proteins present at particular developmental stage of a plant". It involves a cell type, organ or tissue, and individual organelles in a cell, and their proteomic analyses can tell us what kind of proteins are expressed during plant growth and development (Fig. 1). It can be stated "development proteomics is an essential part of plant proteome investigation, and that forms a base for extended proteomic studies, such as those involving protein changes due to unfavorable conditions for plant". Two-dimensional gel electrophoresis (2-DGE [1–3]) and mass spectrometry (MS [4–6]) have been instrumental in these studies; these core technologies have been discussed in detail in part I of the review [7]. In 1995, initial protein profiles of the dicotyledoneous (dicot) model *Arabidopsis thaliana* (ecotype Columbia) were presented on five different tissues (leaf, stem, root, seed and callus), resolving a total of 4763 proteins spots from all these tissues using 2-DGE and silver staining [8]. N-terminal amino acid sequences of 57 proteins were obtained, and 46 proteins were found to be blocked at the N-terminus out of a total of 101 proteins spots analyzed. This study comprised the first proteomic dataset in *Arabidopsis*. In the following sections, individual tissues/organelles used for their proteomes, have been discussed.

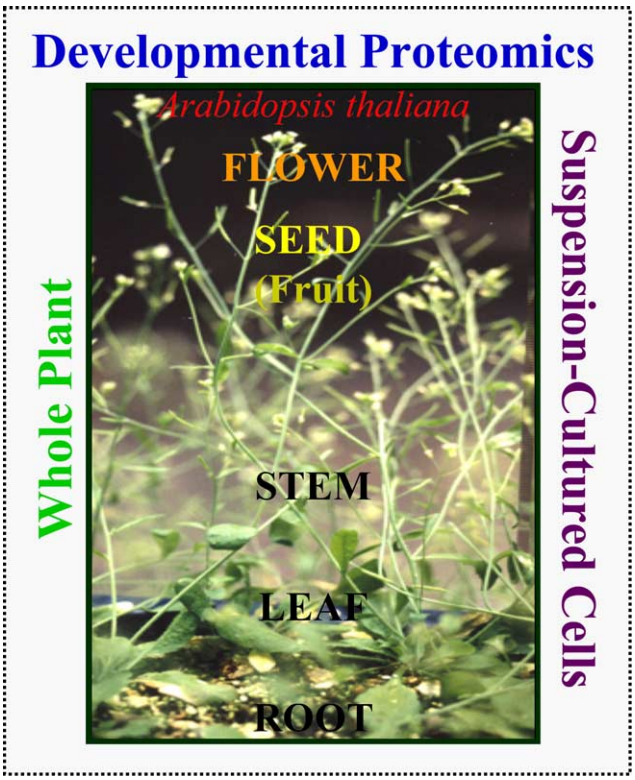

Fig. 1. Developmental proteomics of dicot plant materials. As an example, the *Arabidopsis thaliana* (ecotype Columbia) plant, its tissues, organs, and suspension-cultured cells, which have been (and will be) used for studying the plant growth and development, is depicted. Photograph of the *Arabidopsis* plant (ecotype Columbia) was from Dr. Akihiro Kubo (NIES).

1.1. Tissue specific

1.1.1. Leaf

Among the leaf tissues, the leaf proteomes of *Medicago truncatula* and *Prunus armeniaca* (apricot) were the first to be established. The *M. truncatula* leaf proteins were extracted from leaves (top two apical unfolded trifoliates) of 8-week-old cultivar (cv.) Jemalong A17 plants [9], resulting in the identification of 64 proteins from a total of 84 protein spots (76% success rate). These were grouped into 11 functional categories: energy (47%), metabolism (11%), protein synthesis (11%), protein destination/storage (9%), signal transduction (6%), disease/defense (5%), cell growth/division (5%), transcription (5%), cell structure (5%), unclear (3%), and transporters (2%). In case of *P. armeniaca*, three different extraction methods [trichloroacetic acid (TCA)/acetone, sodium dodecyl sulfate (SDS) and Tris] were used to isolate leaf proteins from variety Canino clone A 1343, to know which extraction buffer is the most suitable [10]. 2-DGE coupled with silver staining resolved 517, 416, and 744 protein spots after extraction with TCA/acetone-, SDS-, and Tris-extraction buffers, respectively. It was concluded that the Tris-based extraction buffer is very suitable for extraction of apricot leaf proteins.

1.1.2. Stem

Again, the stem proteomes are only available for *M. truncatula* and *P. armeniaca*. *M. truncatula* stem proteins were extracted from the first two apical internodes of 8-week-old plants (cv. Jemalong A17) [9]. Forty-six proteins were identified from a total of 94 protein spots (49% success rate), and grouped into 12 functional categories: energy (31%), protein destination/storage (13%), transcription (11%), metabolism (11%), secondary metabolism (7%), cell structure (7%), signal transduction (4%), disease/defense (4%), protein synthesis (4%), unclear (4%), intracellular traffic (2%), and cell growth/division (2%). On the other hand, apricot (Canino clone A 1343) bark (separated into phloem and parenchyma fractions) proteins, extracted using TCA/acetone-, SDS- or Tris-buffers, were resolved by 2-DGE, coupled with silver staining [10]. This resulted in the detection of 434/233, 500/303, and 715/334 protein spots in phloem/parenchyma fractions, with TCA/acetone-, SDS- and Tris-extraction buffers, respectively. The Tris-based extraction buffer was also found to be suitable for extraction of bark proteins, even if they are rich in polyphenolic compounds.

1.1.3. Root

Root proteome is available for *M. truncatula* (2-week-old cv. Jemalong A17 grown in perlite) [9]. Forty proteins were identified from a total of 94 protein spots (43% success rate), and grouped into 7 functional categories: disease/defense (24%), energy (20%), metabolism (15%), unclear (15%), protein destination/storage (15%), secondary metabolism (8%), and cell structure (3%).

1.1.4. Flower

A flower proteome for all stages of the flower, from buds until petal browning and all parts, except the peduncles of 8-week-old plants (cv. Jemalong A17) has also been established [9]. Forty-three proteins were identified from a total of 94 protein spots (46% success rate), and grouped into 12 functional categories: energy (38%), protein destination/storage (16%), disease/defense (9%), metabolism (7%), signal transduction (7%), protein synthesis (5%), cell growth/division (5%), transcription (5%), cell structure (2%), secondary metabolism (2%), transporters (2), and unclear (2%).

