

Dynamic Proteomics Emphasizes the Importance of Selective mRNA Translation and Protein Turnover during *Arabidopsis* Seed Germination*

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During seed germination, the transition from a quiescent metabolic state in a dry mature seed to a proliferative metabolic state in a vigorous seedling is crucial for plant propagation as well as for optimizing crop yield. This work provides a detailed description of the dynamics of protein synthesis during the time course of germination, demonstrating that mRNA translation is both sequential and selective during this process. The complete inhibition of the germination process in the presence of the translation inhibitor cycloheximide established that mRNA translation is critical for *Arabidopsis* seed germination. However, the dynamics of protein turnover and the selectivity of protein synthesis (mRNA translation) during *Arabidopsis* seed germination have not been addressed yet. Based on our detailed knowledge of the *Arabidopsis* seed proteome, we have deepened our understanding of seed mRNA translation during germination by combining two-dimensional gel-based proteomics with dynamic radiolabeled proteomics using a radiolabeled amino acid precursor, namely [³⁵S]-methionine, in order to highlight *de novo* protein synthesis, stability, and turnover. Our data confirm that during early imbibition, the *Arabidopsis* translato- me keeps reflecting an embryonic maturation program until a certain developmental checkpoint. Furthermore, by dividing the seed germination time lapse into discrete time windows, we highlight precise and specific patterns of protein synthesis. These data refine and deepen our knowledge of the three classical phases of seed germination based on seed water uptake during imbibition and reveal that selective mRNA translation is a key feature of seed germination. Beyond the quantitative con-

trol of translational activity, both the selectivity of mRNA translation and protein turnover appear as specific regulatory systems, critical for timing the molecular events leading to successful germination and seedling establishment. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M113.032227, 252–268, 2014.

Seed germination is a vital stage in the plant life cycle during which embryo cells experience a programmed transition from a quiescent to a highly active metabolic state. Accordingly, seed quality in terms of germination vigor is of paramount importance for both ecological aspects and practical applications, as it influences the level, timing, and uniformity of seedling emergence in a wide range of environmental conditions (1, 2). In efforts to decipher the complex molecular mechanisms that control seed germination, genetic, genomic, and postgenomic analyses have been developed on the model plant *Arabidopsis thaliana* (3–6). Germination is classically described as a sequential time course divided into three major phases of seed water uptake (7). Phase I is characterized by rapid seed imbibition, which is crucial for the transition from the quiescent metabolic state of the dry seed to the high metabolic activity of the hydrated seed. Phase II corresponds to a period during which the seed imbibition level remains constant or slowly increases. Although this step occurs without visible morphological changes of the seed, it is characterized by germination-specific metabolism that prepares the seed for radicle protrusion and seedling growth. Phase II is achieved when the radicle (or a part that extends from the growing embryo) emerges through the seed coat thanks to cell elongation. Both Phase I and Phase II define germination *sensu stricto*, a developmental phase that occurs without cell division (8). Finally, Phase III corresponds to the resumption of water uptake when embryonic cells start to divide for seedling establishment. During the past decade, segregation-based genetic mapping approaches have enabled the characterization of several quantitative trait loci related to *Arabidopsis* seed germination quality (9–13). Also, numerous *Arabidopsis* mutant lines have

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been deeply exploited for the functional analysis of genes involved in seed germination (3–6). In addition, high-throughput functional genomic methods, such as transcriptomics and proteomics, uncovered key gene products involved in the germination process (3–6). Finally, thanks to a large set of transcriptomic data, a network model (SeedNet) describing global transcriptional regulations and interactions occurring in *Arabidopsis* seed germination has been created (14). This network model has proved helpful in efforts to understand how the transition from a quiescent to a germinated seed operates at the transcriptional level, and it also allowed the identification of positive and negative regulators of *Arabidopsis* seed germination.

A high proportion of the genome is already expressed in the mature dry *Arabidopsis* seed; over 10,000 transcripts have been detected (15–19). Proteomic studies disclosed that only protein translation is required for radicle protrusion, indicating that germination-specific proteins are translated from stored mRNAs (20, 21). Thus, these long-lived stored transcripts in mature dry seeds were proposed to play a critical role in seed germination, emphasizing the significance of post-transcriptional and translational controls (16, 20, 22, 23). Furthermore, proteomic approaches underlined the seed proteome's diversity as a result of numerous post-translational protein modifications (24). To date, differential transcriptomic and proteomic experiments have provided an overview of the accumulation patterns of mRNAs and proteins during the germination process, but without distinction between their stored and *de novo* synthesized counterparts. The present study highlights the dynamic regulation of protein translation and turnover during the time course of seed germination. To this end, we employed a combined approach involving two-dimensional gel-based differential proteomics and dynamic radiolabeled proteomics. The integration of these proteomic results with publicly available transcriptomic data (18) provides an original perspective on the contribution of dynamic protein turnover and selective mRNA translation in seed germination.

EXPERIMENTAL PROCEDURES

Plant Material and Germination Experiments—Nondormant seeds of *Arabidopsis thaliana*, accession Landsberg *erecta* (Ler), were used in all experiments. Germination assays were carried out at 25 °C, and seeds imbibed for 48 h under continuous light on three sheets of absorbent paper (Roundfilter paper circles, 45-mm diameter, Schleicher & Schuell, Dassel, Germany) and a black membrane filter with a white grid (ME 25/31, 45-mm diameter, Schleicher & Schuell) wetted with 1.3 ml of distilled water in covered plastic boxes (50-mm diameter).

Radiolabeling of Neosynthesized Proteins—To label neosynthesized proteins *in vivo*, six seed lots (50 mg; approximately 2,800 seeds; three biological replicates) were allowed to imbibe for, respectively, 8 h, 16 h, 24 h, 32 h, 40 h, and 48 h in the presence of [³⁵S]-Met (1.85 MBq; MP Biomedicals SARL, Illkirch, France) for a period of 8 hours after imbibition (HAI)¹ (i.e. from 0 to 8 h, 8 to 16 h, 16 to 24 h,

24 to 32 h, 32 to 40 h, and 40 to 48 h, respectively). Radioactive Met ([³⁵S]-Met) has been used successfully to label neosynthesized proteins in germinating *Arabidopsis* seeds (20, 25, 26) and in other species (e.g. 27–29). Furthermore, there is ample circumstantial evidence that exogenously applied Met can easily penetrate into plant cells; indeed, altered plant phenotypes caused by the inhibition of Met biosynthesis and/or accumulation can be alleviated upon Met supplementation (30–34). Following incubation, total protein extracts were prepared as previously described (35). In order to prevent unspecific protein labeling by free [³⁵S]-Met, seed protein extracts were precipitated by trichloroacetic acid/acetone before two-dimensional electrophoresis separation (35). The amount of protein synthesis was measured from trichloroacetic acid precipitation of aliquots of reaction mixtures spotted on Whatmann GF/C filters; after eight washing steps in cold 5% trichloroacetic acid and 0.04 M sodium pyrophosphate and two washing steps in absolute methanol, filters were dried and counted for radioactivity in a liquid scintillation counter (36). Protein extracts were also submitted to two-dimensional gel electrophoresis as described elsewhere (35).

Detection of Total Seed Proteins—Proteins on the two-dimensional gels were stained by silver nitrate (35). Then, stained two-dimensional gels were dried for 2 days at room temperature in a sandwich composed of, from bottom to top, one sheet of cellophane model Gel Dryer (Bio-Rad), two-dimensional gel, one sheet of Saran Wrap (VWR International SAS, Fontenay-sous-Bois, France), and one sheet of cellophane model Gel Dryer (Bio-Rad). After drying, silver-stained gels were scanned with a Sharp JX-330 scanner (Sharp Electronics, Roissy, France). Image analysis was carried out with ImageMaster 2D Elite version 4.01 software (Amersham Biosciences, Les Ulis, France).

Detection of Neosynthesized Proteins—The upper sheet of cellophane and the Saran Wrap sheet were peeled and exposed for 15 days to storage phosphor screens (GE Healthcare). These screens were submitted for phosphorimager analysis (Molecular Dynamics Storm 840 phosphorimager, Amersham Biosciences). Then, neosynthesized proteins revealed by autoradiography were matched with proteins detected on silver-stained two-dimensional gels according to both the position (molecular weight and pI) and the protein spot shape (supplemental Fig. S1). Proteins of interest were identified through comparison with the reference protein maps for the *Arabidopsis* seed proteome available online <http://www.seed-proteome.com/>. The amount of *de novo* synthesized proteins was calculated based on densitometric analyses of the spots on the autoradiography using ImageMaster 2D Elite version 4.01 software (Amersham Biosciences).

Protein Neosynthesis Clusters and Normalization—For protein neosynthesis clustering, the protein neosynthesis intensity of the different protein isoforms was first summed. Then, the protein neosynthesis amount of the resulting 158 nonredundant proteins was first normalized by their maximum value across the different time lapses. Finally, these protein-neosynthesis normalized intensities were clustered via the K-means method (eight clusters, 10 iterations) based on Pearson correlations using MeV software (37).

Functional Analysis of the Proteome Data—The Munich Information Center for Protein Sequence Functional Catalogue Database of the *Arabidopsis* genome (38) was used to annotate the 202 2DE protein spots characterized in the *de novo* synthesized proteome, as well as

nine; AGI, *Arabidopsis* Gene Index; ENO, enolase; ER, endoplasmic reticulum; FER, ferritin; GABA, γ -aminobutyrate; GLYR, glyoxylate reductase; Ler, Landsberg *erecta*; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDAR, monodehydroascorbate reductase; PBP, PYK10-binding protein; PDI, protein disulfide isomerase; ROS, reactive oxygen species; SSP, seed storage protein; TCA, tricarboxylic acid; THF, tetrahydrofolate; TPP, tripeptidyl peptidase.

