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# Proteome analysis for the study of developmental processes in plants

Loïc Rajjou, Karine Gallardo, Claudette Job and Dominique Job

## 7.1 Introduction

Proteome analysis, which involves the identification and characterization of expressed proteins, is a powerful tool for determining the biological roles and functions of individual proteins. Furthermore, by providing a systematic and without any *a priori* mean for large-scale identification of cellular proteins, proteomics is expected to accelerate discoveries in complex processes such as development. Allied to other genome-scale gene expression profiling methods as transcriptomics and metabolomics, proteomics also constitutes a powerful tool toward the development of modern systems biology (Ge et al., 2003; Patterson and Aerborsold, 2003), an emerging approach that seeks to describe (model) biological systems through integration of diverse types of massive data and which will ultimately allow computational simulations of these complex systems, notably in plants (Gutiérrez et al., 2005). With the completion of genome sequencing projects and the constitution of large expressed sequence tags (ESTs) collections for several reference (*Arabidopsis*, rice, *Medicago truncatula*, poplar, *Physcomitrella patens*) and crop (e.g. maize, wheat, barley, soybean) plants and the development of analytical methods for protein characterization (see Hirano et al., 2004; Newton et al., 2004), proteomics has become a major field of functional genomics, allowing large-scale analysis of protein sequences deposited into databases (e.g. *Arabidopsis* genome initiative, 2000; Goff et al., 2002; Yu et al., 2002; Li et al., 2003; Gutiérrez et al., 2004; Sarnighausen et al., 2004; Stauber and Hippler, 2004; Zhang et al., 2004; Giavalisco et al., 2005; <http://www.tigr.org/tdb/tgi/plant.shtml>; <http://mips.gsf.de/proj/sputnik/>). For example, the rice proteome database based on two-dimensional gel electrophoresis (2-DE) presently comprises about 12,000 identified proteins from different tissues and organelles, corresponding to more than 4000 separate entries (Komatsu et al., 2003, 2004; <http://gene64.dna.affrc.go.jp/RDP/>). Also, a 2-DE proteomics reference map comprising 1367 identified proteins has been established from a cell suspension culture of the model legume *M. truncatula* (Lei et al., 2005).

Agrawal et al. (2005b) proposed that developmental proteomics be defined 'as a set of proteins present at particular developmental stage of a plant'. Therefore, two general proteomics approaches have been used to investigate plant development (see Baginsky and Gruissem, 2004). The one, which can be defined as 'Shotgun proteomics' or 'Tissue-specific proteomics', aims at providing an exhaustive overview

of the proteins present in various organs and organelles. At the transcript level, there is evidence that each organ or tissue-type representing the life cycle of *Arabidopsis* has a defining genome expression pattern. Therefore, the knowledge of organ-specific expression patterns and their response to the changing environment provides a foundation for dissecting the molecular processes underlying development (Ma et al., 2005). The other approach, which can be referred to as 'Functional proteomics', concentrates on the analysis of specific proteins related to specific biological processes. In particular, this latter approach aims at the identification of proteins that are subject to post-translational modifications or whose accumulation level changes during specific stages of development. To date, this relies heavily on differential 2-DE, a robust technique used since three decades (O'Farrell, 1975). An example is presented in Plate 7.1 in Plate section, showing the tremendous change in the proteome during *Arabidopsis* embryo development. Such gels can typically resolve more than 1500 proteins following staining by silver nitrate, out of which the accumulation level of several hundreds may be observed to vary during seed development (Plate 7.1). Here, it is expected that the identification of all these changes will provide clues toward understanding seed development.

Recent advances in proteomics include the refinement of 2-DE techniques and the development of sensitive methods for protein visualization and mass spectrometry (MS) analysis. Owing to these progresses, proteomic studies in plants have proliferated during the recent years (reviewed by Hirano et al., 2004; Newton et al., 2004; Rose et al., 2004; Agrawal et al., 2005a; Peck, 2005). A number of excellent papers and reviews are available in several areas of plant proteomics, dealing with the establishment of reference protein maps in plant tissues and organelles (Bardel et al., 2002; Koller et al., 2002; Baginsky and Gruissem, 2004; Heazlewood et al., 2004; Sarnighausen et al., 2004; Stauber and Hippler, 2004; Agrawal et al., 2005b), the identification of proteins that would serve as possible markers of different genotypes and phenotypes and their use in determination of phylogenetic relationships (Thiellement et al., 1999, 2002; Chevalier et al., 2004), the importance of protein modifications ('modificomics') in plants (Hirano et al., 2004; Laugesen et al., 2004), the characterization at the protein level of plant-microorganism interactions (Rolfe et al., 2003; Bestel-Corre et al., 2004; Cánovas et al., 2004; Colditz et al., 2004), or the characterization of the influence of the environment on plant proteomes (Agrawal et al., 2005c). Particularly interesting in the context of plant proteomics is the genetic approach based upon comparison of protein profiles from cultivars and recombinant inbred lines showing diversity for agronomic and developmental traits and enabling the discovery of the genetic factors accounting for the accumulation of the proteins related to these traits (Thiellement et al., 1999, 2002).

The 'Tissue-specific proteomics' approach for the study of plant development has been covered by several reviews (Thiellement et al., 2002; Hirano et al., 2004; Newton et al., 2004; Agrawal et al., 2005b). Therefore, the present analysis will mainly concentrate on the 'Functional proteomics' approach, taking as an example seed development and germination, in which we have been particularly involved.

## 7.2 Examples of proteome analyses of plant development

### 7.2.1 Seed development and germination

The new plant formed by sexual reproduction starts as an embryo within the developing seed, which arises from the ovule. The development of the embryo proceeds through histodifferentiation and seed filling and terminates, for most species growing in temperate climates, with a desiccation phase, after which the embryo enters in a quiescent state, thereby permitting its storage and survival for many years in various environmental conditions. The seed, therefore, occupies a central position in the higher plant life cycle. Dry mature seeds are resting organs, having low moisture content (5–15%) with metabolic activity almost at a standstill. In the absence of dormancy (a physiological process conditioning seed germination not considered here), for germination to occur seeds need to be hydrated under conditions that encourage metabolism, for example a suitable temperature and the presence of oxygen. Broad proteomic analyses of seed development and germination have been initiated using reference plants, as well as with seeds of commercial importance as barley, maize, or tomato. The general aim is to identify characteristic proteins involved in seed development and germination, which will help understanding the biochemical and molecular processes underlying seed quality and vigor. Additionally, these specific proteins might help optimizing industrial seed treatments such as germination enhancement (priming) or malting.

#### 7.2.1.1 Proteomics of developing seeds

Seed development in *M. truncatula* (cv. Jemalong J5) was investigated at specific stages of seed filling corresponding to the acquisition of germination capacity and protein deposition (Gallardo et al., 2003). Individual flowers were tagged on the day of flower opening, and the pods were harvested between 8 and 44 days after pollination (DAP). One hundred and twenty proteins differing in kinetics of appearance were subjected to matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS. These analyses provided peptide mass fingerprint data that identified 84 of them. Some of these proteins had previously been shown to accumulate during seed development in legumes (e.g. legumins, vicilins, convicilins, and lipoxigenases), confirming the validity of *M. truncatula* as a model for analysis of legume seed filling. The study also revealed proteins presumably involved in cell division during embryogenesis (tubulin and annexin). Their abundance decreased before the accumulation of the major storage protein families, which itself occurs in a specific temporal order: vicilins (14DAP), legumins (16DAP), and convicilins (18DAP). Furthermore, the study showed an accumulation of enzymes of carbon metabolism (e.g. sucrose synthase, starch synthase) and of proteins involved in embryonic photosynthesis (e.g. chlorophyll *a/b* binding), which may play a role in providing cofactors for protein/lipid synthesis or for CO<sub>2</sub> refixation during seed filling. Correlated with the reserve deposition phase was the accumulation of proteins associated with cell expansion (actin 7 and reversibly glycosylated polypeptide). This study had not only cataloged proteins but has also described their accumulation patterns at specific stages during seed

development, before and during protein deposition. These findings, therefore, contribute to our understanding of how metabolic networks are regulated at the protein level during reserve deposition in seeds of a legume species. Finally, this work revealed a differential accumulation of enzymes involved in methionine metabolism (*S*-adenosylmethionine (AdoMet) synthetase and *S*-adenosylhomocysteine (AdoHcy) hydrolase; see Figure 7.2) and proposed a role for these enzymes in the transition from a highly metabolically active to a quiescent state during seed development (see Section 7.2.1.4). This knowledge will support further attempts to engineer legume seed composition for added end user value.

In parallel, Watson et al. (2003) 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). 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%).

A high-throughput proteomic approach was recently employed to determine the expression profile and identity of hundreds of proteins during seed filling in soybean (*Glycine max*) cv. Maverick (Hajduch et al., 2005). Soybean seed proteins were analyzed at 2, 3, 4, 5, and 6 weeks after flowering (WAF) using 2-DE and MS. This led to the establishment of high-resolution proteome reference maps and expression profiles of 679 spots. These corresponded to 422 proteins representing 216 non-redundant proteins, which were classified into 14 major functional categories. Proteins involved in metabolism, protein destination and storage, metabolite transport, and disease/defense were the most abundant. To further detail global expression trends of proteins involved in different processes, the authors established composite expression profiles by summing protein abundance, expressed as relative volume, for each protein in each functional class for the five seed stages (Hajduch et al., 2005). Relative abundances of metabolic proteins decreased during the experimental period, suggesting metabolic activity curtails as seeds approach maturity. Interestingly, proteins involved in lipid and sterol metabolism decreased from 2 to 4 WAF, but after 4 WAF their abundance slightly increased. The protein destination and storage class of proteins increased during late seed filling, and this was due to the preponderance of seed storage proteins. The transporter class of proteins, which includes the ubiquitous Suc-binding proteins, exhibited almost constant but slightly increased expression during the experimental period. Disease- and defense-related proteins were highly abundant at the early stage of seed filling; later their abundance decreased to about 50% and from 4 WAF was stable. Proteins involved in energy production increased in abundance during seed filling, whereas cell growth and division proteins as well as signal transduction proteins each had decreasing expression profiles. Proteins involved in protein synthesis and secondary metabolism decreased in abundance during the experimental period. A user-intuitive database (<http://oilseedproteomics.missouri.edu>) has been developed to access these data for soybean and other oilseeds currently being

investigated. In summary, an overall decrease in metabolism-related proteins versus an increase in proteins associated with destination and storage was characteristic of seed filling in soybean.