1.1.5. Seed

Seed is the tissue where the most extensive proteomic studies have been carried out among all the tissues examined so far in dicots. A total of four proteomic studies have been undertaken to understand the seed germination and development processes, two each in *Arabidopsis* [11,12] and *M. truncatula* [9,13]. In the first study, a proteome analysis of *Arabidopsis* (cv. Landsberg *erecta*) seeds was undertaken to examine the germination process [11]. Total and water-soluble protein extracts were prepared from dry mature seeds, seeds at different stages of germination and from primed seeds. The 2-DGE gel profiles visualized by either Coomassie brilliant blue (CBB) or silver staining revealed 1272, 1338, 1461 and 1133 protein spots using dry mature seeds, 1-, 2- and 3-day imbibed seeds, respectively. Changes in the abundance (up- and down-regulation) of 74 proteins were observed during germination sensu stricto (prior to radicle germination) and the radicle protrusion step. Based on their accumulation patterns, the seed proteins were classified into 12 types: during germination (types 0–4), during imbibition drying (types 5 and 6), and during priming (types 7–12). Furthermore, these protein types were discussed according to their characteristics, such as those involved in mobilization of stored seed reserves (types 0, 1, 3–5, 7 and 11), germination sensu stricto (types 1, 2, 5–7 and 11), radicle emergence (types 3 and 4), imbibition (type 5), desiccation (type 6), and priming (types 7, 9–11). The identification of 12S globulins (cruciferins) as the abundant seed storage proteins and several enzymes involved in the catabolism of major seed storage lipids (triacylglycerols) reveals their role in the mobilization of storage lipids during germination. Proteins associated with germination sensu stricto, correlate with initial events in the mobilization of protein and lipid reserves, and the resumption of cell cycle activity, such as WD-40 repeat protein, tubulin and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH). During radicle emergence, proteins mostly involved in defense mechanisms to protect the future seedlings against herbivores, pathogens and other stresses were identified, such as myrosinase, jasmonate-induced myrosinase-binding proteins, *S*-adenosyl-Met (Ado-Met) synthetase, LEA, and heat shock (HSP70) proteins. In addition, a seed maturation protein, probably involved in sequestering biotin, and a chloroplast translation elongation factor (EF-Tu) were found. Among a total of 19 imbibition-associated proteins, 7 proteins were identified, including actin 7 (ACT 7) and WD-40 repeat proteins. Accumulation of the WD-40-repeat protein during imbibition provides first evidence for its role in the germination process. Remarkably, all protein changes associated with the desiccated state of seeds corresponded to proteins already present in the dry mature seeds. One of the three detected desiccation-specific proteins was the cytosolic GAPDH, whose induction during desiccation is a conserved feature among different tissues and organs in plants. Two priming treatments, hydro- and osmo-priming, were also employed to examine the priming-associated proteins that resulted in the identification of a total of nine proteins, such as tubulin, 12S-criciferin β-subunits, catalase, and low molecular weight HSPs.

In the second study, the role of gibberellins (GAs) in germination of *Arabidopsis* seeds using a GA-deficient *ga1* mutant, and wild type seeds treated with paclobutrazol, a specific GA biosynthesis inhibitor was investigated [12]. Results revealed that GAs do not participate in many processes involved in germination sensu stricto, i.e. the initial mobilization of seed protein and lipid reserves. Changes in 46 proteins were detected at this stage, however, only one protein (α-2,4 tubulin) was suggested to depend on the action of GA, as it was not detected in the *ga1* mutant seeds. In contrast, it was suggested that GAs might be involved, directly or indirectly, in

controlling the abundance of several proteins (two isoforms of Ado-Met synthetase and β-glucosidase) associated with radicle protrusion and post-germination processes.

Gallardo and co-workers also investigated the seed development in *M. truncatula* (cv. Jemalong J5) at specific stages of seed filling corresponding to the acquisition of germination capacity and protein deposition [13]. Individual flowers were tagged on the day of flower opening, and the pods were harvested between 8 and 44 days after pollination. One hundred and twenty proteins differing in kinetics of appearance were subjected to MALDI-TOF-MS, resulting in the identification of 84 proteins, with characteristic developmental patterns of accumulation during protein deposition, such as annexin (cell division), ACT7 and RGP1 (cell expansion), and Ado-Met synthase and Ado-Hcy hydrolase (metabolic activities). In parallel, Watson and co-workers also reported a proteome of seed proteins from a variety of developmental stages (including very young pods to those with maturing seeds) of 3-month-old *M. truncatula* plants (cv. Jemalong A17) [9]. Sixty-one proteins were identified from a total of 91 protein spots (67% success rate), and grouped into 10 functional categories: protein destination/storage (60%), energy (15%), disease/defense (7%), signal transduction (5%), unclear (3%), protein synthesis (2%), cell growth/division (2%), transcription (2%), secondary metabolism (2%), and transporters (2%).

1.1.6. Fruit

To date, the fruit proteomes for latex (milky-like fluid within laticifer cells) from opium poppy (*Papaver somniferum*) [14], and grapevine (*Vitis vinifera* L.) ripe berries mesocarp [15] are known. In the case of latex, the cytosolic serum and the sedimented fraction containing the alkaloid-accumulating vesicles were used [14]. Of the serum, representing the protein-rich part of the latex, around 300 protein spots were resolved by 2-DGE and silver staining, which were similar to the protein patterns obtained from the soluble proteins of the cytosolic fractions. A total of 75 protein spots were analyzed by internal peptide microsequencing, and the function of 69 proteins could be assigned, including a codeinomne reductase, an enzyme involved in the morphine biosynthesis. In the vesicle-containing pellet, around 280 proteins spots were visualized, and 23 protein spots were characterized. The identified proteins were broadly categorized as enzymes involved in glycolysis, oxidative decarboxylation and citric acid cycle, in protection function glutathione *S*-transferase, chaperones (HSP70 and the 14-3-3 protein family), regulatory proteins (inositol 1,3,4-triphosphate 5/6 kinase), and the major latex proteins.

On the other hand, major soluble proteins (mesocarp) of grapevine ripe berries were extracted from six different cultivars, including non-vinifera, using TCA/acetone procedure [15]. About 300 proteins spots were resolved by 2-DGE and detected by CBB staining. A total of 67 proteins were identified using MALDI-TOF-MS, out of which 34, 19, and 13% proteins play, respectively, a role in energy, defense/stress/disease, and metabolism. Comparative analysis of the six cultivars revealed that differences between cultivars were low, but different isoforms of alcohol dehydrogenase and of a transcription factor of hexose transporter could be detected.