¹ The abbreviations used are: HAI, hours after imbibition; 2DE, two-dimensional gel electrophoresis; AdoMet, S-adenosylmethio-

TABLE I
Incorporation of [³⁵S]-Met in neosynthesized proteins during the time course of *Arabidopsis* seed germination

Hours after imbibition (HAI) cpm/μg protein	0–8	8–16	16–24	24–32	32–40	40–48
	48 ± 7	531 ± 43	498 ± 23	322 ± 19	353 ± 25	293 ± 24

Note: After each 8-h time window of [³⁵S]-Met incorporation during seed germination, total proteins were extracted and the amount of [³⁵S]-Met taken into proteins was quantified as counts per minute (cpm) per microgram (μg) of protein.

the 273 2DE protein spots characterized in the total proteome but not in the *de novo* synthesized set.

Heat Maps of Transcript Abundance, Protein Neosynthesis, and Protein Abundance—An Affymetrix ATH1 data set focused on *Arabidopsis* seed physiology between harvest and seedling establishment stages was retrieved (18). Microarray Analysis Suite 5.0 (Affymetrix, Santa Clara, CA) normalization was first performed on raw CEL files to determine present/absent/marginal calls for each probe set (39). Probe sets were considered present if they were detected in at least two replicates before GC-Robust Multiarray Average normalization was performed to obtain normalized intensities (39). From the 20, 24, 18, 20, 15, 10, 27, and 13 unambiguous *Arabidopsis* Gene Indexes (AGIs) of, respectively, Cluster 1 through Cluster 8 (supplemental Table S8), transcriptome data could be found for, respectively, 19, 22, 16, 17, 12, 6, 24, and 12 AGIs in the *Arabidopsis* transcriptome data set (18). For each gene, the corresponding normalized transcript value was divided by its maximum to reach a transcript abundance between 0 and 1. The probe intensities for *GAPC1* (At3g04120) and *ACT7* (At5g09810) in Cluster 8 were averaged. Similarly, the protein neosynthesis intensity and the total protein abundance of the corresponding AGI were also retrieved and normalized by their maximum intensity along the germination time course. Heat maps were built using R (40). All normalized data can be found in supplemental Table S11.

RESULTS

From the Full to the Neosynthesized Proteome—A proteomic analysis of *Arabidopsis* germination was carried out on nondormant seeds from the *Ler* accession displaying a high germination quality. Indeed, T_1 and T_{50} values were achieved at 28 and 32 HAI, respectively (T_1 and T_{50} correspond to the times at which, respectively, 1% and 50% of the seeds had germinated). In addition, the final percentage of germinated seeds reached 100%. *Arabidopsis* seed proteome analyses via 2DE revealed about 1,500 protein spots (2DE protein spots), of which 475 were identified via LC-MS/MS (supplemental Table S1 (41)). Because of mRNA processing, protein proteolysis, and chemical protein modification, one gene can produce many different protein species, resulting in wide proteome diversity (24). From one polypeptidic sequence corresponding to a single AGI, many different 2DE protein spots (protein isoforms) were visualized. Indeed, each protein isoform can display distinct electrophoretic properties as a result of variations in their molecular weight and/or isoelectric point (pI). These 475 2DE protein spots corresponded to 257 nonredundant proteins (241 unique AGIs and 16 multiple hits).

During the time course of seed germination, differential proteomics using silver-stained 2DE gels pointed out 241 and 220 2DE protein spots that were differentially accumulated (up or down) from 0 to 24 HAI (germination *sensu stricto*) and from 24 to 48 HAI (radicle protrusion), respectively (supple-

mental Table S2). Still, 147 2DE protein spots did not display abundance variations from dry seed up to radicle protrusion (supplemental Table S2). To our knowledge, no data are available regarding the turnover of these proteins. Thus, pulses of [³⁵S]-Met were applied within six different 8-h time windows during the time course of seed germination (see “Experimental Procedures”). After each period of labeled precursor incorporation into newly synthesized proteins, total proteins were extracted and the amount of [³⁵S]-Met incorporated into proteins was quantified (Table I). For each time window, the total proteins were then separated via 2DE and revealed by silver-staining, and the pattern of neosynthesized proteins was revealed by means of autoradiography (Fig. 1). We highlighted a *de novo* synthesis pattern for 202 2DE protein spots detected as radiolabeled during the germination process (Fig. 2A, supplemental Table S3). These 2DE protein spots contained some isoforms and corresponded to 158 nonredundant proteins (Fig. 2A, supplemental Table S3). It follows that out of the 475 2DE protein spots from the total proteome, 273 2DE protein spots were not detected as radiolabeled. Thus, the proteins corresponding to these 273 2DE protein spots can be considered as not *de novo* synthesized during germination (Fig. 2A, supplemental Table S4). Interestingly, among these 2DE protein spots, 123 were up-accumulated in the full proteome during seed germination (Fig. 2A, supplemental Table S5), a finding that emphasizes the importance of post-translational regulation of seed proteins in the germination process. Furthermore, 81 2DE protein spots showed neither *de novo* protein synthesis nor differential accumulation during *Arabidopsis* germination (Fig. 2A, supplemental Table S6). This suggests that the corresponding proteins had high stability and hence low turnover. Both the neosynthesized and the non-neosynthesized proteome appear to be complementary to ensure seed vigor.

The frequency distribution of functional categories according to FunCat classification (38) was assessed for the 202 2DE protein spots that were radiolabeled and for the 273 2DE protein spots that were not radiolabeled (Fig. 2B, supplemental Table S7) during *Arabidopsis* seed germination. These 202 and 273 2DE protein spots corresponded to 158 and 140 nonredundant proteins, respectively (Fig. 2A). Among these nonredundant proteins, 39 were found in both *de novo* synthesized and non-*de novo* synthesized protein spots, and thus 101 proteins were never detected as *de novo* synthesized during seed germination (Fig. 2A). The frequency distribution of functional categories according to FunCat classification (38) was also assessed for the 158 nonredundant *de*

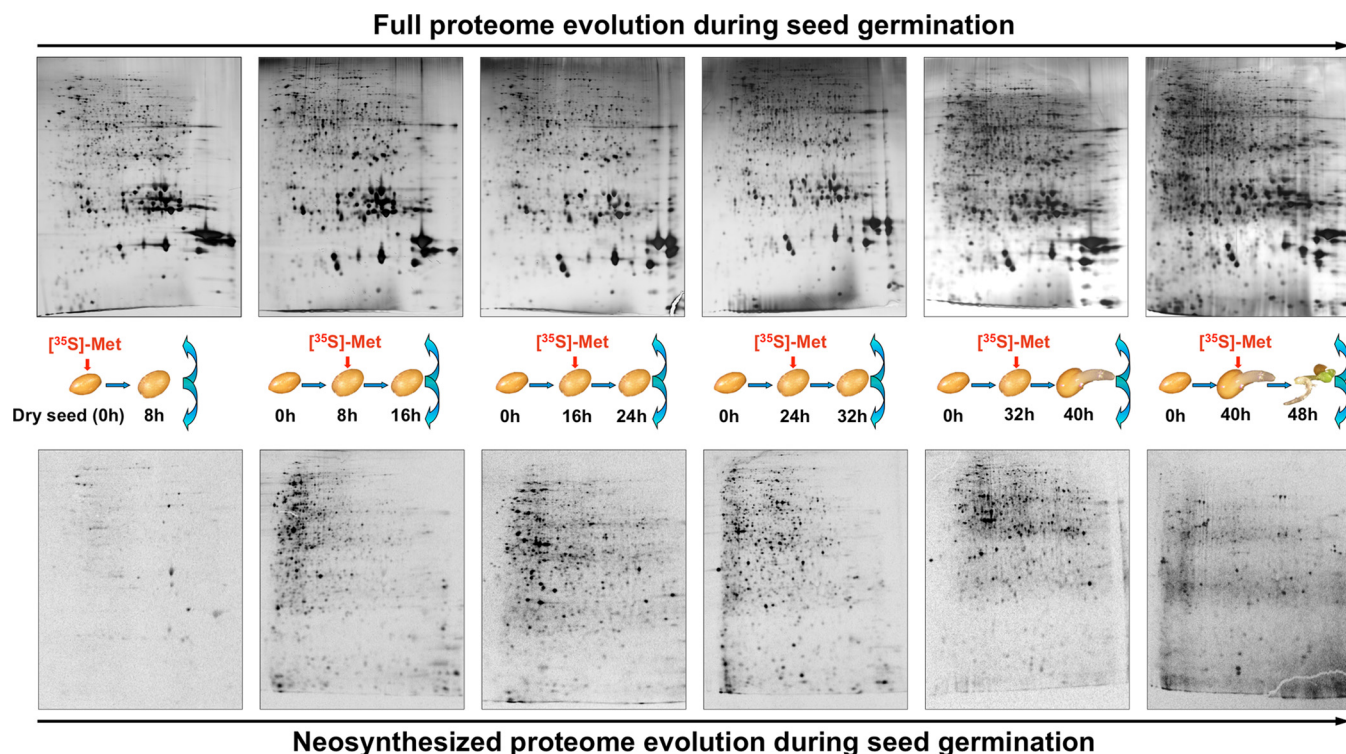


FIG. 1. 2DE patterns of total seed proteome (top row) compared with *de novo* synthesized proteome (bottom row) during *Arabidopsis* seed germination. Total proteins were separated via 2DE, and the patterns of the neosynthesized proteome were revealed by autoradiography of [^{35}S]-Met pulse-chase time windows (0–8 HAI, 8–16 HAI, 16–24 HAI, 24–32 HAI, 32–40 HAI, and 40–48 HAI) during the time course of seed germination.

de novo synthesized proteins and the 101 nonredundant proteins detected as not *de novo* synthesized (Fig. 2C, [supplemental Table S7](#)). Most of these proteins were distributed among the six major following categories: “Cell Rescue, Defense and Virulence,” “Energy,” “Metabolism,” “Protein Fate,” “Protein Synthesis,” and “Storage Protein” (Fig. 2B). As expected, the “Storage Protein” category was more represented in the non-neosynthesized proteome than in the neosynthesized proteome of germinating seeds (Fig. 2B). The FunCat classification of the 202 radiolabeled protein spots revealed enrichment of the “Energy,” “Metabolism,” “Protein Fate,” “Protein Synthesis,” and “Storage Protein” categories while the “Energy” and “Storage Protein” categories were overrepresented for the 273 nonradiolabeled protein spots (Fig. 2B, [supplemental Table S7](#)). In the “Metabolism” category, the presence of several *de novo* synthesized proteins involved in sulfur amino acid metabolism (e.g. cysteine and methionine), which is crucial for seed germination, was evidenced ([supplemental Table S3](#) (6, 34)).