Proteomic approaches were also used to investigate embryo development in monocots. Recently, expressions of more than 400 polypeptide spots during rice caryopsis development, in response to temperature treatments or between varieties were monitored (Lin et al., 2005). Among them, more than 70 differentially expressed polypeptides were analyzed, allowing identifying 54 proteins with known functions. Of these, 21 were involved with carbohydrate metabolism, 14 with protein synthesis and sorting, including glutelins and prolamins, the major storage proteins in rice seeds and 9 with stress responses, most of them being heat-shock proteins (HSPs). The data indicate that changes in the expression of these proteins can be used to mark the physiological development stage of rice caryopses. Waxy (Wx) proteins and glutelins were the most significant spots, which increased significantly during development. Allergen-like proteins, pyruvate orthophosphate dikinase (PPDK) and NADP<sup>+</sup>-dependent sorbitol dehydrogenase (NADH-SDH), also were expressed during development, implying their physiological roles in caryopsis. Concerning PPDK, its prominent and consistent expression suggested that it might be involved in CO<sub>2</sub> fixation or amino acid biosynthesis. The observed accumulation of NADH-SDH was the first report of its expression in rice, although its role in development remains to be clarified. Expression of large isoforms of Wx proteins was correlated with the amylose content of rice caryopses. High temperature (35/30°C) decreased the expression of Wx proteins, allergen-like proteins, and elongation factor 1b, but increased the expression of small heat-shock proteins (sHSP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and prolamin. sHSP was positively correlated with the appearance of chalky kernels. During development, four Wx proteins isoforms were phosphorylated, while several glutelins were phosphorylated and glycosylated. Although the reason of this post-translational modification is unknown, this is an interesting observation, since there are only few reports on post-translational regulation of rice seed proteins. It is noted that phosphorylation of rice glutelins, which was not reported before, was directly demonstrated in this careful study using immunological and LC-MS/MS analyses (Lin et al., 2005).

2-DE was used for a time-resolved study of the changes in proteins that occur during seed development in barley (*Hordeum vulgare*) (Finnie et al., 2002). Protein spots were divided into six categories according to the timing of appearance and disappearance during the 5-week period of comparison. Thirty-six selected spots were identified by MALDI-TOF MS or by nano-electrospray tandem MS/MS. Some proteins were present throughout development (e.g. cytosolic malate dehydrogenase), whereas others were associated with the early grain filling (ascorbate peroxidase), desiccation (cold-regulated protein Cor14b) or late (embryo-specific protein) stages. Most noticeably, the development process was characterized by an accumulation of serpin, low-M<sub>r</sub> α-amylase inhibitors (presumed to defend the starch reserves of the seed against invading insect pathogens), serine protease inhibitors, and enzymes involved in protection against oxidative stress (glyoxalase I, ascorbate peroxidase,

1cys-peroxyredoxin). The expression of this last class of proteins throughout the development process most presumably reflects the importance of protection against reactive oxygen species (ROS) produced during seed development (see Section 7.2.1.7). The study also presented examples of proteins not previously experimentally observed (e.g. Cor14b), differential extractability of thiol-bound proteins, and possible allele-specific spot variation (e.g. at the level of  $\beta$ -amylase).

A proteomic approach was utilized to identify over 250 proteins of wheat (*Triticum aestivum* L., cv. Butte 86) starchy endosperm that participate in 13 biochemical processes: ATP interconversion reactions, carbohydrate metabolism, cell division, cytoskeleton, lipid metabolism, nitrogen metabolism, protein synthesis/assembly, protein turnover, signal transduction, protein storage, stress/defense, transcription/translation, and transport (Vensel et al., 2005). Endosperm protein populations were compared at early (10 days post-anthesis, dpa) and late (36 dpa) stages of grain development. Analysis of protein number and spot volume revealed that carbohydrate metabolism, transcription/translation, and protein synthesis/assembly were the principal endosperm functions at 10 dpa followed by nitrogen metabolism, protein turnover, cytoskeleton, cell division, signal transduction, and lipid metabolism. Carbohydrate metabolism and protein synthesis/assembly were also major functions at 36 dpa, but stress/defense and storage were predominant.

#### 7.2.1.2 Proteomics of somatic embryogenesis

Somatic embryogenesis is a process analogous to zygotic embryogenesis, in which a single cell or a small group of vegetative (i.e. somatic) cells are the precursors of the embryos. This phenomenon can be divided into four major steps: (i) initiation of proembryogenic masses, (ii) proliferation of embryogenic cultures, (iii) maturation of somatic embryos, and (iv) regeneration of whole plants. Somatic embryogenesis is widely investigated in several plants because, on one hand, it provides useful systems for plant propagation (e.g. conifer biotechnology for reforestation programs) and, on the other hand, it allows fundamental studies on embryo development (Zimmerman, 1993).

A recent proteomic study used leaf explants from the mutant line 2HA of *M. truncatula*, which presents a 500-fold greater capacity to regenerate plants in culture by somatic embryogenesis than the wild-type Jemalong cultivar chosen as reference to produce extensive genomic sequences (Imin et al., 2005). Both 2HA and Jemalong leaf explants were grown on media containing the auxin 1-naphthalene-acetic acid and the cytokinin 6-benzylaminopurine. Proteins were extracted from the cultures at different time points (2, 5, and 8 weeks), separated by 2-DE, and detected by silver staining. More than 2000 proteins could be reproducibly resolved and detected on each gel. Statistical analysis showed that 54 protein spots were significantly changed in expression (accumulation) during the 8 weeks of culture. They were subjected to MALDI-TOF or LC-MS/MS analyses. This allowed identifying 16 differentially expressed proteins. More than 60% of the differentially expressed protein spots had very different patterns of gene expression between 2HA and Jemalong during the 8 weeks of culture.

Among the identified proteins, RuBisCo small-chain proteins were gradually decreased in both Jemalong and 2HA during explant cultures. As such, the decreased trend of RuBisCo small-chain proteins can be used as a marker for the dedifferentiation and proliferation of the mesophyll tissues. Two of the most abundant proteins were an abscisic acid (ABA)-responsive protein with homology to the pathogenesis-related protein PR10-1 and PR10-1 itself in both Jemalong and 2HA. They were not detected in the young leaves from which the explant cultures originated. Interestingly, they changed little throughout the 8 weeks of culture, suggesting a general role for ABA-responsive proteins and PR10 proteins in cell maintenance or cell defense. Chaperone proteins (dnaK-type HSP70 and luminal binding), in general, showed a decrease in the 8-week-old cultures in both Jemalong and 2HA, although their expression levels were different. This may imply that a higher level of expression of the chaperones is required for the maintenance of cells during early culture. It is noteworthy that this study also identified proteins involved in seed formation as being expressed only in the highly embryogenic 2HA of 8-week-old cultures. These included a seed maturation protein and a vicilin. Since the same behavior was observed during seed development in *M. truncatula* (Gallardo et al., 2003), it can be concluded that somatic embryogenesis closely resembles the zygotic embryogenesis, and that the approach of inducing embryogenesis in 2HA was a valid comparison of the two systems. One of the most interesting proteins identified was thioredoxin *h* (Imin et al., 2005). It appeared early in both protoplast and explant cultures and became undetectable at later stages of cell proliferation. Furthermore, its expression was not detected in leaf tissue or isolated protoplasts, suggesting it is induced only after putting the tissues in culture. There was a higher expression in 2HA than in Jemalong in this early induction process. These results suggest that in *M. truncatula* thioredoxin *h* plays an important role during early stages of commitment from the vegetative stage to a pathway of cellular differentiation and proliferation. As discussed below (Section 7.2.1.6), thioredoxin *h* regulates a myriad of post-transcriptional biological functions in plant cells (Buchanan and Balmer, 2005). Another interesting protein found in this study is the 1cys peroxiredoxin, which remained at a minimum level in Jemalong during 8 weeks of culture. By contrast, it showed a slight increase at 5 weeks before reaching a much higher level of expression at 8 weeks in the highly embryogenic line 2HA, clearly indicating a role for this enzyme in embryo development (Imin et al., 2005).

Forests play a vital role in both the stability of global ecosystems and in the economy of countries that retain large stands of timber. Sustainable forest management is therefore of considerable interest worldwide. In North America, white spruce is an important component of the forest landscape, not only for its abundance, but also for its high value for the solid wood industry and for pulp and paper production. Somatic embryogenesis of white spruce has the potential to deliver a stable supply of superior seedlings on a large scale for forest plantations. In a recent study (Lippert et al., 2005), a proteomic approach was employed to quantitatively assess the expression levels of proteins across four stages of somatic embryo maturation in white spruce (0, 7, 21, and 35 days post-ABA treatment). The increasing cellular complexity of the

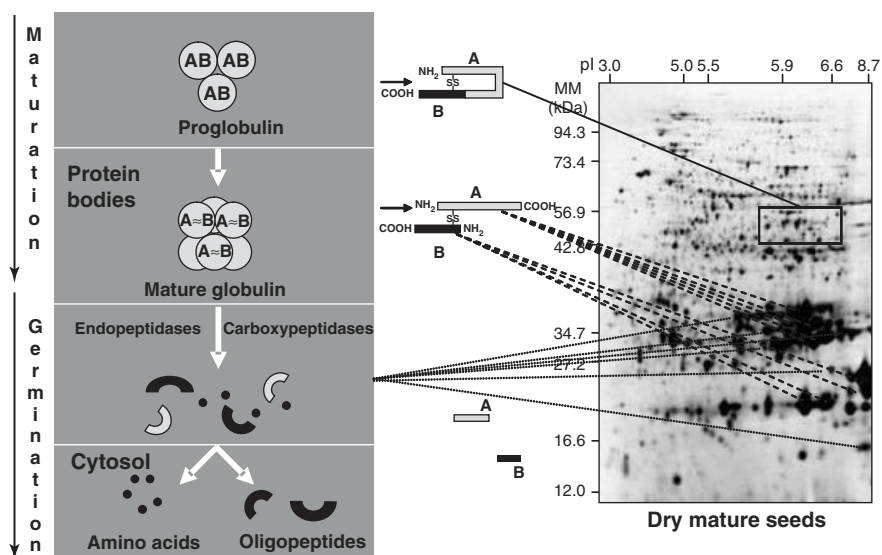


developing embryos was clearly evident at the molecular level. Measurement of protein content in these samples revealed a 5-fold increase in total protein content based on fresh weight between the early and late stages of development. Subsequent analysis of these tissues by 2-DE showed a concomitant increase in the total number of distinct protein species which could be resolved as the embryos developed. This number rose from 696 distinct protein species in the immature stage 0 embryos to 1250 distinct protein species in the mature embryos at day 35, presumably reflecting the added complexity arising as tissues begin to differentiate.