1.2. Suspension-cultured cells

Suspension-cultured cells, abundant and reproducible experimental source material, from *Arabidopsis* [16] and *M. truncatula* [9] have been widely used for proteomic studies. Prime and co-workers introduced the use of *Arabidopsis* callus culture using roots of different ecotypes as a system for proteomic analysis of plant organelles [16]. Using 2-DGE, a database of marker proteins from the endoplasmic reticulum (ER), Golgi apparatus/prevacuolar compartment, mitochondria, and plasma membrane (PM) was prepared. The enrichment of organelles was demonstrated using the PM. In *M. truncatula* (cv. Jemalong A17), root suspension-cultured cells were used for proteomic analysis [9]. Fifty proteins were identified from a total of 94 protein spots (53% success rate), and grouped into 9 functional categories: energy (24%), protein destination/storage (24%), metabolism (22%), disease/defense (18%), cell transcription (4%), growth/division (2%), unclear (2%), and transporters (2%).

1.3. Senescence

Senescence, a final stage of development of leaves, comprises the highly regulated and coordinated activation of self-destruction mechanisms that require expression of specific genes, ultimately resulting in necrosis of the organ [17,18]. In clover (*Trifolium repens*, Grasslands Challenge, genotype 10F), quantitative analysis of 590 consistently detected leaf protein spots, separated by 2-DGE, revealed approximately 40% protein spots to be senescence-related [19]. Among these 178 protein spots were also found on 2-DGE gels of the chloroplast fraction, representing a major portion of the proteins showing senescence-related declines in abundance. Identified chloroplast proteins by MALDI-TOF-MS included ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large- and small-subunits, and RuBisCO activase, and the 33 kDa (10^4 molecular masses) photosystem II (PSII) oxygen evolving complex protein, indicating that photosynthetic apparatus was degraded. A chloroplast glutamine synthase showed partial decline in abundance during late senescence, but was maintained at levels that may support provision of glutamine for export to other tissues. This study emphasizes the importance of proteolysis and chloroplast degradation, and remobilization of nitrogen reserves in senescing leaves of white clover.

1.4. Organelles

To determine the proteome of a cell is a challenge, and which is complicated by proteome dynamics, complexity and

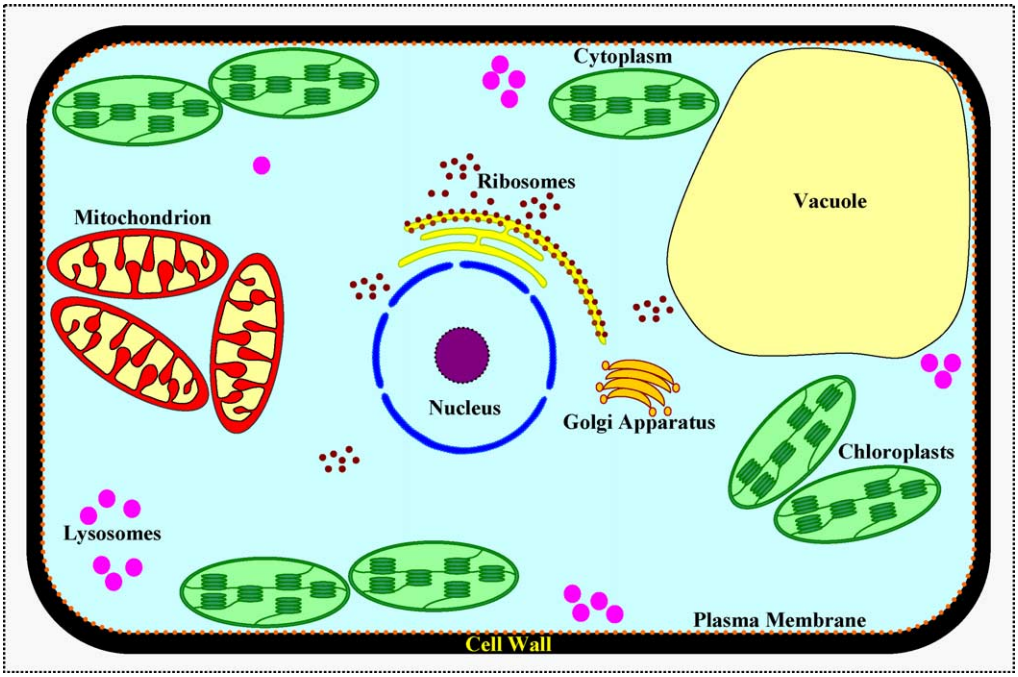

Fig. 2. Plant cell and its organelles.

ambiguous identification of the organelles therein. Therefore, as a next step, the study of the subcellular proteomes is not only essential, but will be required to appropriately address all levels of the organization of the proteome. The subcellular components/organelles of a cell are presented in Fig. 2.

1.4.1. Cell wall

Enriched cell wall fractions prepared from *Arabidopsis* (cv. L. *erecta*) suspension-cultured cells were used to sequentially extract proteins using $CaCl_2$ (ionically bound proteins) and urea (covalently bound and other proteins) buffers [20]. More than 300 abundant protein spots were revealed by 2-DGE, and 111 proteins were identified by MALDI-TOF-MS. These identified proteins represented a total of 69 different proteins with unique gene identities, including the classical cell proteins of known biological function and novel extracytoplasmic, cell wall bound signaling proteins (protein kinases). Interestingly, a number of proteins that were previously associated with cell compartments other than cell wall, were found to have unusual cell wall localization.

1.4.2. Chloroplast

1.4.2.1. Thylakoid lumenal, peripheral and integral proteins.

A first systematic study on the thylakoid lumenal proteins was done in *Arabidopsis* (ecotype Columbia) using 2-DGE [21]. Later, a similar work appeared in 2002 [22]. The thylakoid lumen was isolated from leaves of 10- [21] and 13- [22] week-old plants grown hydroponically. Silver stained 2-DGE gel of the soluble lumenal fraction revealed around 300–700 protein spots in these two studies. In the former study, N-terminal or MALDI-TOF-MS analysis resulted in the identification of eight proteins, including plastocyanin, PsbO, PsbP, PsbQ, and TL29 (showing strong similarity to ascorbate peroxidase) [21]. In the later study, 36 proteins were identified, such as peptidyl-prolyl cis-trans isomerases, proteases, and a family of novel PsbP domain proteins [22]. It was found that 19 proteins possessed a twin-arginine (TAT) motif, suggesting their import by the TAT pathway.