De Novo Protein Synthesis Clustering—Stored mRNAs in dry seeds are accumulated during seed maturation, and some of them are required for germination completion. The application of cycloheximide, a known inhibitor of mRNA translation ([supplemental Fig. S2](#)), blocks seed germination (20). Thanks to the incorporation of [^{35}S]-Met into newly synthesized proteins, the protein synthesis rate could be measured

for the 158 nonredundant proteins. A K-means clustering analysis led to the identification of eight distinct kinetic patterns (Cluster 1 through Cluster 8) of *de novo* synthesized proteins (Fig. 3, [supplemental Table S8](#)).

Comparison of Transcriptome and Proteome Data from *Arabidopsis* Seed Germination—From the 147 unique AGIs, an analysis of mRNA abundances, protein neosynthesis rates, and protein abundances was carried out by comparing available transcriptome data (18) with the present proteomic results (Fig. 4, [supplemental Table S11](#)). In most cases, the heat map representation illustrated that the protein synthesis appeared to be independent of the RNA abundance, but the correlation with the protein abundance was higher.

DISCUSSION

The present results shed new light on the mechanisms involved in the three classical phases of germination based upon water uptake by imbibing seeds (Fig. 5 (7)). The comparison of mRNA level, translational activity, and protein abundance emphasized that selective mRNA translation is a major regulatory mechanism of seed germination (Figs. 3 and 4). This process is both sequential and selective. Thus, it is tempting to speculate that a large set of seed-specific genes is regulated at the level of translation. In particular, these new data on protein turnover during *Arabidopsis* seed germination revealed contrasting protein behaviors ([supplemental Tables](#)

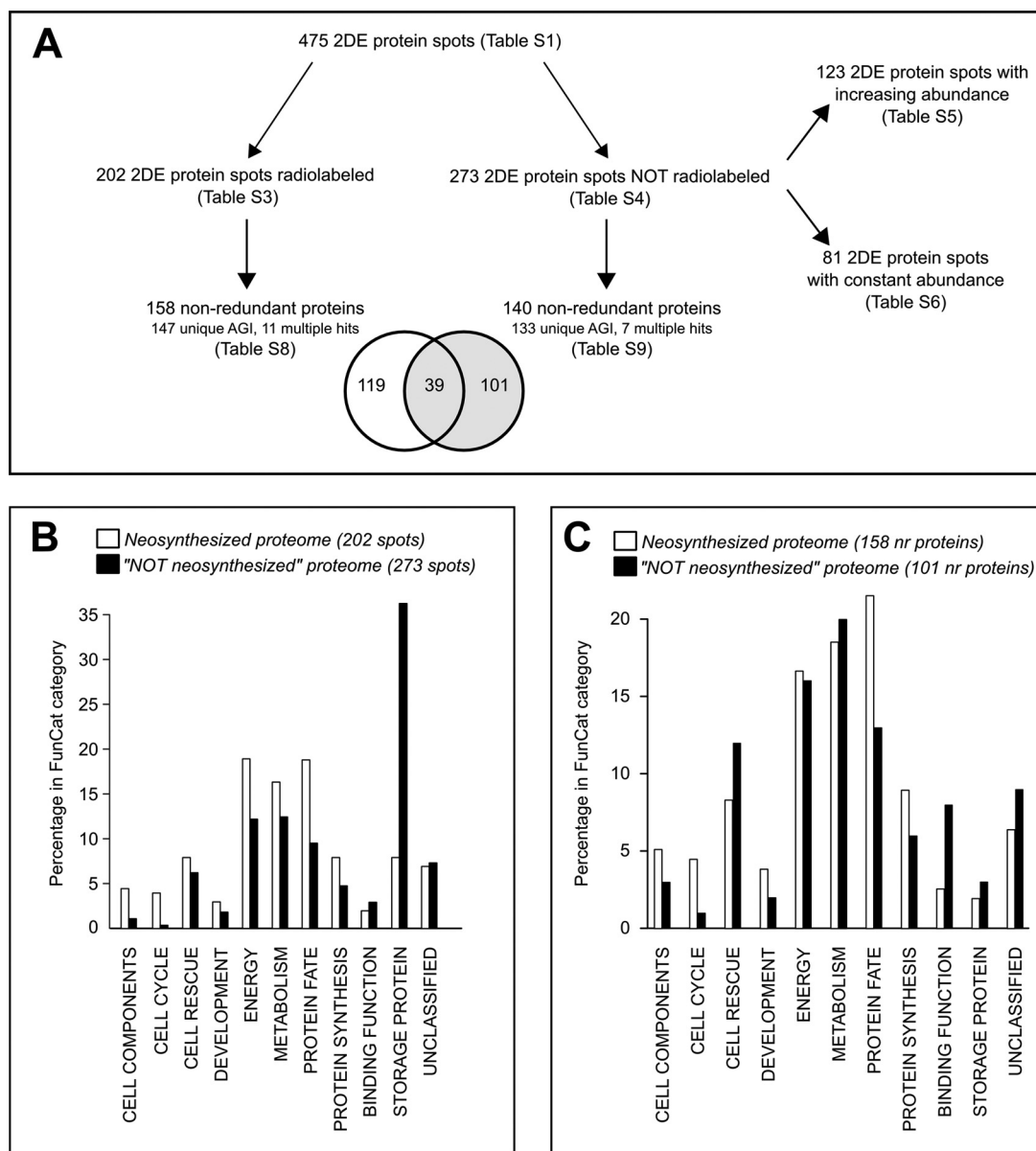


FIG. 2. Functional analysis of the proteins neosynthesized or not neosynthesized in *Arabidopsis* germinating seeds. The analysis workflow applied to the present proteomic data is shown in A. The proteins corresponding to the 202 2DE radiolabeled protein spots and the 273 2DE protein spots detected as not radiolabeled in *Arabidopsis* germinating seeds were classified following FunCat classification (B). The same classification was then applied to the 158 and 101 nonredundant proteins neosynthesized or not during seed germination (C).

S3–S6). This temporal profiling highlights that *Arabidopsis* seed germination consists of a series of sequential events that overlap the three canonical phases of seed germination (7). In the following, we discuss the observed eight different mRNA translation patterns in detail.

Very Early Neosynthesized Proteins—Among the proteins synthesized in the 0–8 HAI time window, several proteins involved in desiccation tolerance were revealed, such as late embryogenesis-abundant proteins (At3g51810, At3g17520, At2g42560) and the RAB18 dehydrin (RAB18, At5g66400), all of which could help to maintain desiccation tolerance in seeds up to radicle protrusion (42–45). Late embryogenesis-abun-

dant proteins are known to accumulate during seed maturation (acquisition of desiccation tolerance) and protect biomolecules (e.g. inner mitochondrial membrane) from dehydration-induced damages.

Seed Storage Proteins—Unexpectedly, several protein spots corresponding to seed storage proteins (SSPs) displayed a similar neosynthesis pattern. SSPs are proteins accumulated mainly during seed maturation and are typically described as a nitrogen and amino acid source for seed germination and seedling establishment. There are three 12S cruciferin annotated genes (*CRA1*, At5g44120; *CRB*, At1g03880; *CRC*, At4g28520) in the *Arabidopsis* Col-0 genome (46). Pre-