Forty-eight differentially expressed proteins have been identified by LC-MS/MS, which displayed a significant change in abundance as early as day 7 of embryo development. These proteins are involved in a variety of cellular processes, many of which have not previously been associated with embryo development. The identification of these proteins was greatly assisted by the availability of a substantial EST resource developed for white, sitka and interior spruce. It must be stressed that the combined use of these spruce ESTs in conjunction with GenBank accessions for other plants improved the rate of protein identification from 38% to 62% when compared with GenBank alone using automated, high-throughput techniques. This underscored the utility of EST resources in a proteomic study of any species for which a genome sequence is unavailable. Interestingly, as in zygotic embryogenesis (Gallardo et al., 2003), AdoMet synthetase was detected at high level in the immature stage 0 embryos and decreased to background level in mature embryos (Lippert et al., 2005), suggesting common features of metabolic regulation in somatic and zygotic embryogenesis (see Section 7.2.1.4). Furthermore, as in zygotic embryogenesis, developing somatic embryos proved capable of accumulating storage proteins as vicilins (Lippert et al., 2005). One potential application of these proteomic data would be in the assessment of seedling performance during the biotechnological process of somatic seedling production.

### 7.2.1.3 *Proteomes of mature seeds and their evolution during germination*

The storage compounds found in most mature seeds accumulate during seed filling. These reserves are of paramount importance for two reasons: (a) they support early seedling growth when mobilized upon germination, and (b) they are widely used for human and animal nutrition. Thus, plants provide over 70% of the protein in the human diet, most of which is constituted by the storage proteins of cereal and legume seeds. Among them, 11–12S globulins are abundant seed storage proteins, being widely distributed in higher plants. They are synthesized during seed maturation on the mother plant in a precursor form consisting of a single protein chain of about 60 kDa. At later stages, the precursor form is cleaved, yielding the mature globulins generally found in dry mature seeds (Figure 7.1). These are composed of six subunit pairs that interact non-covalently, each of which consists of an acidic A-subunit of  $M_r \approx 40,000$  and a basic B-subunit of  $M_r \approx 20,000$  covalently joined by a single disulfide group. These storage proteins are subsequently broken down during germination and used by the germinating seedling as an initial food source (Bewley and Black, 1994; Adachi et al., 2001, 2003) (Figure 7.1).



**Figure 7.1** Proteomics of the mature dry *Arabidopsis* seed, with emphasis on the identification of 12S cruciferins, the major seed storage proteins in crucifers. Main features of 12S cruciferin synthesis and mobilization during seed maturation and germination are shown on the left. The figure also shows on the right a 2-D gel of total soluble proteins from dry mature seeds. Proteins were first separated by electrophoresis according to charge. Isoelectric focusing (IEF) was carried out with protein samples with an equivalent to an extract of approximately 100 seeds, corresponding to about 200  $\mu$ g protein for all samples. Proteins were then separated according to size by SDS-PAGE. Proteins were visualized by silver nitrate staining (for details on protein extractions and proteomic analyses, see Job et al., 2005).

Contrasting with these well accepted findings, a proteomic approach (Gallardo et al., 2001) revealed that total soluble protein extracts from dry mature *Arabidopsis* seeds contain three forms of 12S globulins (cruciferins): (a) residual precursor forms (shown with solid lines in Figure 7.1), (b) A- and B-subunits (shown by dashed lines in Figure 7.1), and (c) proteolysed forms of A- and B-subunits (dotted lines in Figure 7.1). The presence of some residual precursor forms in the dry mature *Arabidopsis* seeds was unexpected (Bewley and Black, 1994). A possible explanation could be that the maturation process giving rise to the formation of the A- and B-chains was not fully completed when developing seeds entered into quiescence. Another unexpected finding was to recover fragments of A- and B-subunits in the soluble protein extracts from the dry mature *Arabidopsis* seeds. This behavior presumably reflects an early mobilization of the cruciferins during the maturation phase. Thus, proteomics enlightened new features of 12S globulin structure in dry seeds, showing that the anabolic processes that occur before germination and the catabolic processes that normally occur during germination are not fully separated developmentally in *Arabidopsis*.

During germination, the residual cruciferin precursor forms disappeared, suggesting that processing of pro-cruciferins during maturation is not required for their mobilization (see also Section 7.2.1.8). Also, a systematic analysis of all cruciferin fragments appearing and disappearing during germination indicated that initial mobilization of the cruciferins preferentially begins with proteolysis of the A-chains (Gallardo et al., 2001). This hypothesis is consistent with the determination of cleavage sites under limited trypsinolysis of 11–12S globulins; all of these sites being located within the A-chains (Adachi et al., 2001, 2003). Likewise, during germination *sensu stricto* (prior to radicle protrusion) and during the radicle emergence step fragments of both A- and B-chains were released. Thus, once initiated at the level of A-subunits, mobilization of cruciferins continues during germination at the level of the B-subunits, indicating that the initial proteolysis of A-chains increased the sensitivity of B-chains toward further proteolytic attacks (Gallardo et al., 2001).

Besides the 12S cruciferins, changes in the abundance (up- and down-regulation) of a number of proteins were observed during germination *sensu stricto* and the radicle protrusion step (Gallardo et al., 2001, 2002b; Rajjou et al., 2004; Job et al., 2005). 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). Proteins associated with germination *sensu stricto* correlated 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 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, AdoMet synthetase, late embryogenesis abundant (LEA), and heat shock (HSP70) proteins. In addition, a seed maturation protein, probably involved in sequestering biotin (Alban et al., 2000), 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 provided 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-cruciferin B-subunits, catalase, and low-molecular-weight HSPs (Gallardo et al., 2001).

In order to further characterize seed vigor, we have submitted *Arabidopsis* seeds to a controlled deterioration treatment (CDT), and analyzed the consequence of this treatment on the seed proteome by differential proteomics based on a comparison of

eight seed lots treated for different periods of time, up to 7 days (Rajjou et al., 2006). Germination tests showed a progressive decrease of seed vigor depending on the duration of CDT, as described (Tasnier et al., 2002). Proteomic analyses revealed that CDT-induced qualitative and quantitative protein modifications in dry mature seeds. Moreover, during germination, the proteome evolution with the deteriorated seeds appeared to be very different from that with the non-deteriorated control seeds. Therefore, loss in seed vigor was accounted for by specific protein changes in the dry seeds and by an inability of low-vigor seeds to display a normal proteome during germination. By following [ $^{35}\text{S}$ ]Met incorporation during germination, we also observed that the loss of germination vigor was associated to a strong reduction in the protein neosynthesis capacity. This finding is in agreement with other results showing the importance of protein synthesis in germination (Rajjou et al., 2004; see Section 7.2.1.5). This differential proteome analysis also revealed 183 proteins involved in seed vigor, all of them being identified by MS sequencing, and raised the possibility of using plant seeds as a very general model for the understanding of cellular aging.

A database based on clickable protein reference maps (<http://seed.proteome.free.fr>) has been developed to access all these *Arabidopsis* data.

Proteomics was also used to identify major proteins in extracts of mature barley seeds and to follow their fate during germination (Østergaard et al., 2004). To overcome the problem of working with total protein extracts which are dominated by storage proteins and to identify new proteins involved in seed germination, a low-salt extraction buffer was used. At this point, it is worth mentioning that the systematic study of plant proteins dates from the nineteenth century and in particular from the work of Osborne (Osborne, 1924). Osborne pioneered the systematic study of plant proteins and introduced the widely used classification into solubility groups based on their sequential extraction in water (albumins), dilute salt solutions (globulins), alcohol–water mixtures (prolamins), and dilute acid or alkali solutions (glutelins). As stressed by Shewry (1995), this classification was, of course, introduced before we had a detailed understanding of protein structure and characterization, and it was therefore inevitable that some modifications have proved necessary (e.g. the use of reducing agents to cleave the interchain disulfide bonds). Nevertheless, it had formed an invaluable framework for the modern study of seed proteins, notably for the establishment of seed proteomes.

The isolation of the albumin fraction of the mature barley seeds resulted in 198 identifications of 103 proteins in 177 spots (Østergaard et al., 2004). These included housekeeping enzymes (glycolysis, starch metabolism, and citric acid cycle), chaperones, defense proteins (including enzyme inhibitors presumably involved in defense of the germinating seed against pathogens), and proteins related to desiccation and oxidative stress. It is noted that a number of the identifications were made using ESTs. Numerous spots in the 2-D gel pattern changed during germination (micro-malting) and an intensively stained area which contained large amounts of the serpin (serine protease inhibitor) protein Z appeared centrally on the 2-D gel. Spots containing  $\alpha$ -amylase also appeared. Identification of 22 spots after 3 days of germination represented 13 different database entries and 11 functions including hydrolytic

enzymes, chaperones, housekeeping enzymes, and inhibitors. Despite the fact that cereal seed proteins have been studied for decades, new proteins have been identified on 2-D gels. Several of the identified proteins have homologs in other organisms but their function in barley seeds is not clear. Yet, their identification will facilitate the analysis of the changes in the proteome that occur during seed development and germination of cereal grains. Furthermore, by combining proteomics with malting quality analysis and genetics, 2-D gel patterns can be related to cultivar characteristics (Finnie et al., 2004a, b).

