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Cold Stratification and Exogenous Nitrates Entail Similar Functional Proteome Adjustments during *Arabidopsis* Seed Dormancy Release

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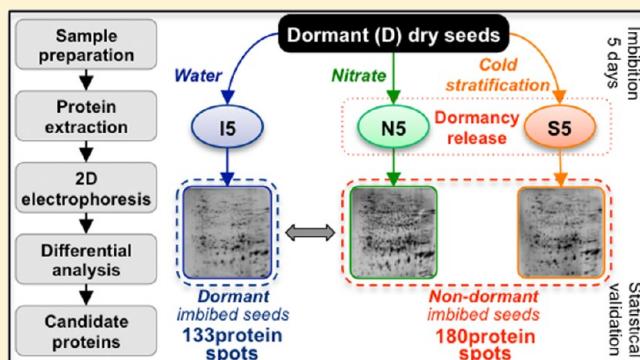
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S Supporting Information

ABSTRACT: Despite having very similar initial pools of stored mRNAs and proteins in the dry state, mature *Arabidopsis* seeds can either proceed toward radicle protrusion or stay in a dormant state upon imbibition. Dormancy breaking, a prerequisite to germination completion, can be induced by different treatments though the underlying mechanisms remain elusive. Thus, we investigated the consequence of such treatments on the seed proteome. Two unrelated dormancy-releasing treatments were applied to dormant seeds, namely, cold stratification and exogenous nitrates, in combination with differential proteomic tools to highlight the specificities of the imbibed dormant state. The results reveal that both treatments lead to highly similar proteome adjustments. In the imbibed dormant state, enzymes involved in reserve mobilization are less accumulated and it appears that several energetically costly processes associated to seed germination and preparation for subsequent seedling establishment are repressed. Our data suggest that dormancy maintenance is associated to an abscisic-acid-dependent recapitulation of the late maturation program resulting in a higher potential to cope with environmental stresses. The comparison of the present results with previously published -omic data sets reinforces and extends the assumption that post-transcriptional, translational, and post-translational regulations are determinant for seed germination.

KEYWORDS: plants, proteomics, seed dormancy, seed germination, nitrate, cold stratification, *Arabidopsis*



INTRODUCTION

Mature seeds of many plant species can display a period of dormancy where they fail to germinate under otherwise favorable conditions. This likely corresponds to a safety mechanism allowing to prevent germination when environmental conditions are not suitable for seedling establishment and/or survival.¹ If not completely released, dormancy will negatively influence seed germination, which is detrimental to crop yield. However, from an agronomical point of view, lack of dormancy is not a desirable trait as it may lead to preharvest sprouting² or reduced longevity.³ Therefore, the management of this trait is of fundamental concern for the seed industry and agriculture performance.

By definition, seed dormancy corresponds to a block of the completion of germination of an intact viable seed placed under

favorable conditions.⁴ It may be due to certain properties of the seed coat, mobilization of reserve components, hormone levels, or the joint action of several of these factors.⁵ In *Arabidopsis thaliana* seeds, a dynamic balance between two antagonistic hormones, abscisic acid (ABA) and gibberellins (GA), controls dormancy and germination.^{6,7} ABA synthesis, perception and signaling associated with GA catabolism maintain the dormant state, whereas GA synthesis, perception and signaling/ABA catabolism dominate the transition toward germination. Environmental factors (e.g., temperature, soil moisture content) can affect this balance along with the sensitivity to these two hormones during seed life.^{4,8–10} The complex

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interplay between hormone synthesis, degradation and sensitivities in response to ambient environmental conditions can result in dormancy cycling.⁸

Nitrate is a major nitrogen source for many plant species. It is assimilated via its reduction by nitrate reductase (NR) and other enzymes leading ultimately to the production of nitric oxide, amino-acids and nitrogenous compounds. High nitrate feeding of mother plants is associated with higher nitrate content and lower dormancy of the seed progeny.¹⁰ Furthermore, exogenously applied nitrate promotes dormancy release and germination, possibly by enhancing ABA catabolism and GA synthesis in close interaction with light.^{11,12} Cold stratification, the imbibition of seeds at low temperature during a few days, is also well-known to release seed dormancy and promote germination by modulating the ABA/GA balance.^{7,13} However, whether these two treatments trigger the same underlying mechanisms to release seed dormancy or act through distinct signaling pathways remains elusive.