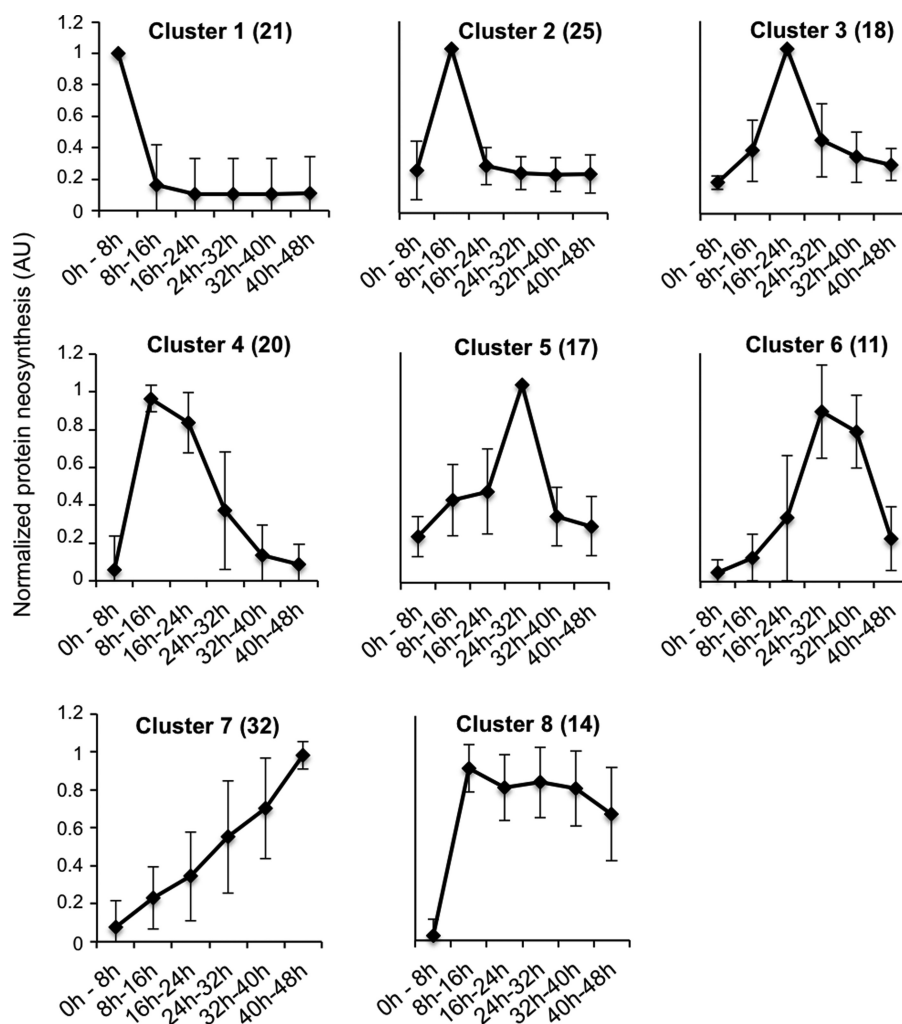


FIG. 3. K-means clustering of the neosynthesized proteins. Protein radiolabeled intensities corresponding to the same protein were summed when multiple protein isoforms were detected. This allowed us to obtain 158 nonredundant protein radiolabeled intensities. Then, for each protein, the protein neosynthesis intensity was normalized by its maximum value over the whole experiment to get a value between 0 and 1. Finally, a K-means clustering (eight clusters, 10 iterations) based on Pearson correlation was performed on the 158 normalized protein intensities using MEV software (37). The numbers in parentheses indicate the number of nonredundant neosynthesized proteins classified in each cluster.

viously, it was shown that the mRNAs corresponding to the three 12S cruciferin genes in *Arabidopsis* are the most abundant in dry mature seeds (15, 16). It is worth noting that the *Ler* genome contains another copy of the *CRA1* gene, called *CRA2*, which represents a tandem duplication (47). The present proteomic data did not allow us to differentiate *CRA1* from *CRA2*, and therefore, in this study, the *CRA1* and *CRA2* protein data are grouped under the same AGI, namely, At5g44120. Interestingly, these four cruciferin genes generate more than 100 protein isoforms because of the high sensitivity of SSPs to post-translational modifications (24). During seed maturation, all four 12S cruciferin genes code for SSPs in the form of ~50-kDa precursors. These precursor forms are then enzymatically processed into acidic α -subunits (~30 kDa) and basic β -subunits (~20 kDa) that remain linked together by a single interchain disulfide bond. The synthesis of the precursor forms encoded by the *CRC* (At4g28520) and *CRB* (At1g03880) genes has been detected only in the very early steps of seed germination (0–8 HAI), whereas the precursor forms encoded by the *CRA1/CRA2* (At5g44120) gene are synthesized up to 16 HAI (supplemental Table S3). Radiola-

belong of the acidic α -subunits and the basic β -subunits was also observed for all these genes up to 16 HAI (supplemental Table S3). This result highlights that the vacuolar-processing enzyme involved in post-translational processing of the precursor cruciferin forms into the corresponding acid and basic 12S cruciferin subunits is functional during the very early steps of seed germination. Vacuolar-processing enzyme is essential for the correct protein storage vacuole structure and compartmentalization of SSPs, and a seedling growth delay was reported in rice *vpe* mutants (48, 49). This indicates that SSP processing can affect early seedling development, presumably because such processing is required for the rapid use of these proteins as a source of nitrogen and amino acids. Moreover, SSP functions might not be limited to supplying the embryo with the nutrients required for germination. For example, the abundant 12S cruciferins were shown to display very high sensitivity to reactive oxygen species (ROS) (25, 26, 50) and could therefore represent a major and predominant ROS scavenger in seeds, an organ known to be continuously exposed to oxidative stress during desiccation, dormancy breaking, and germination (51). Thus, the rapid synthesis of

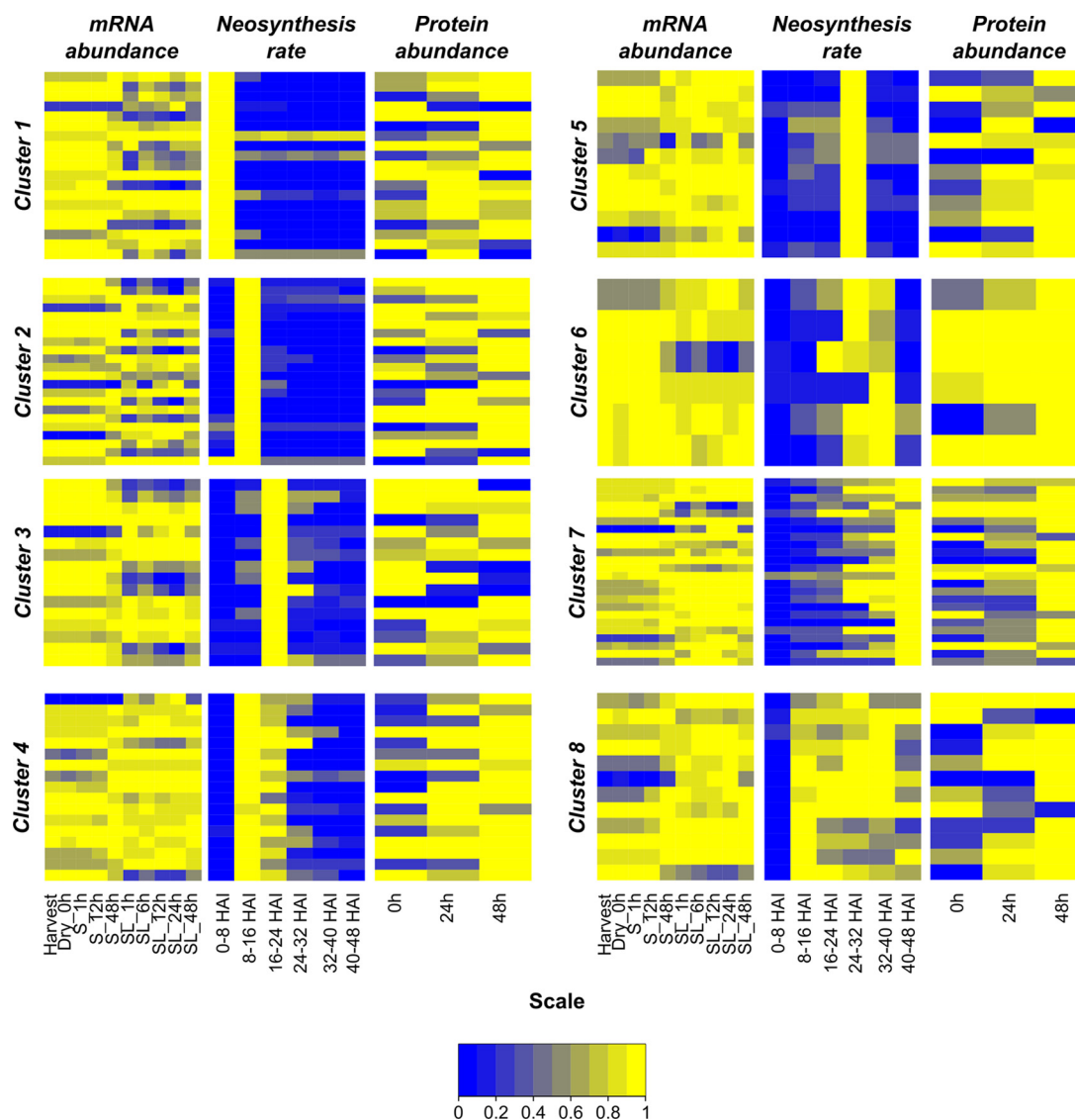


FIG. 4. **Comparison of transcriptome and proteome data during *Arabidopsis* seed germination.** From the 20, 24, 18, 20, 15, 10, 27, and 13 unambiguous AGIs of Cluster 1 to Cluster 8, respectively (supplemental Table S8), transcriptome data could be found for, respectively, 19, 22, 16, 17, 12, 6, 24, and 12 AGIs in a previously published data set (18). The raw Affymetrix CEL data were normalized using the GC-Robust Multiarray Average algorithm (39). For each gene, the corresponding normalized transcript value was divided by its maximum to obtain a transcript abundance between 0 and 1. The probe intensities for *GAPC1* (At3g04120) and *ACT7* (At5g09810) in Cluster 8 were averaged. Similarly, the protein neosynthesis intensity and the total protein abundance of the corresponding AGI were also retrieved and normalized to their maximum intensity along the germination time course. Heat maps were built using R (40). S: seeds stratified during the indicated number of hours; SL: seeds stratified and then germinated under continuous light for the indicated number of hours.

SSPs upon imbibition could help the seed reconstitute protein reserves and cope with strong oxidative stress during natural imbibition/drying cycles in the soil.