In a more systematic study, to provide a 2-DGE map of the lumenal and peripheral proteins of the thylakoids, Peltier and co-workers used pea (*Pisum sativum* var De Grace) as the plant species [23], and were able to visualize more than 400 lumenal and thylakoid peripheral protein spots. After correction for possible isoforms and post-translational modifications (PTMs), at least 200–230 different lumenal and peripheral proteins were calculated to be present. Of the 61 distinct protein sequences identified by MS and Edman sequencing, 33 had a clear function or functional domain, whereas no function could be assigned for 10 proteins. The identified proteins were assigned to nine functional categories: energy (21%), transcription/translation (2%), metabolism (2%), growth and division (10%), protein destination and storage (12%), transport (2%), defense (3%), no assigned function (17%), no identified gene (31%).

In 2002, Peltier and co-workers [24] combined experimental proteome analysis, using 2-DGE and MS, with a genome wide prediction screen to characterize the protein content of the thylakoid lumen of *Arabidopsis* (ecotype Columbia) chloroplasts, to assemble a set of lumenal proteins that would allow determination of the parameters and thresholds for theoretical predictions. The identities of 81 proteins were established, and N-termini were sequenced

to validate localization prediction. Gene annotation of the identified proteins was corrected by experimental data, and an interesting case of alternative splicing was discovered for a putative protein with a pentapeptide repeat (At2g44920). This serves as a good example for correctly annotating a gene based on the experimentally obtained proteome data, in conjunction with the expressed sequence tag (EST) database. Expression of a surprising number of paralogs, such as for the oxygen-evolving complex proteins (OCE16, 23 and 33), plastocyanin and five isomerases of different classes, was detected. Based on identified proteins, it was implied that prime functions of the lumenal proteome include assistance in the folding and proteolysis of thylakoid proteins as well as protection against oxidative stress. Interestingly, more than 50% of the identified lumenal proteins have a typical TAT motif. These independent studies strongly demonstrate that more than half of the lumenal proteins from *Arabidopsis* might be routed across the thylakoid membrane via the TAT pathway.

An extensive analysis of the *Arabidopsis* peripheral and integral thylakoid membrane proteome was carried out by sequential extractions with salt, detergent, and organic solvents, followed by multidimensional protein separation steps (RP-HPLC and 1- and 2-DGE, different enzymatic and non enzymatic protein cleavage techniques, MS, and bioinformatics) [25]. A set of 154 proteins were identified, of which 76 (49%) were α-helical integral membrane proteins, and 27 were new proteins of unknown function but with predicted chloroplast transit peptides, of which 17 (63%) were integral membrane proteins. A plastid proteome database (PPDB) with multiple search functions (http://cbsusrv01.tc.cornell.edu/users/ppd) was created with an objective to provide a centralized data deposit for both predicted and experimentally identified plastid proteins, their annotated functions, as well as their molecular and physiological properties. The workflow used in establishing the chloroplast proteomes in the aforementioned studies is schematically presented in Fig. 3.

1.4.2.2. Chloroplast envelope membrane proteins. To enhance the understanding on the biochemical machinery of plastid envelope membrane, various extraction procedures (chloroform/methanol extraction and NaOH and NaCl treatments) were applied on *Arabidopsis* (ecotype Wassilewskija-2 (Ws-2)) to get an exhaustive array of the chloroplast envelope membrane proteins [26]. More than 100 envelope components of various hydrophobicities, such as ion and metabolite transporters, proteins involved in fatty acid, glycerolipids, vitamins, and pigments metabolism, components of the protein import machinery, proteases, as well as many proteins of unknown function and of previously unknown subcellular localization, were separated by SDS–PAGE and identified by LC-Q-TOF-MS and MS/MS. It was concluded that about 80 and 50% of the identified proteins are very likely located, and have functions known or very likely to be associated with the chloroplast envelope, respectively. In a parallel study, two alternatives to traditional 2-DGE, namely off-line MudPIT and SDS–PAGE followed by LC–MS/MS, alternatively referred to as Gel-C-MS/MS, were used to identify 392 non-redundant proteins from the chloroplastic envelope membrane of *Arabidopsis* (ecotype Columbia) [27]. These two complementary approaches are very sensitive techniques that do not require an enormous amount of starting material (as low as 200 μg of mixed envelopes), and hence are very promising techniques.

1.4.2.3. Chloro-ribosome proteins. Three studies on the chloroplast ribosome (chloro-ribosome) of a higher plant (*Spinacia oleracea*) resulted in its comprehensive proteome [28–30]. These studies revealed that the chloro-ribosomal 30S subunit contains four chloroplast/plastid-specific ribosomal proteins (PSRPs, named PSRP1–4) in addition to the orthologs of the full complement of *Escherichia coli* 30 S subunit ribosomal proteins. The chloro-ribosomal 50S subunit comprised the orthologs of 31 *E. coli* 50S subunit ribosomal proteins (only two *E. coli* ribosomal proteins were unrepresented, L25 and L30), 2 additional PSRPs (namely,

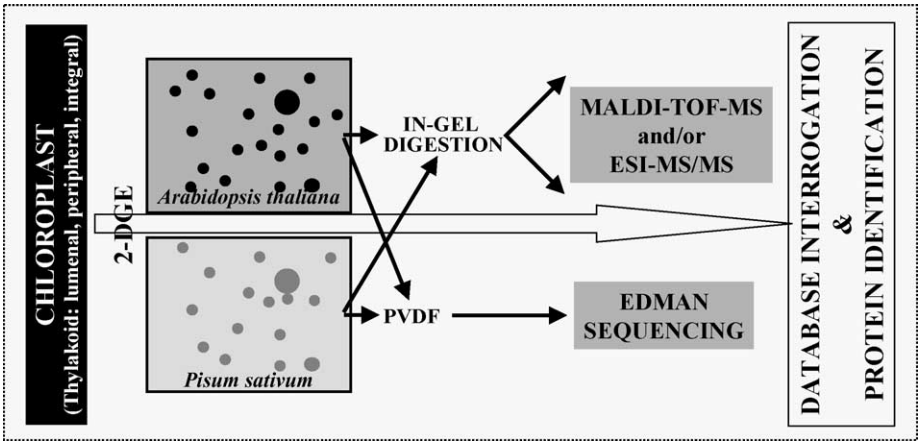

Fig. 3. Chloroplast proteomics. The flow chart schematically depicts the steps from 2-DGE gel-based separations of *Arabidopsis thaliana* and *Pisum sativum* thylakoid lumenal, peripheral, and intergral proteins, to the analysis of in-gel digested or electroblotted proteins on PVDF membranes, by MS or Edman sequencing, respectively, followed by unambiguous protein identification upon database interrogation/searches.