The above studies have been carried out with whole seeds, which comprise several tissues and organs: the embryo itself containing an embryonic axis (radicle) and one or two cotyledons, a storage tissue (either an endosperm, a perisperm, or the cotyledons), and the seed envelopes (Bewley and Black, 1994). Therefore, it is worth noting that proteome analysis of germinating tomato seeds has only recently been carried out from isolated embryo and endosperm tissues. This revealed several spots with different abundance in these tissues in the mature dry state and during germination. In particular, a number of protein spots present mainly in the endosperm were identified as coat protein and chain-P of tomato mosaic virus, suggesting their possible role in defense against such viruses during germination (Sheoran et al., 2005). It is also noted in this context a detailed proteome analysis of the endoplasmic reticulum (ER) from germinating castor seeds including separation of ER proteins into luminal, peripheral membrane and integral membrane subfractions. Most of the proteins identified are concerned with roles in protein processing and storage, and lipid metabolism which occur in the ER (Maltman et al., 2002). Both proteomic studies on tomato and castor seeds help demonstrating the usefulness of tissue dissection and separation technologies for a better understanding of the germination process.

The defense response of plants against fungal infection has been intensively studied in vegetative organs, but quite surprisingly not in seeds. This question was addressed for the first time in germinating maize embryos by a proteomics approach (Campo et al., 2004). Among the proteins induced in response to infection are proteins involved in protein synthesis, folding and stabilization, proteins involved in oxidative stress tolerance, as well as the specific pathogenesis-related proteins. Altogether, the available proteomic data highlighted the unexpected occurrence of a defense response during germination, which most presumably is required for protection of the germinating seed and the establishment of a vigorous plantlet.

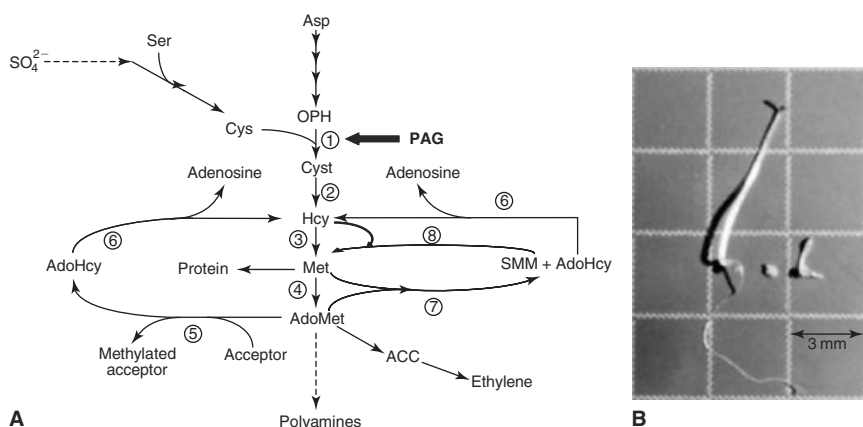
In summary, these facts highlighted the power of proteomics to unravel specific features of complex developmental processes such as germination and to detect protein markers that can be used to characterize seed vigor of commercial seed lots and to develop and monitor priming treatments.

#### *7.2.1.4 Proteomics unravels metabolic control of seed development and germination*

Proteomics of *Arabidopsis* and *M. truncatula* seeds revealed the differential accumulation during germination and development of housekeeping enzymes involved in Met metabolism (Gallardo et al., 2001, 2002b, 2003; Rajjou et al., 2004; Job et al.,

2005). Among the essential amino acids synthesized by plants, Met is a fundamental metabolite because it functions not only as a building block for protein but also as the precursor of AdoMet, the primary methyl-group donor and the precursor of polyamines and the plant-ripening hormone, ethylene (Ravanel et al., 1998, 2004). In plants, Met can be synthesized through two pathways (Figure 7.2). In the *de novo* biosynthetic pathway, *O*-phosphohomoSer is transformed to cystathionine in a reaction catalyzed by cystathionine  $\gamma$ -synthase, then to Hcy in a reaction catalyzed by cystathionine  $\beta$ -lyase, and finally to Met in the presence of the cobalamin-independent Met synthase. In the Met recycling pathway, *S*-methylmethionine (SMM), a compound unique to plants, is synthesized by a methyl transfer from AdoMet to Met, in a reaction catalyzed by AdoMet:Met *S*-methyltransferase. SMM can then be reconverted to Met by transferring a methyl group to Hcy in a reaction catalyzed by SMM:Hcy *S*-methyltransferase. These reactions, together with the reactions catalyzed by AdoMet synthetase and AdoHcy hydrolase, constitute the SMM cycle, which may be the main mechanism in plants for short term control of AdoMet level (Ranocha et al., 2001).

During *Arabidopsis* seed germination, two enzymes in this pathway showed differential expression (Gallardo et al., 2002b). The first corresponded to Met synthase. This protein was present at low level in dry mature seeds, and its level was increased



**Figure 7.2 Methionine metabolism in seed development and germination** (from Gallardo et al., 2002b). **A:** Met metabolism in plants. *Reaction intermediates:* ACC: 1-aminocyclopropane-1-carboxylic acid; AdoHcy: *S*-adenosylhomocysteine; AdoMet: *S*-adenosylmethionine; Cyst: cystathionine; Hcy: homocysteine; OPH: *O*-phosphohomoserine; SMM: *S*-methylmethionine. *Enzymes:* 1: cystathionine  $\gamma$ -synthase; 2: cystathionine  $\beta$ -lyase; 3: cobalamin-independent methionine synthase; 4: AdoMet synthetase; 5: AdoMet-dependent transmethylation; 6: AdoHcy hydrolase; 7: AdoMet: methionine *S*-methyltransferase (MMT); 8: SMM: Hcy *S*-methyltransferase (HMT). DL-propargylglycine (PAG) blocks methionine synthesis by inhibiting cystathionine  $\gamma$ -synthase. **B:** Effect of DL-PAG on seed germination and seedling growth. Morphology of seedlings after 10 days germination on water (Left), 1 mM PAG (Middle) or 1 mM PAG + 10  $\mu$ M Met (Right). The photograph shows a typical result observed with all seeds in each germination conditions. Square size: 3 mm  $\times$  3 mm.

strongly at 1-day imbibition, prior to radicle emergence. Its level was not increased further at 2-day imbibition, coincident with radicle emergence. However, its level in 1-day imbibed seeds strongly decreased upon subsequent drying of the imbibed seeds back to the original water content of the dry mature seeds. The second enzyme corresponded to AdoMet synthetase. In this case, this enzyme was detected in the form of two isozymes with different pI and  $M_r$ . Both proteins were absent in dry mature seeds and in 1-day imbibed seeds but specifically accumulated at the moment of radicle protrusion. As transcriptomics, proteomics can only suggest candidate proteins whose function in development remains to be established. To this end, we analyzed the effect of DL-propargylglycine, a specific inhibitor of Met synthesis on *Arabidopsis* seed germination and seedling growth (Gallardo et al., 2002b). Seed germination was strongly delayed in the presence of this compound. Furthermore, this compound totally inhibited seedling growth. These phenotypic effects were largely alleviated upon methionine supplementation in the germination medium (Figure 7.2).

These results therefore validated the proteomics data and established that Met synthase and AdoMet synthetase are fundamental components controlling metabolism in the transition from a quiescent to a highly active state during seed germination. Moreover, the observed temporal patterns of accumulation of these proteins were consistent with an essential role of endogenous ethylene in *Arabidopsis* only after radicle protrusion (Gallardo et al., 2002b).

Strikingly, many proteins whose abundance varied during *M. truncatula* seed filling also corresponded to enzymes involved in Met biosynthesis (Gallardo et al., 2003). Consistent with the high demand for protein synthesis between 12 and 20 DAP, two spots detected in seed extracts throughout this period corresponded to Met synthase. In addition, one spot corresponded to AdoMet synthetase. Interestingly, the level of AdoMet synthetase fell sharply at the 16-DAP stage and remained low up to desiccation. This result was supported by the observation that ESTs corresponding to AdoMet synthetase were only found in cDNA libraries corresponding to early stages of seed development, and is in keeping with the finding that this enzyme is absent from dry mature *Arabidopsis* seeds (Gallardo et al., 2001, 2002a). Recent studies also disclosed a sharp decrease of Met synthase during soybean seed filling (Hajduch et al., 2005) and of both Met synthase and AdoMet synthetase during wheat endosperm development (Vensel et al., 2005). Therefore, proteomics unraveled a characteristic feature of embryo development across plant species.

In summary, these proteomic data revealed a strong correlation between metabolic activity in embryonic cells and accumulation of key enzymes of Met metabolism. Hence, these data provided the first demonstration of a metabolic control of seed development and germination. This type of control might be a more general feature, as inferred from the observation that relative abundances of metabolic proteins steadily decreased during soybean seed filling, this trend being established for 82 identified proteins associated with metabolism (Hajduch et al., 2005). Several studies also documented that Lys metabolism and catabolism are tightly regulated during seed development (Zhu and Galili, 2003).