Genetic screens and quantitative trait loci (QTL) analyses for dormancy phenotypes have provided a valuable list of genes involved in the control of dormancy.^{14,15} In addition, recent findings support an implication of chromatin assembly in dormancy release.^{16–18} However, these approaches did not allow yet reconstituting the whole signaling pathways involved. Taking advantage of the deeply dormant *Arabidopsis* accession Cvi originating from Cape Verde Islands, several transcriptomic studies have been carried out to dissect the molecular mechanisms involved in the control of this germination blockage.^{19–22} In the context of proteomics, although *Arabidopsis* seed germination is well documented,^{23–25} limited information on seed dormancy breaking is as yet available.²⁶ Furthermore, most of the studies realized up to now focused on dormancy release by after ripening (i.e., during dry storage in controlled conditions) and early imbibition times, up to 24-h.

Studying the molecular events allowing seed dormancy release during imbibition is complicated due to the continuum ultimately leading to germination. This requires distinguishing properly the molecular consequences of water uptake from the signalization pathways responsible either for dormancy release and subsequent germination or dormancy maintenance. Therefore, the choices of the imbibition time and of the physiological conditions to compare are of paramount importance to dissect these complex processes. Here, we aim to pursue the study of the physiological primary dormancy of *Arabidopsis* Cvi seeds by proteomics. For that purpose, we use two distinct dormancy-releasing treatments, cold stratification and exogenous nitrate application, as means to highlight the specificities of the dormant imbibed state by the characterization of proteins associated to the maintenance of this germination blockage. The present work also intends to compare the effect of the two treatments to identify the molecular processes triggered in both cases in relation to dormancy release. Thus, through this approach one might expect to unravel the proteome adjustments associated with the transition from dormant (D) to nondormant (ND) imbibed state.

MATERIAL AND METHODS

Plant Material and Germination Assays

Dormant seeds of *Arabidopsis* (*A. thaliana*) L. Heynh, ecotype Cvi, were used in all experiments. Plants were grown in a growth chamber at 19/20 °C under a 16-h photoperiod of

artificial light (Orsam L58/31830 luminux plus Wanton Wan White tubes, 45 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 70% of relative humidity (RH). After full maturation of the seeds, plants were no longer watered, and kept dry during 3 weeks before seed harvest. The collected D seeds were then stored during two months at 20 °C, 50% RH, in darkness prior to any experiment. Germination assays were carried out on three replicates of 50 seeds sown on a basal medium composed of distilled water buffered with Mes (3 mM, pH 5.7) and gelified with agar (7 g/L Noble agar, Difco). When indicated, potassium nitrate (Sigma-Aldrich) was incorporated into the medium at a final concentration of 7 mM. For the cold stratification treatment, seeds were placed 5 days at 4 °C in darkness on the indicated medium. Seeds were then incubated in covered plastic boxes (Ø 50 mm) in a controlled culture room under 16-h photoperiod (Philips TRM HOW/33 RS tubes, 170 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), at 25 °C (light period)/20 °C (dark period) and a constant 70% RH. A seed was regarded as germinated once the radicle protruded through the seed coat and the endosperm.

Total Soluble Protein Extraction and Two-Dimensional Gel Electrophoresis

Total soluble protein extract preparation from mature imbibed seeds and subsequent separation by two-dimensional (2D) electrophoresis were carried out as previously described.²⁷ For each condition analyzed, 2D gels were made in three biological replicates (see Material S1 in Supporting Information).