PSRP5 and 6), and a plastid ribosome-recycling factor, pRRF. In all, the chloro-ribosome proteome is composed of 59 distinct proteins: 6 PSRPs, a bacteria-type pRRF, and 52 orthologs of eubacterial ribosomal proteins. With these reports, the characterization of all six PSRPs (nuclear encoded) in spinach chloro-ribosome was completed. Interestingly, orthologs of all six PSRPs are present in the complete genome sequence of *Arabidopsis*, and in the higher plant EST database.

1.4.2.4. Thioredoxins. A thioredoxin (Trx) affinity purification method [31] was used for an extensive search of Trx-targeted chloroplast proteins [32]. The mutant Trx *f* and *m* proteins bound to affinity columns were used to trap the proteins from spinach chloroplast stroma, followed by separation and analysis by 2-DGE and MS, respectively (Fig. 4). Fifteen potential targets were identified that function in 10 chloroplast processes (isoprenoid, tetrapyrrole and vitamin biosynthesis, protein assembly/folding, protein and starch degradation, glycolysis, HCO_3^-/CO_2 equilibration, plastid division, and DNA replication/transcription) not known to be Trx linked. It appeared that these proteins function in plastid-to-nucleus signaling and in a previously unrecognized type of oxidative regulation. Moreover, 20 proteins were also identified as members of pathways regulated by Trx. In all, a total of 35 Trx-linked proteins, participating in 18 different chloroplast processes were identified.

1.4.3. Endoplasmic reticulum

The ER of the developing oilseeds is central to the synthesis, sorting and storage of protein and lipid reserves, while the germinating seed is concerned with their degradation. ER fraction was isolated from castor (*Ricinus communis* L. var Hale) bean seed (5-day germinated and 25 days after flowering, stage VI) using sucrose density gradient [33]. SDS–PAGE of both developing and geminating ER fractions led to the identification of at least three proteins (disulphide isomerase, calrecticulin, and a developing-ER-specific oleate-12-hydroxylase involved in the biosynthesis of ricinoleic acid). 2-DGE and silver staining manifested about 300 protein spots in each developmental fraction, revealing significant differences between germinating and developing ER. From germinating ER, 20 protein spots were selected and analyzed by Q-TOF-MS/MS, of which functions of 10 proteins could be assigned. Most of these proteins were found to have roles in protein processing and storage, and lipid metabolism. Two proteins, aspartate proteinase precursor and *N*-carbamyl-L-aminohydrolase-like protein were found to be absent from the developing profiles.

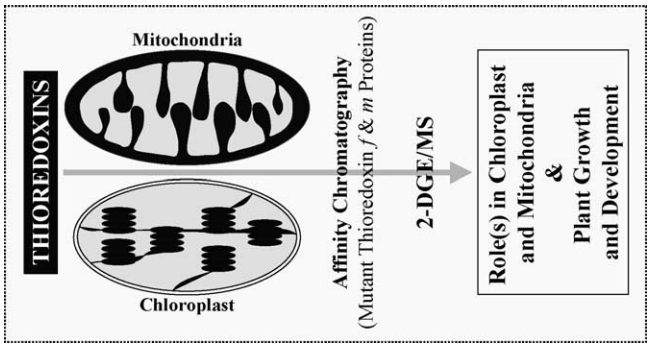

Fig. 4. Proteomics approach to identify chloroplast and mitochondrial thioredoxins from dicot plants. The thioredoxins enriched/separated from plant chloroplasts and mitochondria using affinity chromatography (mutant thioredoxin *f* and *m* proteins) are separated and analyzed by 2-DGE and MS, respectively, revealing their potential role/function in these organelles and during plant growth and development.

1.4.4. Microtubule

For identification of plant microtubule-associated proteins (MAPs), Chan and co-workers adapted the method developed for carrot cell suspensions to *Arabidopsis* suspension-cultured cells [34]. MAPs were trysinized, and their PMFs were analyzed by MALDI-TOF-MS, resulting in identification of a range of proteins, such as Spc98p, MOR1, elongation factor 1α, MAP65, several kinesins, and several unknown proteins.

1.4.5. Mitochondria

Detailed studies on the mitochondrial proteome have been reported for *Arabidopsis* [35–40], pea [41], beans and potato [39]. The *Arabidopsis* mitochondrial proteome project was started for a comprehensive investigation of mitochondrial functions in plants. Mitochondria were prepared from *Arabidopsis* leaves and stems or from suspension-cultured cells [35]. The purity (>90%) of the generated fractions was tested by the resolution of organelle protein complexes applying 2-DGE blue native (BN)/Tricine SDS–PAGE. The mitochondria prepared from suspension-cultured cells (grown in the dark) were very pure, and hence used as a source material for establishing the mitochondrial proteome. The mitochondrial proteome was analyzed by 2-DGE (using Tricine SDS–PAGE in the second dimension) and 650 different proteins in a p*I* range of 3–10 were separated. The majority of proteins had molecular masses between 30 and 60 kDa, and p*I* between pH 4.5 and 8. Forty dominant protein spots were the most abundant. Moreover, by varying solubilization conditions, pH gradients for isoelectric focusing (IEF), and gel staining procedures, about 800 proteins could be resolved. Immunoblotting, direct protein sequencing, and mass spectrometry identified a total of 52 proteins spots. The identified proteins were localized in all four mitochondrial subcompartments: the inner (>15 proteins) and outer (≥5 proteins) mitochondrial membranes, the mitochondrial intermembrane space (≥2 proteins), and the mitochondrial matrix (≥20 proteins). Except for the α-subunit of the mitochondrial ATP synthase complex, all the identified proteins are nuclear encoded and imported into the organelle. Identified proteins were grouped into 10 functional categories, such as respiration, citric acid cycle, amino acid and nucleotide metabolism, protection against O_2, mitochondrial assembly, molecular

transport, and protein biosynthesis. Thirty and 25% of the proteins were shown to have a role in respiration, and in primary metabolism (pyruvate decarboxylation, citric acid cycle and amino acid and nucleotide metabolism), respectively. Five proteins represented chaperones (HSP60/HSP70, prohibitin, CPN10, and putative chaperone for complex I assembly), whereas two proteins were involved in anti-oxidative defense (superoxide dismutase (SOD) and putative peroxyredoxin). Interestingly, 20% of the identified proteins are not known as plant mitochondrial proteins.