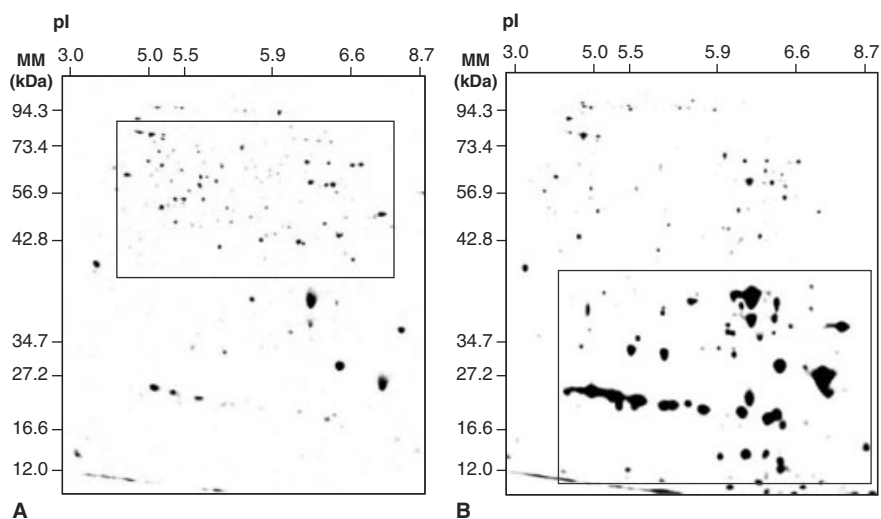
#### 7.2.1.5 The neosynthesized proteome during germination

Functional proteomics aims at detecting proteins whose expression varies in response to a perturbation. As shown above, this is usually achieved by differential 2-DE. However, the amount of a protein spot in a 2-D gel reflects the accumulation level (turnover) of that protein and not simply its rate of synthesis. The use of labeled precursors of protein synthesis is necessary to clarify this question (e.g. see the paper by Chang et al. (2000) which nicely documents this point). It is noted that combined with 2-DE, this radiolabeling technique has been widely used in the past (Cuming and Lane, 1979; Dure et al., 1981), leading to the foundation of major concepts in seed biology, for example, the role during germination of stored and nascent mRNAs in coding developmentally regulated proteins as Em or germin (reviewed by Lane, 1991) or the mechanisms of cellular desiccation and hydration through the discovery of the LEA proteins (Dure et al., 1989). Since mature dry seeds contain mRNAs stored during maturation there are two mechanisms to account for the synthesis of a protein during germination, which depend on the use of stored or nascent mRNA as template. Earlier studies based on the use of metabolic inhibitors (cycloheximide, actinomycin D) had supported the view that protein synthesis occurs on such stored templates during the early stages of seed germination and that *de novo* transcription is not necessary (Dure and Waters, 1965; Waters and Dure, 1966).

To investigate the role of stored and neosynthesized mRNAs in *Arabidopsis* seed germination, the effect of  $\alpha$ -amanitin, a transcriptional inhibitor targeting DNA-dependent RNA polymerase II (de Mercoyrol et al., 1989), was examined on the germination of non-dormant *Arabidopsis* seeds (Rajjou et al., 2004). *Transparent testa* mutants were used of which seed coat is highly permeable (Debeaujon et al., 2000) to better ascertain that the drug can reach the embryo during seed imbibition. Even with the most permeable mutant (*tt2-1*), germination (radicle protrusion) occurred in the absence of transcription, while subsequent seedling growth was blocked. In contrast, germination was abolished in the presence of the translational inhibitor cycloheximide. Taken together, the results highlighted the role of stored proteins and mRNAs for germination in *Arabidopsis* and showed that in this species the potential for germination is largely programmed during the seed maturation process. In the aim of characterizing the *de novo* synthesized proteome during germination, proteomic studies were carried out with this system, in the presence of [ $^{35}\text{S}$ ]Met as a labeled precursor for protein synthesis (Figure 7.3).

The radioactive protein patterns differed remarkably depending on the presence or absence of the transcriptional inhibitor in the germination assay (Figure 7.3). Upon incubation for 24 h on water, seeds synthesized a number of proteins of molecular mass larger than 30 kDa and of pI less than 7.3. Under the same conditions but in the presence of  $\alpha$ -amanitin a large number of these protein spots were no more detectable or were only present at a much lower level than in the water control (rectangle in Figure 7.3A). Proteins for which *de novo* synthesis was repressed by  $\alpha$ -amanitin are involved in reactivation of metabolic activity during germination; for example, mitochondrial enzymes as the mitochondrial processing peptidase and succinate dehydrogenase, Met biosynthesis, triacylglycerol metabolism, and hexose assimilation, a





**Figure 7.3 Influence of  $\alpha$ -amanitin on *de novo* protein synthesis during 24-h germination of the *tt2-1 Arabidopsis* mutant** (from Rajjou et al., 2004). Seeds were incubated for 24 h in the presence of [ $^{35}$ S]Met, in the absence (A) or presence (B) of  $\alpha$ -amanitin. Proteins were extracted, submitted to 2-DE, and the radiolabeled proteins revealed by Phosphorimager analysis. The labeled protein spots (rectangle in (A), proteins that showed reduced accumulation levels in the  $\alpha$ -amanitin-incubated seeds; rectangle in (B), proteins that showed increased accumulation levels in the  $\alpha$ -amanitin-incubated seeds) were identified either by MALDI-TOF analysis, by Edman protein sequencing, or by comparison with *Arabidopsis* seed protein reference maps (Gallardo et al., 2001, 2002a,b; Rajjou et al., 2004; Job et al., 2005; <http://seed.proteome.free.fr>).

finding which provides another indication of the importance of metabolic control of seed germination. Interestingly, a comparison of silver-nitrate-stained gels and radioactive gels revealed a number of spots whose levels apparently remained constant during germination as inferred by quantification from the silver-nitrate-stained gels, although the proteins were labeled with [ $^{35}$ S]Met, thereby indicating the occurrence of protein turnover during germination. This finding therefore reveals the existence of regulatory mechanisms to maintain constant the accumulation levels of some enzymes during the germination process, necessary for proper functioning of metabolic pathways. More generally, these data illustrate the power of combining classical proteomics with dynamic proteomics in the interpretation of protein accumulation patterns. Besides these metabolic enzymes, proteins for which *de novo* synthesis was strongly depressed by  $\alpha$ -amanitin during germination corresponded to translation elongation and initiation factors a finding which is in agreement with the observation that cycloheximide fully inhibited seed germination and seedling establishment in *Arabidopsis*.

Very surprisingly, in the presence of  $\alpha$ -amanitin the seeds massively synthesized proteins of molecular mass smaller than 40 kDa, which were only detected at low

level from the radiolabeled proteome obtained in the absence of the fungal toxin (rectangle in Figure 7.3B). The majority of these proteins corresponded to proteins normally synthesized during seed development, such as 12S globulin subunits and members of the dehydrin family. In other words, it seemed that transcriptional inhibition by  $\alpha$ -amanitin somehow allowed the germinating seeds to recapitulate part of the maturation program, by favoring the use of stored mRNAs encoding maturation proteins. This finding is in keeping with the work of Lopez-Molina et al. (2002) showing that *Arabidopsis* seed germination comprises a short window of time after the start of imbibition during which the seed can still recruit late maturation programs in order to remain osmo-tolerant. This ABA-mediated developmental checkpoint has physiological significance since control of this developmental transition to auxotrophic growth may enable seeds to monitor environmental water status and to mount appropriate adaptive responses (Lopez-Molina et al., 2002). It must be stressed, however, that our data showing an apparent re-induction of the embryonic maturation program during germination were obtained in the presence of  $\alpha$ -amanitin, raising the hypothesis that the fungal toxin repressed the synthesis of factor(s) preventing the use of some of the stored mRNAs during germination (Rajjou et al., 2004). Taken together, these results enlightened the importance of protein synthesis from the pool of stored mRNAs for radicle protrusion. The proteomic data suggested that commitment to seedling growth occurs after radicle protrusion. That stored mRNAs encoding maturation proteins (e.g. cruciferin subunits) can be translated during normal germination is indicated by the data in Figure 7.3 showing that common protein spots are detected in the *novo* protein patterns obtained in the absence or presence of  $\alpha$ -amanitin. This last finding raises an old, yet hitherto unanswered, question (Bewley and Black, 1994) that commitment to germination and plant growth requires transcription of genes allowing the imbibed seed to discriminate between mRNAs to be utilized in germination and those to be destroyed.

#### 7.2.1.6 *Proteomics of the thioredoxin protein-protein interaction in developing and germinating seeds*

The reversible formation of disulfide bonds is important in the regulation of a growing number of enzymes and regulatory proteins such as transcription factors. The thiol-disulfide redox state of these proteins is modulated and maintained by cellular redox agents. Paramount among these is thioredoxin, a 12-kDa protein with a conserved catalytically active disulfide group found in virtually all organisms. The thioredoxin occurring in plant cell cytoplasm (*h*-type) has been found to function in several capacities, including germination and early seedling development (Buchanan and Balmer, 2005).

The role of thioredoxin in wheat starchy endosperm was investigated by proteomics (Wong et al., 2004b). In one approach, thioredoxin targets were isolated from total soluble extracts of endosperm and flour and separated by 2-DE following reduction of the extract by the NADP/thioredoxin system and labeling the newly generated sulfhydryl (SH) groups with monobromobimane (mBBBr). In parallel, an elegant method has been developed based upon trapping covalently interacting proteins on an