Protein Staining and 2D Gel Analysis

2D gels were stained with silver nitrate as previously described²⁸ using the Hoefer Automated Gel stainer apparatus (Amersham Pharmacia Biotech). Silver-stained gels were scanned with the Sharp JX-330 scanner equipped with the Labscan v3.00 (Amersham Pharmacia Biotech). Quantitative analysis of the 2D gels was then achieved using the software Progenesis SameSpot (v3.2, NonLinear Dynamics). Briefly, after 2D gel alignment and subsequent spot detection, the software calculates background corrected abundance by determining the lowest intensity value of the image pixels outside the spot's outline and subtracting it from the intensity value of every pixel inside the spot outline. These abundances are then normalized compared to a reference gel in order to obtain a normalize intensity value for each spot. The spot volume ratios between the different conditions were calculated using the average spot normalized volume of the three biological replicates.

Spot Selection and Statistical Analyses

The SameSpot software was used to perform a one-way ANOVA for each spot returning a p-value that takes into account the mean difference, variance and sample size. The criteria retained for the selection of differentially abundant spots were a maximum fold change higher than 1.5 (up and down accumulation) and a p-value lower than 0.05 in the comparison between all the established groups of biological replicates. In addition, a visual validation was carried out to avoid false positives/negatives due to partial overlapping of the proteins spots on the 2D gels. The SameSpot software was then used to generate the principal component analysis (PCA) based on the volumes of the selected spots and to establish clusters by correlation analysis (with a distance of 1.5 on the dendrogram). When indicated, pairwise comparisons were also applied to recover the ANOVA p-values (see <http://www.nonlinear.com/products/progenesis/samespots> for further details).

Protein Identification

The spots that corresponded to previously identified seed proteins, localized on 2D *Arabidopsis* seed proteome reference maps²⁶ (<http://www.seed-proteome.com>), were identified by visual comparison. For protein spots previously identified by MALDI-TOF, the identification results were updated from mass lists publicly available using the MASCOT (<http://www.matrixscience.com>; Matrix Science) search engine software in order to check the reliability of the results in lights of the improvement made to the search algorithm and databases (Table S1A,C). The following parameters were used for database search: a peptide mass tolerance of ± 0.2 Da, one missed cleavage allowed and two variable modifications, cysteine carbamidomethylation and methionine oxidation. Otherwise, spots of interest were excised from silver-stained 2D gels, digested with trypsin and identified by LC–MS/MS (See Material S2 in Supporting Information). Database search was performed using XTandem and XTandem Pipeline (<http://pappso.inra.fr/bioinfo/xtandempipeline/>) (Material S2 in Supporting Information, Table S1A,B).

RESULTS AND DISCUSSION

Effect of Nitrate and Cold Stratification on Dormancy Release

Freshly harvested Cvi seeds are deeply dormant and insensitive to most of the treatments known to release this physiological blockage of germination.²⁰ Here, the dry mature seeds were stored during two months at 20 °C and 50% RH in darkness prior to any experiment. Such seeds imbibed on basal medium only reached about 10% germination after 30 days testifying that they were still dormant (Figure 1). Cold stratification of

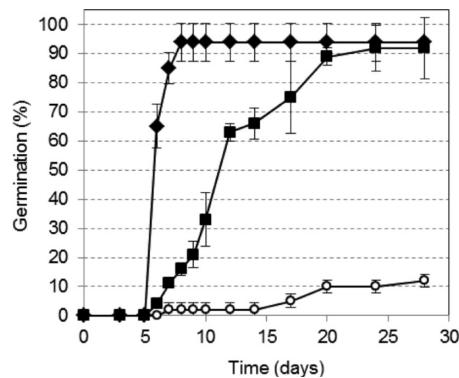


Figure 1. Alleviation of *Arabidopsis* seed dormancy (accession Cape Verde Islands; Cvi) by stratification or nitrate treatments. Mature dormant (D) Cvi seeds were sown on basal germination medium (○) or with 7 mM potassium nitrate (■) at 25 °C (16 h light) or cold stratified (5 days at 4 °C in the dark) and then transferred into the same conditions as the other samples (◆). Germination (considered as radicle protrusion through the seed coat and the endosperm) was scored daily.