In parallel, Millar and co-workers identified approximately 100 abundant and 250 low abundance mitochondrial proteins, derived from suspension-cultured cells (heterotrophic cell culture established from callus of *Arabidopsis* cv. L. *erecta* stem explants), by 2-DGE [36]. Separation and comparison of subfractions of mitochondrial proteins provided information on the soluble membrane (43) and peripheral (21) proteins, or their integral membrane locations (18 proteins). Using MALDI-TOF-MS, spectra for a total of 170 protein spots were obtained, out of which 155 spectra were of high quality. Searching translated *Arabidopsis* genomic databases identified a total of 91 proteins. Of this set, 81 had defined functions based on sequence comparison. These functions included respiratory electron transport, TCA cycle, metabolism, amino acid metabolism, protein import, processing and assembly, chaperonins, degradation, transcription, membrane transport, and anti-oxidant defense. Among the different functional classes, the proteins of the TCA cycle, the electron transport chain, and HSP60/HSP70s dominate the list of identified and highly abundant proteins on 2-DGE. A total of 10 spectra were matched to *Arabidopsis* putative open reading frames (ORFs) for which no specific function has been determined, where 64 spectra did not match to an identified ORF. Moreover, 15 samples had poor spectra, and therefore could not be used for further analysis. Interestingly, analysis of full-length putative protein sequences using subcellular targeting tools revealed significant variation in predictions, and also a lack of mitochondrial targeting prediction for several characterized mitochondrial proteins. Despite identification of numerous proteins, a large number of known abundant mitochondrial proteins (encoded in the mitochondrial genome, and a range of inner membrane carriers) could not be identified. One of the reasons attributed to this was the hydrophobicity and the basic nature of the protein sequences.

The same group (Heazelwood and co-workers) in 2004, revealed novel insight into *Arabidopsis* (L. *erecta* suspension-cultured cells) mitochondrial function from a large experimental proteome derived using direct sample analysis by LC–MS/MS [42]. A total of 416 proteins were identified that includes a significant number of low-abundance proteins involved in DNA synthesis, transcriptional regulation, protein complex assembly, and cellular signaling, and approximately 20% proteins of unknown function. A comparison of the experimental dataset with the predictions of subcellular localizations using five targeting algorithms and putative orthologs from other organisms revealed new metabolic, regulation, and signaling pathways in plant mitochondria. The workflow used in establishing the mitochondrial proteomes in the aforementioned studies is schematically presented in Fig. 5.

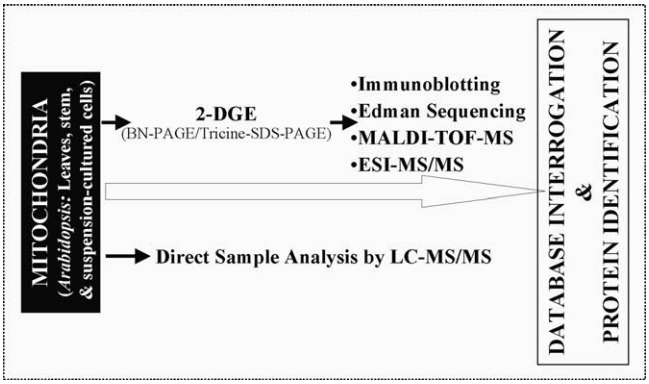

Fig. 5. Mitochondrial proteomics. The *Arabidopsis* tissues and suspension-cultured cells have been used for identifying the mitochondrial proteins, including the low abundance proteins that are enriched in part due to mitochondria purification and subsequent solubilization of individual compartments. Two methods are used: the commonly used 2-DGE separation of purified proteins, followed by immunoblotting, Edman sequencing or MS-based analyses, and a recently developed direct mitochondrial protein sample analysis by LC–MS/MS. Unambiguous protein identification is done by database interrogation/searches.

1.4.5.1. Mitochondrial carrier proteins. The mitochondrial carrier proteins that maintain metabolic communication with the cytosol remain largely unknown. Previously, proteome analysis of *Arabidopsis* mitochondria by 2-DGE failed to identify any mitochondrial carrier proteins, presumably because of their hydrophobicity and basicity [36]. In *Arabidopsis*, a subset of 45 putative genes encoding members of this family have been identified based on generalized mitochondrial carrier features. A comparison of this *Arabidopsis* carrier subset to the yeast gene family (35 genes) also revealed 10 orthologous groups between the two species. For their identification, integral membrane proteins from *Arabidopsis* mitochondria [36] were initially separated from the bulk peripheral membrane protein by Na_2CO_3 treatment, and separated by SDS–PAGE [37]. Ten protein bands (in the range of 28–38 kDa) were subjected to Q-TOF-MS/MS analysis, resulting in the identification of both carrier (adenine nucleotide translocater, dicarboxylate/tricarboxylate carrier, phosphate carrier, uncoupling protein, and a carrier gene of unknown function) and non-carrier proteins. Combined genomic and proteomic analysis led to the conclusion that only a small subset of the carrier family of genes provide the majority of carrier proteins of *Arabidopsis* mitochondria.

1.4.5.2. NADH-ubiquone oxidoreductase complex. The NADH-ubiquinone oxidoreductase of the mitochondrial respiratory chain is a large multisubunit complex in eukaryotes containing 30–40 different subunits. A BN–PAGE coupled to Q-TOF-MS/MS was applied to analyze this complex in

Arabidopsis [40]. A heterotrophic cell culture established from callus of ecotype L. *erecta* stem explants was used for mitochondria isolation according to a previous method [36]. This resulted in the identification of a series of 30 different proteins. Together with a study in rice, it was revealed that plants contain a series of 14 highly conserved proteins of complex I subunits found in other eukaryotic and related prokaryotic enzymes, and a small set of nine proteins widely found in eukaryotic complexes. It was observed that a significant number of proteins present in bovine complex I, but absent from fungal complex I, were also absent from plant complex I, and are not encoded in plant genomes. A series of plant-specific nuclear encoded complex I associated subunits were identified, including a series of ferripyochelin-binding protein-like subunits and a range of small proteins of unknown function.