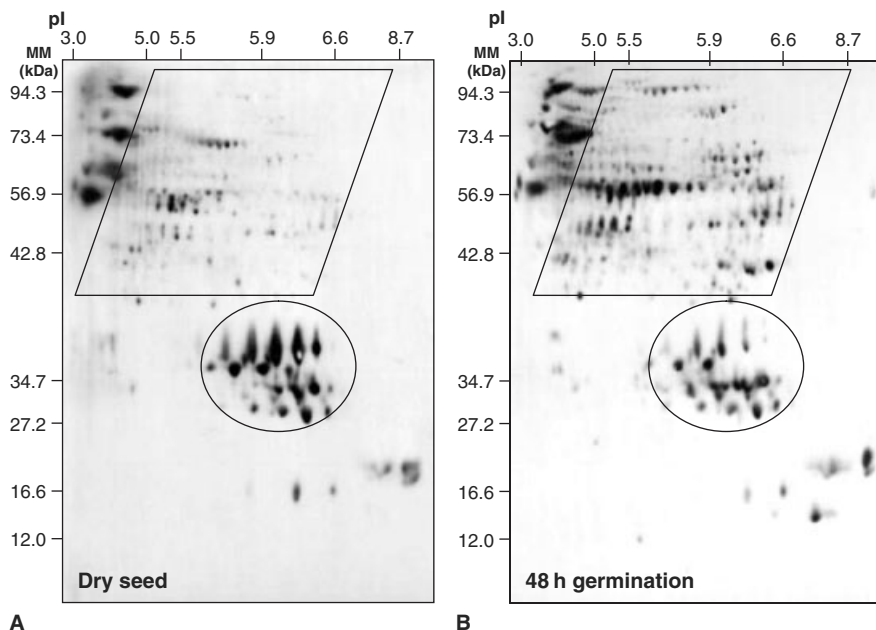
affinity column (Motohashi et al., 2001) prepared with mutant thioredoxin *h* in which one of the active site cysteines was replaced by serine. The two procedures were complementary: of the total targets, one-third was observed with both procedures and one-third was unique to each. Altogether 68 potential targets were identified, for which almost contained conserved cysteines. In addition to confirm known interacting proteins, this study identified 40 potential targets not previously described in seeds. A comparison of the results obtained with young endosperm isolated 10 days after flowering (DAF) to those with mature endosperm, isolated 36 DAF, revealed a unique set of proteins functional in processes characteristics of each developmental stage. Targets of thioredoxin in young endosperm emphasized the importance of biosynthesis (protein synthesis and nitrogen metabolism) and cell division at this stage. Targets of mature endosperm, on the other hand, highlighted functions related to stress, protein storage, and degradation. Indeed, the shift in the processes regulated by thioredoxin reflects the evolution of major biological events characterizing seed development: an emphasis in young endosperm on building the machinery needed for grain filling, growth, cell expansion, and division versus storing and potentially degrading protein in the mature endosperm. The increase in stress- and defense-related proteins in the older tissue could account for the seed's need to survive trash conditions until and when germination commences.

The NADP/thioredoxin system, composed of NADPH, thioredoxin *h* and NADP-thioredoxin reductase (NTR), was also shown to function in the reduction of the major storage proteins of the grain endosperm, gliadins and glutenins in wheat, converting disulfide (S—S) bonds to the reduced (SH) state during the germination process (Kobrehel et al., 1992; Lozano et al., 1996). The soluble proteins (albumins/globulins) and the major insoluble storage proteins (hordeins) of barley endosperm resembled their counterparts in other cereals in undergoing reduction (conversion of S—S to —SH) during germination and early seedling development (Marx and Buchanan, 2003). Recent improvement for identification of thioredoxin targets in mature and germinating barley seeds was achieved by application of a highly sensitive Cy5 maleimide dye and large-format 2-D gels, resulting in a 10-fold increase in the observed number of labeled spots (Maeda et al., 2004). This study confirmed  $\alpha$ -amylase/subtilisin inhibitor, several  $\alpha$ -amylase/trypsin inhibitors and cyclophilin as thioredoxin targets. Furthermore, lipid transfer protein, embryo-specific protein, three chitinase isoenzymes, a single-domain glyoxalase-like protein and superoxide dismutase were novel identifications of putative target proteins, suggesting new physiological roles of thioredoxins in barley seeds, related to seed defense against pathogens and oxidative stress, or protein folding.

In summary, this work revealed new functions for thioredoxin in seeds, and conclusively documented the general occurrence of a sequence of redox changes taking place in cereal endosperm: proteins are synthesized in the reduced state early in seed development and oxidized during maturation and drying. Upon germination, thioredoxin reduces the oxidized proteins, thereby leading to increased solubility (Wong et al., 2004a), proteolysis and, ultimately, nitrogen and carbon mobilization.

### 7.2.1.7 The oxidized (carbonylated) proteome in developing and germinating seeds

Increased cellular levels of ROS are known to occur during seed development and germination (Bailly, 2004), but the consequences in terms of protein oxidation and damage are poorly characterized. Protein carbonylation is a widely used marker of protein oxidation and sensitive methods for its detection have been developed (Levine et al., 1994; Nyström, 2005). It occurs by direct oxidative attack on lysine, arginine, proline, or threonine residues of proteins, thus inhibiting or altering their activities and increasing their susceptibility toward proteolytic attack (Levine et al., 1994; Nyström, 2005). A proteomic investigation showed that protein carbonylation can be detected in dry mature *Arabidopsis* seeds and during the first stages of germination (Job et al., 2005) (Figure 7.4), which was a direct demonstration of the accumulation of ROS in seed development and germination.



**Figure 7.4 The oxidized proteome of *Arabidopsis* seeds** (from Job et al., 2005). Characterization of the carbonylated proteins in dry mature seeds (A) and in germinating (2-d imbibed) seeds (B). Proteins were separated by 2DE. Following transfer to nitrocellulose, the appearance of carbonyl groups in proteins was analyzed by immunodetection of protein-bound 2,4-dinitrophenylhydrazones (DNP) after derivatization with the corresponding hydrazine, as described (Job et al., 2005). Proteins shown in the oval correspond to 12S globulin (cruciferin) subunits. Those shown in the diamond correspond to various albumins, including a large number of glycolytic enzymes, mitochondrial ATP synthase, chloroplastic ribulose biphosphate carboxylase large chain, aldose reductase, methionine synthase, translation factors and several molecular chaperones.

In the dry mature seeds, the legumin-type globulins (12S cruciferins) were the major targets (Figure 7.4A). During imbibition various carbonylated proteins accumulated (Figure 7.4B). This oxidation damage was not evenly distributed among seed proteins but targeted specific proteins as glycolytic enzymes, mitochondrial ATP synthase, chloroplastic ribulose biphosphate carboxylase large chain, aldose reductase, Met synthase, translation factors, and several molecular chaperones. Among the specific targets of protein oxidation detected in *Arabidopsis* seeds (Job et al., 2005), HSP70 chaperones, aconitase, translation initiation and elongation factors, ATP synthase  $\beta$ -subunit, actin, or GAPDHc have also been shown to be highly sensitive to oxidation either in bacterial, yeast, or animal cells, pointing out common features in various living systems. Furthermore, the patterns of protein oxidation observed during *Arabidopsis* seed germination are strikingly reminiscent of those described for *Escherichia coli* or *Saccharomyces cerevisiae* cells exposed to oxidative stress (Tamarit et al., 1998; Cabiscol et al., 2000), lending further support to the contention that oxidative stress normally accompanies seed germination (Bailly, 2004).

Although accumulation of carbonylated proteins is usually considered in the context of aging in a variety of model systems (Berlett and Stadtman, 1997), this was clearly not the case for the *Arabidopsis* seeds since they germinated at a high rate and yielded vigorous plantlets. Thus, the results supported the proposal of Johansson et al. (2004) that the progression of oxidative damage in the life cycle of plants and animals is fundamentally different, and that the observed specific changes in protein carbonylation patterns are probably required for counteracting and/or utilizing the production of ROS caused by recovery of metabolic activity in the germinating seeds (Job et al., 2005). In agreement with a previous proposal (Côme and Corbineau, 1989), the results suggested that blocking glycolysis could be beneficial during conditions of oxidative stress since it would result in an increased flux of glucose equivalents through the pentose phosphate pathway, thus leading to the generation of NADPH. This could provide the reducing power for antioxidant enzymes, including the thioredoxin and GSH/glutaredoxin systems. As mentioned above the NADP/thioredoxin system composed of NADPH, thioredoxin *h* and NTR plays a crucial role in seed germination since it functions in the reduction of the major storage proteins in seeds, converting disulfide S—S bonds to the reduced SH state, thereby leading to increased solubility and mobilization (Wong et al., 2004a). In this context, it is also worth noting that many of the carbonylated proteins detected in *Arabidopsis* seeds (Job et al., 2005) have been identified as thioredoxin targets in wheat seeds (Wong et al., 2004b), lending further support for the existence of a link between ROS and redox regulatory events catalyzed by thioredoxin in seeds (Wong et al., 2004a).

If not correlated to aging, then what might be the role of protein carbonylation in seed physiology? Since carbonylation of proteins increases their susceptibility to proteolytic cleavage, one possibility might be that carbonylation of 12S cruciferin subunits occurring during seed development facilitates their mobilization during germination, which might be advantageous for seedling establishment. Another possibility might be that the 12S cruciferins being the most abundant proteins in the *Arabidopsis* seeds act as scavengers of ROS generated during seed development and

germination, thereby counteracting their deleterious effects. A key question is whether the carbonylation events occurring during germination play a role in seedling establishment or if they are merely the consequence of cells undergoing intense metabolism activity in the awakening from quiescence. In view of the specificity of protein oxidation observed and the fact that the patterns of oxidized proteins closely resemble those of bacterial or yeast cells exposed to oxidative stress, it appears more likely that protein carbonylation does not simply reflect secondary epiphenomena but is used as a means to adapt embryo metabolism to the oxidative conditions encountered during germination. The biochemical basis of such specific carbonylation of proteins remains to be elucidated.

#### 7.2.1.8 Proteomics of developmental mutants in seeds

Comparison of the 2-DE pattern of a mutant with that of the wild-type has often been used to evaluate the effects of a mutation, or to characterize the protein(s) encoded or influenced by the mutated gene. Examples of such proteomic analyses can be found in the reviews by Thiellement et al. (1999, 2002) and Cánovas et al. (2004). Here, we discuss some proteomic studies carried out with seed developmental mutants.

The one concerns *Arabidopsis* mutants deficient in (*aba*) or insensitive to (*abi3*) ABA, and the double mutant (*aba,abi3*) (Meurs et al., 1992). 2-D protein patterns demonstrated that in seeds of the double mutant from 14 to 20 DAP, there was only very low amount of various maturation-specific proteins whereas many proteins similar to those occurring during germination were induced, although no germination was apparent. It appears that in the *aba,abi3* double mutant seed development is not completed and the program for seed germination is initiated prematurely. 2-D protein patterns also demonstrated that exogenous ABA can complete the program for seed maturation. Thus, by providing clear and contrasted protein profiling signatures, this study paved the way for further characterization of important mechanisms occurring in seed development as development of desiccation tolerance or the shift from maturation to germination developmental programs, and that are under ABA control. It will be extremely interesting to revisit these mutants with the modern tools of proteomics toward the characterization of the protein targets of ABA during seed development.