Remodeling/Repair Mechanisms and Control of Water Uptake—Cluster 1 brings out proteins mostly neosynthesized during the first 8 HAI in the very early steps of seed germination (supplemental Table S8). Strikingly, most of these proteins are known to be associated with the seed maturation program. Our results are in accordance with those showing that *Arabidopsis* seed germination takes place in a short window of time after the start of imbibition during which the

seed can still recruit its late maturation program (52). Such proteins are presumably translated from stored mRNAs (22), as this neosynthesized proteome is reminiscent of the reinduction of the maturation program as previously described in hydrated seeds in the presence of the transcriptional inhibitor α -amanitin (20). Notably, a peptidyl-prolyl cis-trans isomerase (cyclophilin family) named ROC1 (At4g38740) was specifically neosynthesized between 0 and 8 HAI, and its protein level subsequently remained abundant in the hydrated seeds up to radicle protrusion (supplemental Tables S1 and S7). Peptidyl-prolyl cis-trans isomerases catalyze the conversion of pepti-

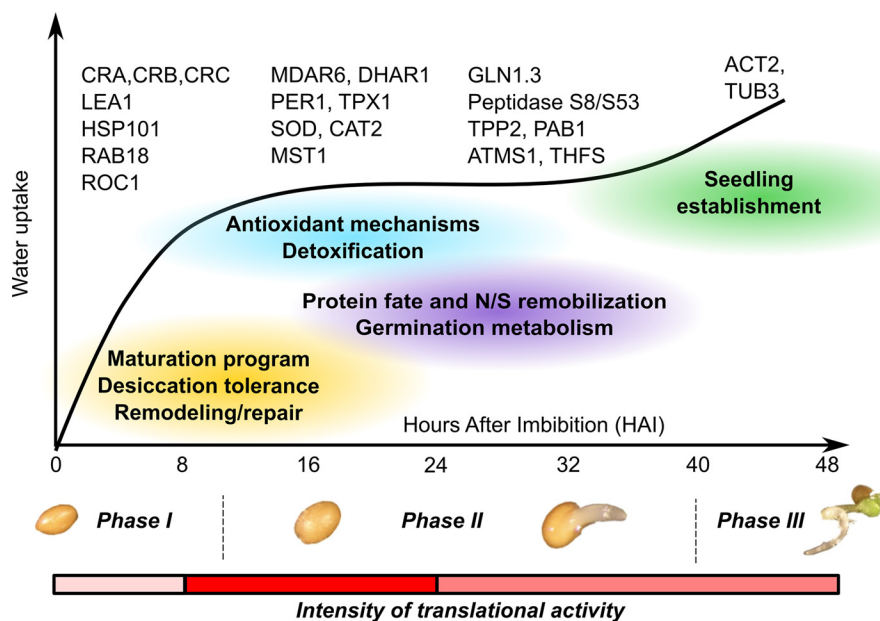


FIG. 5. Metabolic transitions evidenced from *Arabidopsis de novo* synthesized proteins during the three canonical phases of seed germination. During Phase I, the hydrated seed translates stored mRNAs and restarts the late seed developmental program as exemplified by the synthesis of seed storage proteins (e.g. cruciferin (CRA)) and late embryogenesis abundant proteins (e.g. LEA1). The transition from Phase I to Phase II is characterized by the action of important remodeling/repair (e.g. rotamase cyclophilin (ROC1)), antioxidant (e.g. monodehydroascorbate reductase (MDAR6)), and detoxification mechanisms (e.g. mercaptopyruvate sulfurtransferase 1 (MST1)). During Phase II, seed storage and other proteins are degraded by the combined action of the proteasome (20S proteasome subunit PAB1) and peptidases (peptidase S8/S53, tripeptidyl peptidase II (TPPII)) that fuel amino-acid-incorporating metabolism (glutamine synthetase 1.3 (GLN1.3)). Immediately preceding Phase III, seedling establishment is prepared thanks to the mRNA translation cytoskeleton components (Actin2 (ACT2), Tubulin 3 (TUB3)). The vertical dashed lines distinguish the three phases of *Arabidopsis* seed germination based on water uptake kinetics. The red bar represents the mRNA translational activity based on radiolabeled Met incorporation (Table I).

dyl-prolyl imide bonds in peptide and protein substrates. The *Arabidopsis* genome encodes at least five cytosolic cyclophilin family proteins (53). In plants, protein remodeling by proline cis-trans isomerization via ROC1 is emerging as a critical step in signaling cascades, given its role in stem elongation (54), seedling de-etiolation (55), and pollen germination (56). The present results suggest that ROC1 is involved in *Arabidopsis* seed germination.

Moreover, it is noteworthy that the subtilisin-like serine protease SBT1.7 (At5g67360), which is involved in the regulation of pectin methylesterification and therefore mucilage release upon imbibition (57), was also neosynthesized preferentially in the 0–8 HAI time window (supplemental Table S8). Mutants affected in mucilage production exhibit poor germination vigor when water availability is low (58). During the very first hours of seed imbibition, the released mucilage helps to control the speed of water uptake. Recent studies assumed involvement of the seed mucilage in assisting embryonic cells to maintain DNA integrity (59, 60). This supports the assumption that this mucilage contributes to seed environmental adaptation and plays an important role in successful germination.

Neosynthesized Proteins Associated with a Specific Germination Program—Clusters 2, 3, and 5 displayed proteins with a maximum of synthesis at, respectively, 8–16 HAI, 16–24 HAI, and 24–32 HAI, whereas Cluster 4 contained proteins

highly neosynthesized from 8 to 24 HAI (supplemental Table S8). The period from 8 to 32 HAI corresponds to Phase II of seed germination, which leads to increased metabolic activity up to radicle protrusion.

Initiation of the Germination Program by Phosphoenolpyruvate/Oxaloacetate-related Metabolism—The early germination phase is defined as the sum of events occurring before testa rupture (61). Aerobic respiration is resumed rapidly upon imbibition and becomes the main energy source of the germinating seed. In the absence of carbon fixation via photosynthesis, the degradation of molecules for energy production (e.g. glycolysis, glyoxylate cycle, tricarboxylic acid (TCA) cycle) is presumed to be a key determinant of germination vigor. Accordingly, several enzymes involved in these energetic pathways were found neosynthesized during the early hours of the germination process. For instance, a peroxisomal 3-ketoacyl-CoA (PKT3/KAT2, At2g33150) involved in fatty acid β -oxidation was among the first proteins neosynthesized (Cluster 1). It has been reported that this protein positively regulates signaling via the phytohormone abscisic acid (62). In addition, *de novo* synthesis of key enzymes of the glyoxylate cycle, such as isocitrate lyase (At3g21720), aconitase 3 (At2g05710), and malate synthase (At5g03860), were pointed out from the early germination phase (Clusters 7 and 8), supporting early activation of lipid reserve remobilization upon

seed imbibition. The concomitant neosynthesis of cytosolic malate dehydrogenase 1 (At1g04410, Cluster 8) suggests a possible conversion of the malate generated from both the glyoxylate and TCA cycles into oxaloacetate in the cytosol (Fig. 6). The generated oxaloacetate could then fuel amino acid synthesis pathways via aspartate aminotransferase activity (ASP5, At4g31990, Cluster 3; ASP2, At5g19550, Cluster 4) or be converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase 1 (At4g37870), preferentially *de novo* synthesized between 0 and 8 HAI (Cluster 1). Indeed, phosphoenolpyruvate carboxykinase 1 was previously associated with the ability to mobilize storage lipids and proteins (63). The reversible nature of phosphoenolpyruvate carboxykinase 1 activity places this enzyme as a central element for the metabolic adaptation from germinating seeds to seedling establishment (64). Accordingly, *de novo* synthesis was observed for many enzymes involved in neoglucogenesis and/or glycolysis within the first 16 HAI (mainly in Clusters 2, 4, and 8; Fig. 6). For instance, two enolases (ENO1, At1g74030; ENO2, At2g36530) were present in Cluster 2 (Fig. 6, [supplemental Table S8](#)). These enzymes catalyze the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate in the glycolytic/neoglucogenic pathway. In developing *Arabidopsis* embryos, ENO1 and ENO2 are, respectively, responsible for most of the plastid and cytosolic enolase activity (65). The plastid-localized ENO1 has been identified and functionally characterized in *Arabidopsis* (66). The strong accumulation of fructose-6-P and glucose-6-P during the early steps of germination supports neoglucogenesis activity (67). Nonetheless, glycolysis is also presumed to occur, especially within plastids (23). Overall, the respective flux through these metabolic pathways seems to depend on the tissue and cell compartment considered but could also change along the time course of seed germination. Thus, several enzymes involved in reversible reactions of glycolysis/neoglucogenesis might be involved in the metabolic balance controlling germination vigor.

Some of these enzymes also correspond to multifunctional proteins. For instance, ENO2 was described as a bifunctional enolase localized to the cytoplasm and to the nucleus. Indeed, the ENO2 protein sequence contains both DNA-binding and repression domains (68). It has been shown that *Arabidopsis* ENO2 can bind to the promoter of *STZ/ZAT10* (At1g27730), a gene encoding a transcription factor with two Cys(2)/His(2)-type zinc-finger motifs that act as transcriptional repressors for oxidative stress responses, jasmonic acid biosynthesis, and cold responsive genes (68–71). ENO2 has a repressor role in *STZ/ZAT10* expression, and transcriptome data show that the *STZ/ZAT10* transcript is preferentially expressed at the end of seed maturation (*Arabidopsis* eFP Browser (72)). Furthermore, the *STZ/ZAT10* transcript is strongly accumulated (9-fold) in 5-day-old wild-type seedlings treated with H₂O₂ and in seedlings subjected to drought stress (69, 73). Given all of its roles, the *STZ/ZAT10* gene would be part of a late maturation program dedicated to seed

drought resistance. Then, upon imbibition, the ENO2 protein that is preferentially neosynthesized between 8 and 16 HAI would repress *STZ/ZAT10* expression to encourage the seed to exit its maturation program (loss of desiccation tolerance) and commit to seedling establishment.