1.4.5.3. Respiratory supercomplexes. Respiratory protein complexes form supercomplexes in plant mitochondria. A systematic investigation of supercomplexes in mitochondria was initiated using mitochondrial fractions from *Arabidopsis*, potato (*Solanum tuberosum*) and beans (*Phaseolus vulgaris*) plants [39]. Mitochondria were prepared from non-green *Arabidopsis* suspension-cultured cells, potato tubers, and 18-day-old etiolated bean seedlings. Combining gentle protein solubilization with BN–PAGE, the mitochondrial respiratory chain supercomplexes were identified using previously described methods [43,44]. Analysis of proteins was done by MALDI-TOF-MS and nESI-Q-TOF-MS. Three supercomplexes were visualized: (1) dimeric ATP synthase (complex V); (2) a supercomplex formed by dimeric complex III and complex I; and (3) a supercomplex containing two copies of dimeric complex III and two copies of complex I. On the other hand, complexes II and IV, as well as the alternative oxidase did not form part of supercomplexes under all tested conditions. Furthermore, larger and smaller forms of cytochrome c oxidase were found, which differ by at least two protein subunits, and a complex II, comprising of a very unusual subunit composition. Based on these results, it was concluded that the majority of mitochondrial proteins probably form part of protein complexes, and possibly most protein complexes are involved in the formation of even larger supermolecular structures, which remain to be discovered. These data are available at http://www.gartenbau.uni-hannover.de/genetik/braun/AMPP.

1.4.5.4. Ribosomal proteins. Isolation of a fraction enriched for mitochondrial ribosome and the labeling ([^{35}S] methionine) of mitochondrial translation products was used to identify the ribosomal proteins encoded by mitochondrial genome of the broad beans (*Vicia faba*, 12-day-old etiolated seedlings of cv. Exelle) [45]. 2-DGE resolved a total of 26 labeled products. A total of six protein spots identified both by CBB staining and autoradiography, were analyzed by N-terminal amino acid sequencing. Two of the identified proteins corresponded to ribosomal proteins, S10 and S12, and therefore it was concluded that these two proteins are encoded by the mitochondrial genome.

1.4.5.5. Divalent metal cation binding proteins. A range of divalent metal cations interacts with proteins, facilitating biological functions in enzyme catalysis and cell signaling. Using mobility shift assay technique, divalent metal binding proteins in the *Arabidopsis* (L. *erecta* stem explant suspension-cultured cells) mitochondrial proteome were identified [38]. A total of 34 reproducible protein spots were shifted, when various combinations of EDTA and $CaCl_2$ were used between the one (1)- and 2-DGE gel separations. The excised proteins were subjected to Q-TOF-MS/MS, and 23 proteins were identified that represented 11 different *Arabidopsis* gene loci. These included, succinyl CoA ligase β subunit, Mn-SOD, a Fe-S centered component of complex I, and the REISKE iron-sulfur protein of the b/c_1 complex. A further set of four proteins of known function but without known divalent-binding properties, were also identified (Vb subunit of cytochrome c oxidase, a subunit of ATP synthase (orfB), the acyl carrier protein, and the translocase of the outer membrane (TOM20). Three proteins of unknown function were also identified, and may represent novel metal-binding proteins.

1.4.5.6. Thioredoxin. Mitochondria contain Trx, a regulatory disulfide protein and an associated flavoenzyme, NADP/Trx reductase, which provide a link to NADPH in the organelle. Unlike the animal and yeast Trx(s), the function of plant Trx in the mitochondria is largely unknown. Balmer and co-workers used a proteomic approach to identify soluble Trx-linked proteins of mitochondria from both photosynthetic (leaf, spinach and pea) and heterotrophic (tuber, potato) sources [46]. A combination of affinity chromatography as described in their previous report on chloroplast Trx [32] and fluorescence gel electrophoresis in which target proteins are identified visually after reduction of the preparation by Trx [47], was used (Fig. 4). As bait, a chloroplast mutant Trx *m* was used, which was related to mitochondrial Trx *o*. The study led to the identification of 50 potential Trx-linked proteins functional in 12 processes (photorespiration, citric acid cycle and associated reactions, lipid metabolism, electron transport, ATP synthesis/transformation, membrane transport, translation, protein assembly/folding, nitrogen metabolism, sulfur metabolism, hormone synthesis, and stress-related reactions). These results suggest that Trx acts as a sensor for the redox state of the mitochondria, and functions in an inter-organelle network that enables communication between chloroplast and mitochondria. Hence, proteomics has resulted in uncovering the potential role/function of Trx in plant mitochondria and chloroplast, and in plant growth, reproduction and stress.

1.4.6. Nucleus

A first comprehensive study on nuclear proteome was presented in *Arabidopsis* [48]. Nuclear proteins were isolated

from leaves of ecotype Columbia following the method described by Folta and Kaufman [49] with some modifications, and analyzed using 2-DGE in combination with MALDI-TOF-MS. Approximately, 500–700 spots were detected and 184 protein spots were identified that corresponded to 158 different proteins implicated in a variety of cellular functions: DNA replication/repair/modification (38%), signaling and gene regulation (39.7%), protein degradation (6%), protein folding (2.7%), structure (8.7%), RNA metabolism (3.8%), translation (12.5%), carbon metabolism (3.2%), and uncharacterized (19.8%).

1.4.7. Peroxisome

Leaf peroxisome, isolated from greening *Arabidopsis* (ecotype Columbia) cotyledons, was used for proteomic analysis [50]. 2-DGE followed by silver staining, revealed the presence of at least 53 protein spots, which were subjected to MALDI-TOF-MS. Among these 29 proteins were identified that included 5 proteins related to gylcolate pathway, 4 proteins involved in scavenging hydrogen peroxide, and additionally 20 novel leaf peroxisomal proteins (protein kinases and phosphatases, and proteins carrying peroxisomal targeting signals).

1.4.8. Plasma membrane

Initial study on PM was conducted in tobacco by 2-DGE, which led to the tagging of 12 new plant proteins and to the identification of 5 ESTs as encoding putative PM proteins [51]. A more systematic work was carried out using *Arabidopsis* PM, where 82 polypeptides were resolved on 2-DGE [52]. In a later study, *Arabidopsis* (ecotype Columbia) root (700) and leaf (500) PM proteins were resolved, compared and discriminated between integral and peripheral proteins [53]. The majority (60%) of proteins between leaf and root PM were the same. Moreover, only minute differences (1.5%) were observed between the leaf PM protein profiles of Columbia, L. *erecta*, and Ws-2.