In another study, the role of gibberellins (GAs) in germination of *Arabidopsis* seeds using a GA-deficient *gal* mutant, and wild-type seeds treated with paclobutrazol, a specific GA biosynthesis inhibitor was investigated (Gallardo et al., 2002a). With both systems, radicle protrusion was strictly dependent on exogenous GAs, as previously reported (Koornneef and van der Veen, 1980). The proteome analysis revealed that GAs do not participate in many processes involved in germination *sensu stricto*, that is the initial mobilization of seed protein and lipid reserves. Changes in 46 proteins were detected at this stage in the wild-type and mutant seeds. However, only one protein ( $\alpha$ -2,4 tubulin) was suggested to depend on the action of GA, as it was not detected in the *gal* mutant seeds and accumulated in the mutant seeds incubated in the presence of GAs. In contrast, it was suggested that GAs might

be involved, directly or indirectly, in controlling the abundance of several proteins (two isoforms of AdoMet synthetase and  $\beta$ -glucosidase) associated with radicle protrusion and post-germination processes. In conclusion, this proteomic study established for the first time the developmental stage at which GAs exert their action during germination, namely the radicle protrusion step, and unraveled several protein targets that can account for its action at this step.

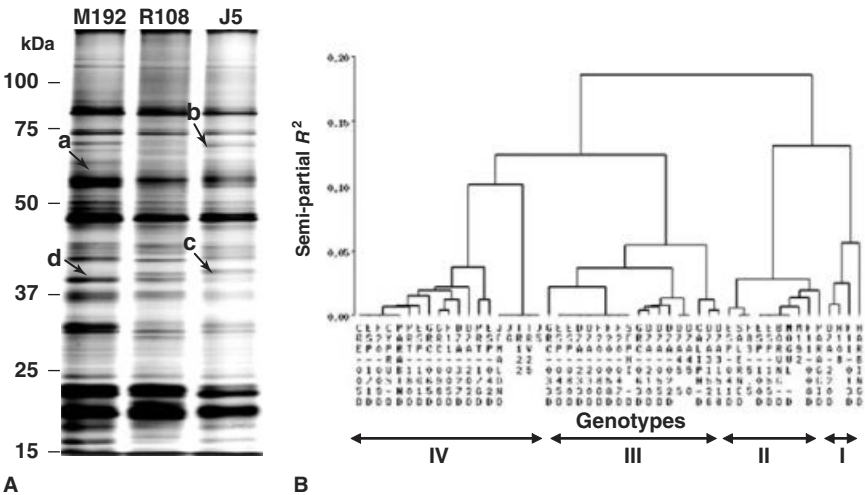
Recently, the role of specific proteases, the seed-type members of the vacuolar processing enzyme (VPE), in seed protein processing during seed filling and maturation has been investigated by proteomics, using knockout mutant alleles of all four members ( $\alpha$ VPE,  $\beta$ VPE,  $\gamma$ VPE, and  $\delta$ VPE) of the VPE gene family in *Arabidopsis* (Gruis et al., 2004). The complete removal of VPE function in the quadruple mutant resulted in a total shift of storage protein accumulation from wild-type processed polypeptides to a finite number of prominent alternatively processed polypeptides cleaved at sites other than the conserved Asn residues targeted by VPE. Although, alternatively proteolyzed legumin-type globulin polypeptides largely accumulated as intrasubunit disulfide-linked polypeptides with apparent molecular masses similar to those of VPE-processed legumin polypeptides, they showed markedly altered solubility and protein assembly characteristics. Instead of forming 11S hexamers (see Figure 7.1), alternatively processed legumin polypeptides were deposited primarily as 9S complexes. However, despite the impact on seed protein processing, plants devoid of all known functional VPE genes appeared unchanged with regard to protein content in mature seeds, relative mobilization rates of protein reserves during germination, and vegetative growth. These findings indicated that VPE-mediated Asn-specific proteolytic processing, and the physiochemical property changes attributed to this specific processing step, are not required for the successful deposition and mobilization of seed storage protein in the protein storage vacuoles of *Arabidopsis* seeds. These results are also in good agreement with the proteomic data of Gallardo et al. (2001) showing that residual precursor forms of 12S cruciferins were mobilized at a high rate during wild-type *Arabidopsis* seed germination (see Section 7.2.1.3).

#### 7.2.1.9 Proteomics of genetic diversity for seed composition

Since the pioneer work of Zivy et al. (1983, 1984), 2-DE and proteomics have been widely used to characterize varieties in several plants (Thiellement et al., 1999, 2002; Chevalier et al., 2004). This is because genetic variations can be evidenced by the position of proteins in 2-D gels, as influenced by sequence polymorphism, and/or by the variation in amount of a given protein in different genotypes (genetically determined quantitative variations). This powerful approach linking plant proteomics and plant genetics can lead to the characterization of PQLs (protein quantity loci) that explain part of the variability in spot intensity (Damerval et al., 1994; de Vienne et al., 1999). Several examples of the use of such an approach can be found in the reviews by Thiellement et al. (1999, 2002). Below we describe a recent proteomic study in this area dealing with genetic diversity for seed protein content and composition in the model legume plant *M. truncatula*. Here, 50 lines of *M. truncatula*, derived from

ecotypes or cultivars of diverse geographical origin, were grown under uniform conditions, and variation in seed protein composition and quantity was investigated (Le Signor et al., 2005). Electrophoretic analyses revealed 46 major seed polypeptides, of which 26 were polymorphic within the collection. The polymorphism for the major seed protein classes (the vicilin/convicilin (7S) and the legumin (11S) type proteins) allowed the clustering of the genotypes into four groups (Figure 7.5).

All lines not belonging to either *M. truncatula* ssp. *truncatula* or ssp. *longispina* were clustered in a single group, demonstrating the value of seed protein profiles in delimiting species boundaries. The Jemalong line group was differentiated early in the dendrogram, and thus represents an ancient clade in the seed diversity of *M. truncatula*. Within-accession variation was investigated for 1-D seed profiles, with additional lines obtained from the same ecotypes. As expected for an autogamous species, within-accession variation was low. Seed protein content was highly variable among the 50 lines examined. Lines contrasting for qualitative traits and seed protein content were identified to allow for the genetic determination of these characters, which will be relevant for legume crop improvement (Le Signor et al., 2005).



**Figure 7.5 Genetic variability for seed protein composition in *M. truncatula*** (from Le Signor et al., 2005). **A:** Examples of globulin polymorphism detected in 1-D protein profiles (silver stained) of dry mature seeds from three *M. truncatula* lines (M192, R108, Jemalong J5). Vicilin-like (7S) and legumin-like (11S) storage proteins were detected after Western-blotting using antibodies raised against pea vicilins and pea legumins, respectively. Arrows indicate four polymorphic bands identified by immunoblotting as vicilin-like (a, b) and legumin-like (c, d) proteins. **B:** Dendrogram of the 1-D profiles of 50 *M. truncatula* lines of identical geographical origin (Ward's minimum variance method). Four groups with distinct protein patterns were identified. The lines M192, R108 and J5, shown in (A), fall into the groups II, I, and IV, respectively.



7.2.1.10 A model of seed development and germination based upon proteomics data

The proteomic data described above can be used to elaborate a model of seed development and germination (Figure 7.6). A first salient feature emerging from these studies is the importance of metabolic control to maintain quiescence in the dry state and conversely to allow awakening from that quiescence during germination. As we have discussed here, this finding seems now well established by studies with different plants (*Arabidopsis*, *M. truncatula*, soybean, and wheat) and with different systems (e.g. somatic and zygotic embryos, whole seeds, organelles). In particular, a well-documented metabolic block concerns the absence of important metabolic enzymes in mature quiescent seeds, such as enzymes involved in Met metabolism. Owing to the central role of this sulfur amino acid in metabolism, a control exerted at the level of an enzyme such as AdoMet synthetase will have an impact on a myriad of processes, not only at the level of metabolism (protein synthesis, methylations) but also in regulation of development mediated by ethylene and polyamines. Contrarily to expectation, the mobilization of seed storage protein reserves cannot support germination and *de novo* Met biosynthesis is required for germination to occur. It would be interesting to investigate whether this requirement is specific to Met or holds more generally for other amino acids.

Another central enzyme involved in metabolic control of quiescence appears to be the mitochondrial processing peptidase. This processing peptidase is a fascinating enzyme that catalyzes the specific cleavage of the diverse pre-sequence peptides from hundreds of the nuclear-encoded mitochondrial precursor proteins that are synthesized in the cytosol and imported into the mitochondrion. Knowing the central

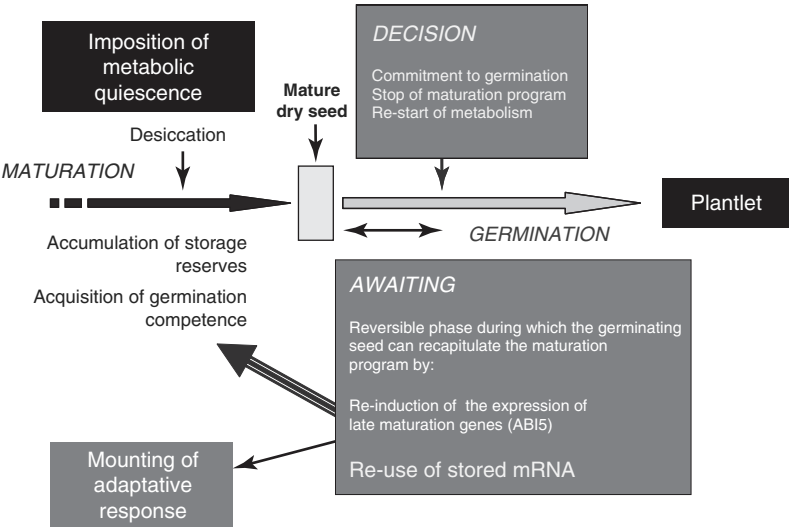


Figure 7.6 A working model of seed development and germination.

role of mitochondria in seed germination and seedling establishment (Bewley and Black, 1994), the possibility of controlling this important enzyme during germination would provide a simple and very efficient means to functionality-control these essential organelles.