Antioxidant Mechanisms and Detoxification—Five proteins related to ROS homeostasis—iron/manganese superoxide dismutase (At3g56350), 1-cysteine peroxiredoxin (At1g48130), catalase 2 (At4g35090), thioredoxin-dependent peroxidase 1 (At1g65980), and ferritin 2 (FER2, At3g11050)—are represented in the neosynthesized proteome. Successful seed germination has been suggested to occur within a certain oxidative window where ROS act as signaling molecules without deleterious consequences for biomolecules (51). Three proteins involved in ROS detoxification (iron/manganese superoxide dismutase, peroxiredoxin, and thioredoxin-dependent peroxidase 1) and one involved in free iron(II) sequestration (FER2) display maximum neosynthesis between 16 and 24 HAI (Cluster 3; [supplemental Table S8](#)). The present results indicate that the synthesis of ROS detoxifying enzymes occurs from 16 HAI in nondormant *Arabidopsis* seeds. Therefore, the above-mentioned oxidative window (51) would take place in the very early steps of *Arabidopsis* germination (before 16 HAI). Finally, the catalase 2 protein showed maximum neosynthesis between 24 and 32 HAI (Cluster 5; [supplemental Table S8](#)) in accordance with the significant production of H₂O₂ at 24 HAI in nondormant *Arabidopsis* seeds (50, 74). Free iron sequestration is also important in controlling ROS production caused by the Fenton reaction. Previously, FER2 was found to be the main ferritin isoform accumulated in dry seeds (75). In hydrated nondormant seeds, FER2 is translated not in the very early steps of seed germination but rather between 16 and 32 HAI, suggesting that this protein is involved in germination before radicle protrusion ([supplemental Table S3](#)). Interestingly, during germination, *fer2* mutant seeds are sensitive to oxidative stress (76). It is important to underline that the FER1 (At5g01600) protein is accumulated in the early steps of seed development but not in dry seeds, as revealed by Western blot (76). However, we can detect the FER1 protein in the dry seed proteome with LC-MS/MS ([supplemental Table S1](#)). Remarkably, both FER1 and FER2 protein abundances strongly decreased during *Arabidopsis* germination to nearly undetectable levels ([supplemental Table S1](#)). Therefore, FER1 and FER2 are probably involved in both free iron and ROS homeostasis during seed germination. Interestingly, ferritins were described as classic examples of translationally regulated genes (77).

Ascorbate (vitamin C) is important for oxidative responses in both plants and animals. Yet the ascorbate pool appears to be relatively low in dry seeds (78). An intermediate during the oxidation process is a short-lived radical, monodehydroascorbate, which can be further enzymatically converted to ascorbate by monodehydroascorbate reductase (MDAR). The *Arabidopsis* genome contains five genes encoding

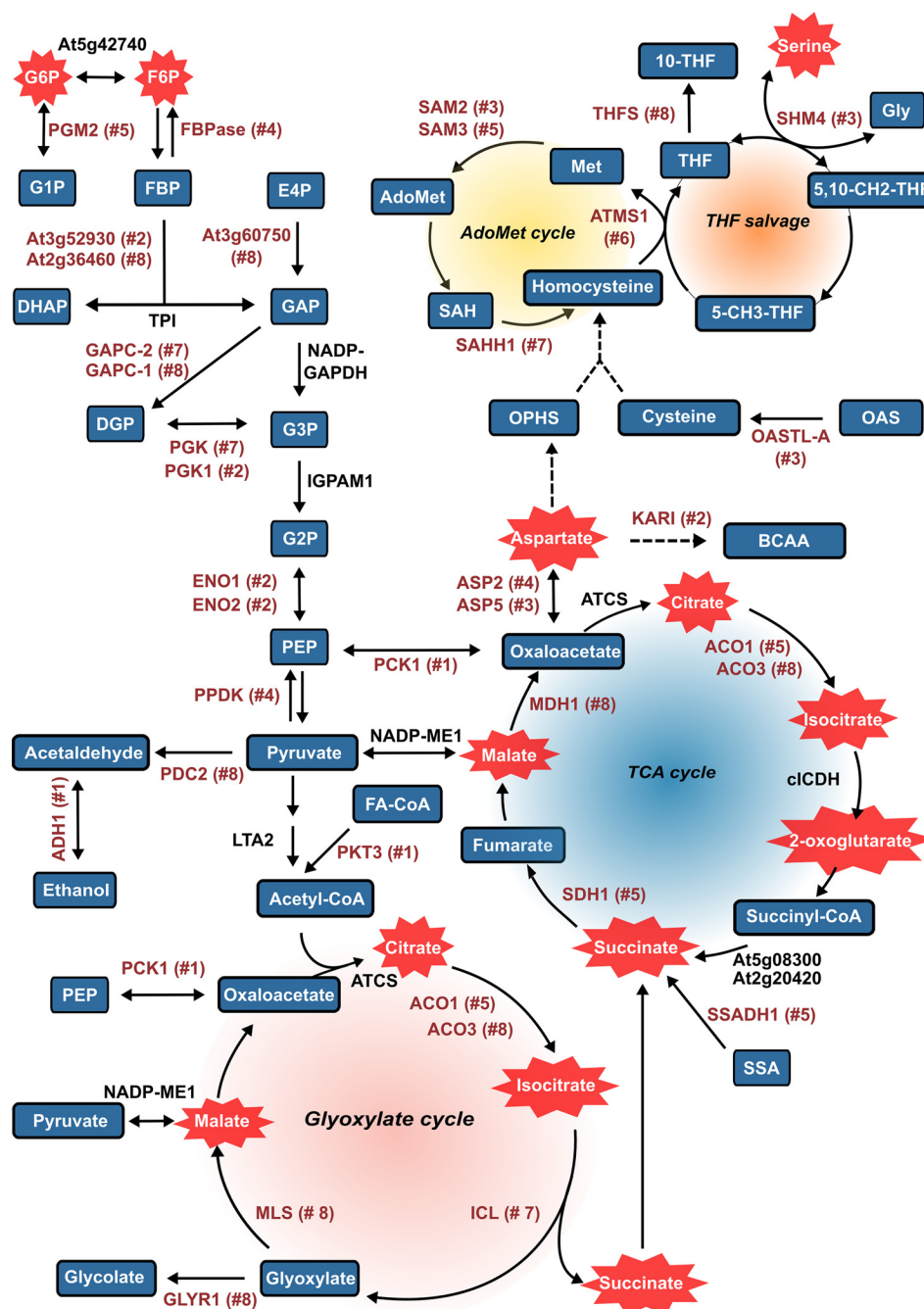


FIG. 6. Neosynthesized proteins related to seed glycolysis/neoglucogenesis, energy, and amino acid metabolisms. The proteins neosynthesized (red) related to seed energy and neoglucogenesis metabolisms are displayed on their corresponding enzymatic reactions with their cluster number between brackets. The metabolites previously found to be up-accumulated during *Arabidopsis* seed germination are displayed within red stars (67). Proteins present in the total seed proteome but not neosynthesized during germination are indicated in black. 5,10-CH₂-THF, 5,10-methylene-THF; ACO1/3, aconitase 1/3; ADH1, aldehyde dehydrogenase 1; ASP2/5, aspartate aminotransferase 2/5; ATCS, citrate synthase; ATMS1, cobalamin-independent methionine synthase 1; BCAA, branched-chain amino acids; CICDH, cytosolic NADP-dependent isocitrate dehydrogenase; DHAP, dihydroxyacetone phosphate; DGP, glycerate 1,3-diphosphate; E4P, erythrose-4-phosphate; ENO1/2, enolase 1/2; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; FBPase, fructose-1,6-bisphosphatase; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G3P, glycerate-3-phosphate; G2P, glycerate-2-phosphate; GAP, glyceraldehyde-3-phosphate; GAPC-2, glyceraldehyde-3-phosphate dehydrogenase C2; Gly, glycine; GLYR1, glyoxylate reductase 1; ICL, isocitrate lyase; IGPAM1, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1; KARI, ketol-acid reductoisomerase I; LTA2, plastid E2 subunit of pyruvate decarboxylase; MDH1, malate dehydrogenase 1; MLS, malate synthase; NADP-GAPDH, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; NADP-ME1, NADP-malic enzyme 1; OAS, O-acetylserine; OASTL-A, O-acetylserine (thiol) lyase cytosolic isoform A1; OPHS, O-phospho-L-homoserine; PCK1, phosphoenolpyruvate carboxykinase 1; PDC2, pyruvate decarboxylase 2; PGK, phosphoglycerate kinase;