1.4.8.1. Glycosylphosphatidylinositol (GPI)-anchored proteins. Glycosylphosphatidylinositol (GPI)-anchoring can determine the subcellular localization of proteins or alter their association with membranes [54]. Proteins with these anchors are often associated integrally with PMs, but become more hydrophilic after specific cleavage of the anchor by phospholipase [55]. Biochemical fractionation, 2-DGE and reference to plant membrane database revealed the existence of multiple GPI-anchored proteins in *Arabidopsis* [56]. At least six of these GPI-anchored proteins, AtGPIP1–4, 9 and 10, were present in the purified PM. Some of these proteins were also found in the extracellular matrix, such as an arabinogalactan protein (AGP), a class of proteins known to be associated with cellular differentiation. Moreover, the amino acid sequence analysis of two novel AGP-like proteins, predicted the existence of consensus signals for GPI-anchor addition. This study supports a model where GPI-anchored proteins are involved in the generation of specialized cell surfaces and extracellular signaling molecules.

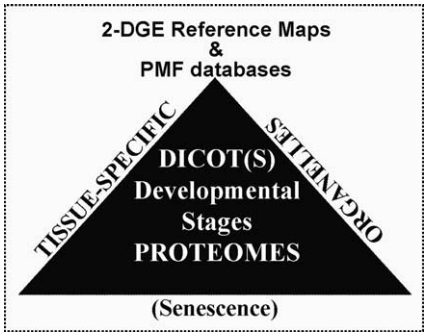

Fig. 6. Dicot developmental stages proteomes are a valuable resource for the plant proteomics community. Experimental proteomes (2-DGE reference maps and PMF databases) are available for various tissues (leaf, stem, root, flower and fruit, and suspension-cultured cells), and specific organelles (cell wall, chloroplast, endoplasmic reticulum, microtubule, mitochondria, nucleus, peroxisomes, and plasma membrane).

2. Conclusions and perspectives

"Good experimental set up leads to good science"—this is evident from the progress made on the proteomics of dicot plant materials presented here. Developmental proteomics, specifically in the two model species, *A. thaliana* and *M. truncatula*, have laid a strong foundation for dicot plant proteome analyses. To date, experimental proteomes (2-DGE reference maps and PMF databases) are available for various tissues (leaf, stem, root, flower and fruit, and suspension-cultured cells), and specific organelles, such as cell wall, chloroplast, ER, microtubule, mitochondria, nucleus, peroxisomes, and PM (Fig. 6). A large number of changes in protein profiles are evident from the tissues-specific, and senescence (where about 40% of the senescence-related proteins are involved in photosynthesis) and organellar proteomes. These studies indicate that proteomic approaches can be successfully applied globally to investigate protein expression patterns in plants. Furthermore, these proteomes are very useful in studying (establishing and comparing) the proteomes of other plant species at the tissue and organellar levels.

An important progress in proteomics of dicot plant materials has come through the analyses of organelle subproteomes. Of these, the chloroplast and mitochondrial proteomes are by far the best characterized, and begin to provide insight into the proteins present and metabolic operations therein. Analyzing the organellar proteomes is a good strategy, as it reduces both sample complexity and the analytical challenge, while enriching the biological information relevant for a particular organelle. It is worth mentioning that in many cases, proteomes have been developed using suspension-cultured cells, specifically the mitochondria, which does not reflect the true physiological conditions. Nonetheless, it is a good start, and has given valuable information on the mitochondrial function

in plants. Moreover, a comparison of proteome maps can tell us how similar or dissimilar these organelles are among the plant species. As for example, a comparison of *Arabidopsis* and pea thylakoid proteins revealed overall resemblance in protein profiles both in terms of expression levels and as coordinates for the different proteins. It was reported that nearly all pairs of *Arabidopsis*/pea proteins matched within a range of ±10 kDa and ±0.75 pH, with a few exceptions. All lumenal proteins identified on the pea maps also were identified on the *Arabidopsis* maps, with the exception of two protein spots, indicating that the lumenal proteomes of the two species are rather similar. On the other hand, proteome has also revealed the specificity of the organelle proteins. For example, the nuclear proteome comprises mostly of proteins implicated in signaling and gene regulation, reflecting the role of nucleus in gene expression and regulation [48]. This is in contrast with the chloroplast and mitochondrial proteomes that revealed the significant fractions of proteins to be involved in energy production, either in electron transport or in ATP production [23,36]. The existence of TAT pathway for protein translocation in plant chloroplast is another interesting finding of organellar proteomes. This finding receives support from functional genomic studies, first in rice [57] and then in *Arabidopsis* [58], where the loss-of function of TAT-like genes affected the seedling growth by disrupting chloroplast development. Additionally, the benefit of using an affinity-based proteomic approach to enrich a specific set of functional proteins can be seen from elegant studies on Trx from chloroplast [32] and mitochondria [46].

The number of proteins identified in these studies shows us just the "tip of the iceberg", and the on-going and future studies will only unravel the true extent (and vast numbers) of proteins waiting to be separated, identified, and functionally catalogued.

3. Nomenclature

ADO-MET *S*-adenosyl-methionine synthase
ATP adenosine triphosphate
BN–PAGE blue native–polyacrylamide gel electrophoresis
CBB Coomassie brilliant blue
ER endoplasmic reticulum
ESI-MS/MS electrospray ionization tandem-mass spectrometry
ESI-Q-TOF-MS ESI-quadrupole-time of flight-tandem MS
EST expressed sequence tags
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GPI glycosylphosphatidylinositol
HPLC high-performance liquid chromatography
HSP heat shock protein
IEF isoelectric focusing
LC–MS/MS LC–tandem MS
MALDI matrix-assisted laser desorption/ionization
MAPS microtubule associated proteins
MS mass spectrometry
nESI-Q-TOF-MS nanospray-ESI-Q-TOF
ORF open reading frame
PAGE polyacrylamide gel electrophoresis
PM plasma membrane
PMF peptide mass fingerprinting
RuBisCO ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS sodium dodecyl sulfate
TAT twin arginine motif
TCA trichloroacetic acid
3-DGE three-dimensional gel electrophoresis
2-DGE two-dimensional gel electrophoresis

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