A second salient feature put forward by the proteomic data was the general importance of protein modification in seed development and germination. It was somewhat amazing to observe the very large number of proteins that proved to be the specific targets in seeds of thioredoxins and of oxidation through carbonylation. Unexpectedly, the proteomic studies unraveled a new role for protein carbonylation in seed development and germination that has no counterpart in microorganisms and animals. Last, the proteomic data revealed a link between ROS leading to protein carbonylation and redox regulatory events catalyzed by thioredoxin in seeds. The results suggest that a tuning of such protein modifications might exert a dramatic control upon seed development and germination, which will be the object of future research.

Finally, the proteomic data documented the distinct role of stored and nascent mRNA pools in germination and seedling growth. Along with other molecular studies (Lopez-Molina et al., 2002; Li et al., 2005; Nakabayashi et al., 2005), the proteomic studies provided the foundation of a new paradigm in seed germination, in that imbibition of the mature dry seed does not irreversibly commit the seed to the germination program, but rather there exists a possibility to reinduce the maturation program in early phase of seed germination. Presumably, the *PKL* gene, which encodes a CHD3-chromatin-remodeling factor preventing expression of the embryonic development state during *Arabidopsis* germination (Ogas et al., 1999), is involved in such control, suggesting the importance of epigenetic regulation in seed developmental programs. In this context, it is worth noting that a significant portion of the *Arabidopsis* genome shows a co-regulated expression pattern (Ma et al., 2005; Nakabayashi et al., 2005).

As discussed above, and as stressed by Lane (1991), this peculiar ‘reversible’ behavior observed during early germination may serve as a barometric control that buffers a germinating plant embryo against instant loss of desiccation tolerance. For example, in response to the vagaries of nature’s water supply, anhydrobiosis proteins may be reversely lost and restored. Plant cell enlargement through water uptake is of paramount importance in plants because it is an analog of mobility in animals in that it allows higher plants to move through the air or the soil, thereby providing the plant’s photosynthetic machinery with access to light (Lane, 1991).

### 7.2.2 Proteomics of other developmental processes in plants

As mentioned in Section 7.1, most developmental studies in plants based on the proteomic approach concern the establishment of lists of specific proteins in organs and organelles (Thiellement et al., 2002; Hirano et al., 2004; Newton et al., 2004; Agrawal et al., 2005b). By the ‘Functional proteomics’ approach, seed and embryo are the tissues for which the most extensive proteomic studies have been carried out

among all the tissues examined so far in dicots (Agrawal et al., 2005b). There are, however, some studies using this approach in other plant tissues. They are described briefly below.

A flower proteome for all stages of the flower, from buds until petal browning has been established in *M. truncatula* (Watson et al., 2003). 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%).

A proteomic approach was also used to study petal development in rose (*Rosa hybrida*) (Dafny-Yelin et al., 2005). Using 2-DE, stage-specific (closed bud, mature flower, and flower at anthesis) petal protein maps with ca. 1000 unique protein spots were generated. Expression analyses of all resolved protein spots revealed that almost 30% of them were stage-specific, with ca. 90 protein spots for each stage. Most of the proteins exhibited differential expression during petal development, whereas only ca. 6% were constitutively expressed. Eighty-two of the resolved proteins were identified by MS and annotated. Classification of the annotated proteins into functional groups revealed energy, cell rescue, unknown function (including novel sequences), and metabolism to be the largest classes, together comprising ca. 90% of all identified proteins. Interestingly, a large number of stress-related proteins were identified in developing petals.

A proteomics study of senescence, a final stage of development of leaves, has been carried out in clover (*Trifolium repens*). This study allowed the quantitative analysis of 590 spots separated by 2-DE (Wilson et al., 2002). Of these, about 40% (178 spots) were found to be senescence-related, most of them being localized to chloroplast. More recently, Schiltz et al. (2004) analyzed quantitative variations in leaf proteins during nitrogen remobilization related to seed filling in pea (*Pisum sativum*) and identified 130 spots implicated in a variety of cellular functions. Altogether, the data emphasized the importance of proteolysis and chloroplast degradation, and remobilization of nitrogen reserves in senescing leaves of white clover and pea.

Two recent studies concerned proteomic investigations of leaves under drought stress, a major limitation to agricultural production worldwide. The one concerned sugar beet leaves (Hajheidari et al., 2005). Out of more than 500 protein spots separated by 2-DE, 79 showed significant changes (up- and down-regulation) under drought. Twenty protein spots were analyzed by LC-MS/MS, leading to the identification of RuBisCo and 11 other proteins involved in redox regulation, oxidative stress, signal transduction, and chaperone activities. Interestingly, a protein was identified as putative nascent polypeptide-associated complex  $\alpha$ -chain (NAC) whose level showed a significant decrease in response to drought. NAC is associated with ribosomes and binds to emergent polypeptides; it prevents inappropriate interactions of nascent polypeptide chains with other factors (Wiedmann et al., 1994). A decrease in  $\alpha$ -NAC levels may reflect a general repression of translation under drought stress

and/or result in the mistranslation, mistargeting, and proteolysis of proteins, decreasing overall plant performance. It is worth noting that a recent proteomic study demonstrated that the NAC was down-regulated in rice roots submitted to a salt stress in the presence of 150 mM NaCl (Yan et al., 2005). It is proposed that some of these proteins could constitute a physiological advantage under drought, making them potential targets in marker-assisted selection programs.

The other dealt with a proteome analysis of water deficit in maize leaves (Vincent et al., 2005). Remarkably, this study provided 2-D protein patterns from leaf segments evenly sampled along the maize leaf at different stages of development (2 cm-long fragments of the sixth leaf). This allowed separating direct effects of water deficit from side effects related to the decrease of growth. The proteomic data lend to draw a hypothesis on the localization of lignin synthesis in leaves and on the effect of drought on lignification. This study, by pointing out the question of heterogeneity in tissue samples, should be taken as an example for future proteomic research in plant development and encourage the use of well-defined plant samples.

A proteome analysis of plant programmed cell death (PCD) has been carried out in *Arabidopsis* cell culture (Swidzinski et al., 2004). This study allowed identifying 11 proteins potentially associated with PCD, including antioxidant enzymes (catalase, superoxide dismutase), indicating that oxidative stress is associated with PCD, and several mitochondrial proteins, which confirmed the importance of this organelle during PCD. These mitochondrial proteins may be involved in redox signaling that triggers PCD or in the release of mitochondrial pro-apoptotic proteins into the cytosol.

A proteome analysis was used to investigate the changing patterns of protein synthesis during pollen development in anthers from rice plants grown under strictly controlled growth conditions (Kerim et al., 2003). More than 150 protein spots separated by 2-DE showed changing accumulation levels during development (six discrete microspore developmental stages were studied, ranging from the pollen mother cell to mature pollen), out of which 40 representing 33 unique gene products were identified. These proteins included regulators of light-mediated signal transduction in plants, enzymes of carbohydrate metabolism, cell wall, and cytoskeleton-associated proteins. Furthermore, new Open Reading Frames (ORFs) were defined.

In summary, these studies demonstrated the utility of the proteomic approach in addressing a biological system in which there is little prior knowledge to form the basis of more hypothesis-driven studies.

### 7.3 Conclusions and perspectives

In conclusion, we have described the usefulness of proteomics to investigate the molecular mechanisms taking place during plant development with an emphasis on two extremely complex processes of the plant life cycle that are seed formation and germination. In this review we have outlined the combination of quantitative

proteomics with the comprehensive analysis of the *de novo* synthesized, carbonylated, and reduced (thioredoxin targets) proteins, which leads to additional information about rates of synthesis and degradation, modifications, and protein interactions that might exert a dramatic influence upon seed development and germination. It should be emphasized that such characterization of the functional status of hundreds of proteins at specific stages of seed development and germination, as well as during other aspects of plant growth, represent an important part of attempts to uncover the dynamic complexity of these biological systems and ultimately create working models of entire 'systems biology' networks in plants. It should also be underlined that in this emerging field of 'systems biology', the integration of transcriptomics and metabolomics with proteomics data will lead to substantial information about the regulation of protein accumulation and activity, and contribute to an essential part of understanding biological functions. The comparative analysis of transcript, protein and metabolite profiling during different aspects of plant growth, development and physiological processes, such as responses to biotic or abiotic stresses, seems therefore to be of great importance.

An additional important focus in plant proteomics is directed toward the identification of candidate proteins governing complex agronomic traits, such as yield, hybrid vigor, disease resistance, and stress tolerance. In this context, the combination of quantitative trait loci (QTL) analyses for these traits and the identification of loci affecting the quantity (PQL), the position shift (PSL) or the functional state of the proteins detected in 2-DE, should help elucidating the link between the protein spots and the traits (Thiellement et al., 2002). For example, co-location of a PQL with the structural gene encoding the protein detected in 2-D gels and a QTL controlling an important agronomic trait would point to an association between the protein spot itself and the variation observed for the trait. Furthermore, co-location of PQLs with agronomic trait QTLs but not with the corresponding structural genes would result in identifying those proteins in 2-D gels whose genetic factors control their quantity. The use of plants that have their genome sequenced should help identifying those regulatory genes in the QTL/PQL regions. To further validate the role of candidate proteins, methodologies of reverse genetics are very useful. In particular, a method for Targeting-Induced Local Lesions In Genomes, or TILLING, overcomes the limitations of knocking out an entire essential gene and expands knowledge of active protein domains. This method uses chemical mutagenesis to yield an allelic series of point mutations in the genome. High-throughput TILLING facilities have been established for a number of species, such as *Arabidopsis*, *M. truncatula*, rice, pea, and maize, to provide a screening resource for isolating different mutant alleles corresponding to genes of interest with known sequences (Henikoff et al., 2004). Recently, TILLING has been extended to the improvement of crop plants since favorable alleles detected in mutant collections of crop plants can be used directly in selection (Slade and Knauf, 2005). Therefore, proteomics combined with genetics and TILLING shows great promise as a general method for functional study and validation of key proteins related to diverse aspects of plant growth, development, and physiological processes.

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