MDARs (MDAR1, -2/3, -4, -5, and -6). These proteins are enzymatic components of the glutathione-ascorbate cycle, one of the major antioxidant systems in plants. In *Arabidopsis*, MDAR4 is required for seed storage lipid hydrolysis and post-germinative growth (79). Our results indicated that MDAR6 (At1g63940) is synthesized in the very early steps of seed germination (Cluster 1; [supplemental Table S8](#)), suggesting a rapid production of ascorbate from monodehydroascorbate during this process. The subcellular localization of MDAR6 has been assigned to the plastid compartment (80). Monodehydroascorbate can also be converted to a divalent oxidation product, dehydroascorbic acid, through spontaneous disproportionation or further oxidation (81). *Fait et al.* (67) described a significant increase in the level of dehydroascorbic acid during *Arabidopsis* seed germination. Dehydroascorbic acid can also be enzymatically reduced to ascorbate by dehydroascorbate reductase in a glutathione-dependent reaction. The enzyme dehydroascorbate reductase 1 (At1g19570) displayed a maximum of synthesis from 8 to 16 HAI in *Arabidopsis* seeds (Cluster 2; [supplemental Table S8](#)). Thus, MDAR6 and dehydroascorbate reductase 1 are key components of the ascorbate recycling system and would maintain significant levels of ascorbic acid in the very early stages of germination. Our results are in accordance with previous works indicating that ascorbate accumulation restarts very early (within a few hours) upon seed imbibition (82, 83). It is worth noting that the exogenous application of low concentrations of ascorbate can partially rescue rice seed germination from abscisic acid treatment (84). Moreover, an examination of the neosynthesized proteome highlights that GDP-D-mannose 3',5'-epimerase (At5g28840), which converts GDP-D-mannose to GDP-L-galactose, exhibits a maximum of synthesis from 8 to 24 HAI in seeds ([supplemental Table S8](#)). Encoded by a single gene in the *Arabidopsis* genome, GDP-D-mannose 3',5'-epimerase is a control point in the ascorbate biosynthetic process (85). Dehydroascorbic acid is derived from the antioxidant ascorbate, which is maintained at a constant steady-state level, apparently because it is replenished from the hexose phosphate pool (83). Taken together, our data indicate that the ascorbate recycling system would act upstream of the ascorbate biosynthetic process during seed germination.

Protein Fate (Folding)—Cluster 4 includes proteins highly neosynthesized from 8 to 24 HAI that are involved in protein fate, such as heat shock proteins (HSP91, At1g79930; HSP70, At3g12580; HSC70.5, At5g09590; HSP60.2, At2g33210) and protein disulfide isomerases (PDI4, At3g16110; PDI6, At1g77510) ([supplemental Table S8](#)). The *Arabidopsis* genome contains 12 PDI genes (86). PDIs are thiodisulfide oxi-

doreductases that catalyze disulfide bond formation and rearrangements in the substrate proteins, facilitating the folding of nascent polypeptides in the endoplasmic reticulum (ER). PDIs also function in the organelles outside the ER and play roles in redox signaling. However, little is known about their functions and their subcellular locations in *Arabidopsis* seeds. It has been reported that PDI5 (At1g21750) chaperones and inhibits cysteine proteases during trafficking to vacuoles prior to programmed cell death of the endothelium in developing seeds (86), and this protein is oxidized during *Arabidopsis* seed germination (50). In rice seeds, a PDI protein (LOC_Os11g09280) prevents proglutelin and prolamin from aggregating by chaperoning their segregation in the ER lumen (87, 88). In addition, *PDI2* is highly expressed in *Arabidopsis* seeds; *PDI2* accumulation is localized to both ER and nucleus, and a slight decrease in the germination rate of *pdi2* mutant seeds relative to wild-type seeds has been observed (89). The strong expression of *PDI2* in the outer cell layers of the seed and the *PDI2* localization to the nucleus are intriguing. Notably, *PDI2* interaction with Maternal Effect Embryo Arrest 8 (At1g25310) was reported, and Maternal Effect Embryo Arrest 8 is proposed to play a role in controlling the expression of genes in maternal tissue required for normal embryo and endosperm biogenesis (90). Moreover, our data disclosed that *PDI11* (At2g47470) was also neosynthesized throughout germination until 40 HAI (Cluster 6). Thus, the present results suggest important roles for *PDI4*, *PDI6*, and *PDI11* in *Arabidopsis* seed germination.

Preparation for Radicle Protrusion and Germination Sensu Stricto—Clusters 5 and 6 contained proteins that displayed maximum neosynthesis in the 24–32 HAI and 24–40 HAI time windows, respectively. Consequently, these proteins are presumably involved in the preparation of radicle protrusion.

Protein Degradation and Nitrogen Remobilization—Cytosolic glutamine synthetase 1–3 (At3g17820) belongs to Cluster 5, which coincides with protein degradation. Glutamine synthetase is a key enzyme for inorganic nitrogen assimilation and remobilization in plants. Indeed, glutamine synthetase catalyzes the ATP-dependent fixation of ammonium to the δ -carboxyl group of glutamate to form glutamine. Remarkably, genetic studies in maize showed the co-localization of a quantitative trait locus for high germination rates with the glutamine synthetase 1–3 gene, suggesting that high levels of glutamine synthetase activity lead to faster germination (91). Consequently, the release of amino acids from SSPs in germinating *Arabidopsis* seeds would start from 24 HAI, therefore anticipating radicle protrusion and seedling growth. This hypothesis is supported by the presence of two proteases, peptidase S8/S53 (At3g14067) and tripeptidyl peptidase II

PGK2, phosphoglycerate kinase 1; PKT3, peroxisomal 3-ketoacyl-CoA thiolase 3; PGM2, phosphoglucomutase 2; PEP, phosphoenolpyruvate; PPK, pyruvate orthophosphate dikinase; SAH, S-adenosyl-L-homocysteine; SAHH1, S-adenosyl-L-homocysteine hydrolase 1; SAM2/3, S-adenosylmethionine synthetase 2/3; SDH1, succinate dehydrogenase 1; SHM4, serine hydroxymethyltransferase 4; TCA cycle, tricarboxylic acid cycle; THF, tetrahydrofolate; THFS, 10-formyltetrahydrofolate (10-THF) synthetase; TPI, triose-phosphate isomerase; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase.

(TPPII) (At4g20850), in Clusters 5 and 6 that are highly neosynthesized from 24 HAI (supplemental Table S8). TPPII is the main exopeptidase involved in the processing of large proteasome products and is probably necessary for efficient protein turnover (92), especially contributing to the preferential catabolism of oxidized (carbonylated) proteins (93). Given that SSPs represent the major forms of carbonylated proteins in dry mature seeds (50), it seems conceivable that TPPII might contribute to the degradation of SSPs during seed germination via the 20S proteasome pathway. In agreement, three protein subunits of the 20S proteasome (PAB1, At1g16470; PAA1, At5g35590; PAE2, At3g14290 or At1g53850) were detected in the dry and germinating seed proteomes (supplemental Table S1). The 20S proteasome subunit PAE2 was constitutively neosynthesized from 8 to 48 HAI, whereas PAB1 was neosynthesized preferentially between 8 and 24 HAI (supplemental Table S8). It has been highlighted that the degradation of carbonylated proteins is partially mediated by the 20S proteasome in accordance with the favored detection of a 20S proteasome/TPPII couple in *Arabidopsis* seeds (93, 94).

Sulfur-containing Amino Acids (Cysteine and Methionine) and Folate Metabolism during *Sensu Stricto* Seed Germination—The *de novo* synthesis of several proteins related to sulfur-containing amino acids, the methionine cycle, and folate metabolism was highlighted in the present data (supplemental Table S3, Fig. 6). First, cytosolic *O*-acetylserine (thiol) lyase protein (At4g14880), contributing to cysteine synthesis (95, 96), was found in Cluster 3. In addition, the cobalamin-independent Met synthase (ATMS1, At5g17920) belongs to Cluster 6, which contains proteins with increasing *de novo* synthesis up to radicle protrusion (40 HAI) followed by a subsequent dramatic decline (supplemental Table S8). This enzyme, involved in the last step of methionine synthesis in plants (30, 97), plays a key role in seed germination because *de novo* Met synthesis is crucial to seed vigor and seedling establishment in *Arabidopsis* (6, 34). Moreover, Met is the precursor of *S*-adenosylmethionine (AdoMet), the universal methyl-group donor in biological reactions (30). The last step in the production of AdoMet is catalyzed by AdoMet synthetase, and the present data indicate that AdoMet synthetase 2 (SAM2, At4g01850) and AdoMet synthetase 3 (SAM3, At3g17390) are highly synthesized from 16 to 24 HAI (Cluster 3) and from 24 to 32 HAI (Cluster 5), respectively. This is in accordance with the characteristic accumulation of AdoMet synthetase just before radicle protrusion (34, 98). In addition, the presence of the *S*-adenosyl-homocysteine hydrolase 1 (At4g13940; Cluster 7) demonstrated the importance of AdoMet recycling, with very important neosynthesis starting from 24 HAI and continuing up to radicle protrusion. This enzyme is required for proper functioning of the methyl cycle to remove *S*-adenosyl-homocysteine, which is a potent inhibitor of the AdoMet-dependent methyltransferases (99). These results add a new line of evidence regarding the importance of methionine metabolism for seed germination *sensu stricto* (6).

Furthermore, of outstanding importance for seed germination, tetrahydrofolates (THFs) are essential co-factors mediating the transfer of one-carbon units (100). For instance, the reaction catalyzed by ATMS1 is a transfer of the methyl group from the 5-methyl-THF to homocysteine, yielding Met. In turn, in conditions where photorespiration is unlikely to take place, as in germinating seeds, 1-carbon formate could be incorporated into THF to form 10-formylTHF and, eventually, 5,10-methylene-THF (101). Finally, as we detected serine hydroxymethyltransferase 4 (At4g13930) in Cluster 4, the 5,10-methylene-THF could be converted to serine that accumulates during the first 24 h of germination (67). The origin of the necessary formate remains unclear, but it could be derived from oxalate metabolism.

Proteins Constitutively Neosynthesized in the Time Course of Seed Germination up to Seedling Establishment: Glucosinolate Remobilization—The proteins belonging to Cluster 7 displayed a progressive increase in protein neosynthesis toward radicle protrusion (supplemental Table S8). In this cluster, two proteins, nitrile-specifier protein 2 (At2g33070) and PYK10 protein (At3g09260), could be involved in glucosinolate catabolism. Glucosinolates are sulfur-rich plant secondary metabolites, and their overall content declines upon seed imbibition (102, 103). The *Arabidopsis* PYK10 protein (At3g09260) was described as a root- and hypocotyl-specific myrosinase (104) whose activity could release glucose and breakdown products, including defense compounds such as isothiocyanates. To date, the physiological role and natural substrate(s) of PYK10 are unknown, but functional β -glucosidase and β -fucosidase activities have been reported for PYK10 (105). In addition, nitrile-specifier protein 2 is abundant in both dry and germinating *Arabidopsis* seeds and is also progressively translated (supplemental Tables S1 and S7). Through *in vitro* assays on mature seed extracts, nitrile-specifier protein 2 was shown to preferentially promote nitrile production upon glucosinolate breakdown, thereby allowing efficient remobilization of the sulfur and nitrogen contents (106). Thus, glucosinolate degradation during *Arabidopsis* seed germination could represent a significant source of sulfur and nitrogen, thereby fueling the sulfur amino acid metabolism essential for germination (107, 108). These results provide further support for the crucial importance of sulfur metabolism in seed germination and vigor (6).

PYK10 is the major component of the ER bodies (109), and it has been shown that the *PYK10* promoter mediates developmentally regulated gene expression that becomes specific to the root in advanced developmental stages and shows no activities in other parts of the mature plant (104). PYK10 is able to interact with PYK10-binding protein 1 (PBP1, At3g16420), which possesses two repeated regions, each of which is highly homologous to the α -chain of jacalin (plant-based lectin), a carbohydrate-binding protein (110). PBP1 was also detected as *de novo* synthesized during germination (Cluster 6), suggesting a marked necessity of the PYK10–

PBP1 complex (β -glucosidase complex) before radicle protrusion. Indeed, PBP1 contributes to PYK10 activity and may act as a molecular chaperone that facilitates the correct polymerization of PYK10 when cells are exposed to stress. The *PYK10* and *PBP1* genes are both regulated by the NAI1 (At2g22770) basic-helix-loop-helix-type transcription factor (105). Moreover, it has also been demonstrated that NAI2 (At3g15950) deficiency in *Arabidopsis* specifically reduces the accumulation of PYK10 protein but does not affect the accumulation of PBP1 (111). *nai1* and *nai2* mutants display an absence of ER bodies, and the consequences of these mutations on seed biology would deserve to be studied in depth.

Cytoskeleton—Cytoskeletal elements involved in cytoplasmic organization, cell morphology, and motility should be essential to restart cellular metabolism during germination. This seems to be the case, as components of microtubules (β -tubulin 3, At5g62700; β -tubulin 8, At5g23860) and actin filaments (Actin 2, At3g18780) were detected as neosynthesized in Cluster 7 (supplemental Table S8). Furthermore, tubulins 3 and 4 (At5g19770; At1g04820) and actin 7 (At5g09810) were also neosynthesized and belonged to Cluster 4 and Cluster 8, respectively (supplemental Table S8). If the *act2* mutation does not cause any obvious phenotypes except reduced fitness, the *act7* mutation impairs germination and seedling and root growth (112, 113). These observations concur with previous works indicating that in germinating seeds, cytoskeleton reorganization is necessary for the large rates of cell elongation that precede radicle protrusion (114–116).

Constitutive de Novo Synthesis of Enzymes Involved in Energy and Neoglucogenesis Pathways—A large number of enzymes involved in the TCA cycle, the glyoxylate cycle, and glycolysis/neoglucogenesis were neosynthesized from the very early steps of seed germination up to seedling establishment (Fig. 6). Specifically, in Clusters 7 and 8, we found *de novo* synthesized proteins involved in energy production (TCA cycle, aconitase 3, malate dehydrogenase 1) or dedicated to the consumption of fatty acid (glyoxylate cycle, aconitase 3, isocitrate lyase, malate synthase). Most of these enzymes are important for seed vigor and seedling establishment (117–119). Cluster 8 contained proteins highly neosynthesized from 8 up to 48 HAI in a constant manner, with the exception of the very early steps of seed germination (supplemental Table S8). Interestingly, glyoxylate reductase 1 (GLYR1) (At3g25530) belongs to this cluster. This enzyme catalyzes the reduction of both glyoxylate and succinate semialdehyde, which are intermediates in the metabolism of glycolate and γ -aminobutyrate (GABA), respectively (120). Preferentially, GLYR1 catalyzes the NADPH-based reduction of glyoxylate into glycolate, and it has been proposed that glyoxylate detoxification by GLYR1 could provide an alternative sink for excess electrons during the recycling of reducing equivalents (121). Yet recently it was shown that the GLYR1 protein is exclusively present in the cytosol and is not found in the peroxisome (122). As GABA is converted to succinate semialdehyde in the mitochondria,

GLYR1 could thus be involved in the detoxification of glyoxylate that accidentally reaches the cytosol. In addition, the protein succinic semialdehyde dehydrogenase 1 (At1g79440) was found to be (i) abundant in dry seeds, (ii) highly *de novo* synthesized between 24 and 32 HAI, and (iii) accumulated to increasing amounts during the germination time course (supplemental Tables S1 and S7). The succinic semialdehyde dehydrogenase 1 enzyme can reduce succinate semialdehyde to γ -hydroxybutyrate. Together, these findings suggest that the detoxification of reactive aldehydes such as glyoxylate and succinate semialdehyde is very important for the metabolism of germinating seeds. The GABA shunt activity could also provide a major supply of succinate to the TCA cycle during germination. This could be related to the established role of the GABA shunt in regulating the C/N ratio in conditions where the carbon supply is limited (123).

Translational Machinery and Selective mRNA Translation—Translational control is exerted in all stages of plant development and affects a wide range of mRNAs (124, 125). Notably, protein synthesis is absolutely required for *Arabidopsis* seed germination (20), and translational activity is strongly reduced in aged seeds that exhibit depressed seed vigor (26, 119). Quantitatively, three distinct steps of mRNA translational activity are detected during seed germination (Table I). Around 10% (15/158 proteins) of the *de novo* synthesized proteins detected in this study are involved in mRNA translation (supplemental Table S10). These proteins were not part of Cluster 1, indicating that the stored translational machinery is already effective during the very first hours upon imbibition (0–8 HAI), as previously described for wheat germ extract (126). Thus, the low translational activity associated with the resumption of the late maturation program could be assimilated as an adaptive mechanism to protect the embryo in the very early steps of seed germination. Indeed, if the environmental conditions are not appropriate for germination and seedling establishment, this late maturation program might be extended, contributing as well to the maintenance of seed dormancy (23). From 8 HAI, both a qualitative and a quantitative failover of the translational program are observed in germinating seeds (Fig. 5, supplemental Table S8). The translational activity peaked between 8 HAI and 24 HAI before declining slightly and finally remaining stable until 48 HAI. The sequential neosynthesis of proteins involved in translational process (supplemental Table S10) strongly suggests an important modulation of mRNA translation. This finding supports previous results showing that a change in mRNA abundance for a given gene does not necessarily result in the expected change in the corresponding protein abundance (127–129). Based on the present results, the selective translation of mRNAs emerges as an important mechanism regulating molecular functions during *Arabidopsis* seed germination (Fig. 4). Moreover, regarding seed biology, the selective recruitment of either stored mRNAs or *de novo* synthesized mRNAs is also a likely mechanism. Indeed, the nuclear cap-binding complex (consisting

REFERENCES

- of CBP20 and CBP80) plays a role in both seed germination and abscisic acid perception (130). The cap-binding complex binds co-transcriptionally to the 5' cap of pre-mRNAs and is involved in the maturation, export, and degradation of nuclear mRNAs. In addition, *in vitro* experiments on maize seed germination showed that stored mRNAs are preferentially translated by the eIF(iso)4E isoform, a component of the cytoplasmic mRNA cap-binding complex (eIF4F) (124, 131). Finally, mRNA decay is of importance because impairment in the 3'-5' exonuclease RRP41L delays germination and inhibits seedling growth (132). In the *rrp41l* knockout mutant, the mRNA levels of genes characteristic of the late maturation program (e.g. SSPs, 9-cis-epoxycarotenoid dioxygenases, oleosins, etc.) are considerably higher than in wild-type plants. Some of the identified checkpoints that regulate *Arabidopsis* seed germination through the modulation of mRNA transcription, selective recruitment of mRNA for translation, mRNA decay, protein translation, and protein activity are summarized in [supplemental Fig. S3](#). As translational regulation is not dependent on mRNA half-lives, genes encoding stable mRNAs can still be dynamically regulated as long as their protein half-lives are short (129).
- CONCLUSION AND PROSPECTS
- Further research will address the question of the spatiotemporal control of mRNA translation during seed germination, notably concerning translational control by small RNAs (133–135) and mRNA oxidation (136). However, an unanswered question regarding oxidized mRNAs and small RNAs concerns the identity and determinants of mRNA decay and translational repression. In the present study, we opted for classic and efficient radiolabeling of proteins, compatible with a 2DE approach. However, technological improvements have recently allowed the use of stable isotopes (e.g. stable isotope labeling by amino acids in cell culture) to analyze the turnover of plant cellular proteomes. This strategy is combined with either 2DE gel-based proteome studies (137) or shotgun proteomic approaches (138). In the future it will be relevant to implement these novel approaches to obtain a more detailed picture of seed proteome turnover. The germinating seed is undoubtedly a relevant model for exploring the precise mechanisms of translational selectivity.
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