these dormant seeds (carried out at 4 °C, in darkness during 5 days) or application of exogenous potassium nitrate (KNO₃ at a concentration of 7 mM) resulted in an almost complete germination of the seeds ($\approx 95\%$ germination in both cases) in agreement with previous results.⁷ Thus, even though germination kinetics were clearly different, both treatments resulted in efficient seed dormancy release (Figure 1). Cold stratification was the most efficient dormancy-releasing treat-

ment with a rapid and homogeneous germination once the seeds were brought back in standard germination conditions (see Materials and Methods). Previous work documented that dormancy breaking either by cold stratification or nitrate is correlated with a decreased ABA content,^{7,12} thereby suggesting the induction of some common molecular mechanisms by these treatments. In both cases, the reinduction of ABA synthesis observed when dormancy is maintained is prevented.⁷ Enhanced ABA catabolism especially via an induction of ABA 8'-hydroxylase genes such as CYP707A2¹² and reduced ABA synthesis could explain this reduction of ABA levels in seeds.

Principal Component Analysis Reveals Similar Patterns of Protein Accumulation in Response to Cold Stratification and Exogenous Nitrate Application

The question of the existence of a common underlying mechanism leading to dormancy release in response to distinct treatments, like cold stratification or exogenous nitrate application, has already been addressed by transcriptomic approaches.²⁰ However, as a single mRNA can lead to many different protein variants, due to proteolysis and post-translational modifications (PTMs), proteomics constitutes a valuable complement and can bring robust information about the biological functions affected in physiological changes.²⁹ Here, we conducted a quantitative proteomic comparison of three distinct samples: seeds imbibed on basal medium during 5 days or with 7 mM potassium nitrate and seeds stratified during 5 days at 4 °C in darkness (conditions subsequently abbreviated IS, NS, or SS, respectively) (Figure 2a).

The choice of these samples was motivated by several reasons. First, most of the previous studies focused on the first 24-h of seed imbibition,^{19,21,26} despite the fact that the reinduction of ABA synthesis associated with the maintenance of dormancy does not occur before 3 days of imbibition.⁷ Second, full dormancy release of Cvi seeds by cold stratification or exogenous nitrate requires at least 4 days of treatment (Figure 1).⁷ In the present experimental conditions, none of the seeds had germinated after 5 days imbibition but, with both dormancy-releasing treatments, the first seeds germinated the next day (Figure 1). This testifies that the seeds had already acquired the potential to germinate by that time. Finally, some processes are directly related to water imbibition regardless of the depth of seed dormancy.³⁰ This led us to keep the same time of imbibition for all seed samples to observe only the effects resulting from the dormancy-releasing treatments.

2D protein patterns of total soluble proteins from IS, NS and SS samples were compared by image analysis (Figure 2a) resulting in the selection of 401 differentially accumulated protein spots, out of nearly 1500 spots detected on the 2D gels (see Materials and Methods). To evaluate differences between the three conditions, the average normalized volumes of these spots were used to plot the three gel replicates for each condition on a PCA. The first dimension of this PCA grouped the NS and SS samples separately from the IS samples and accounted for 62% of the variance (Figure 2b), indicating that this first distinction is directly correlated to the physiological state of the seeds. Otherwise, the second dimension appears to correspond to the specific effect of each treatment establishing a clear separation between NS and SS accounting for 22% of the variance. Thus, in accordance with transcriptome results,²⁰ the grouping on the PCA appears to be related to the depth of seed dormancy rather than treatment-specific.

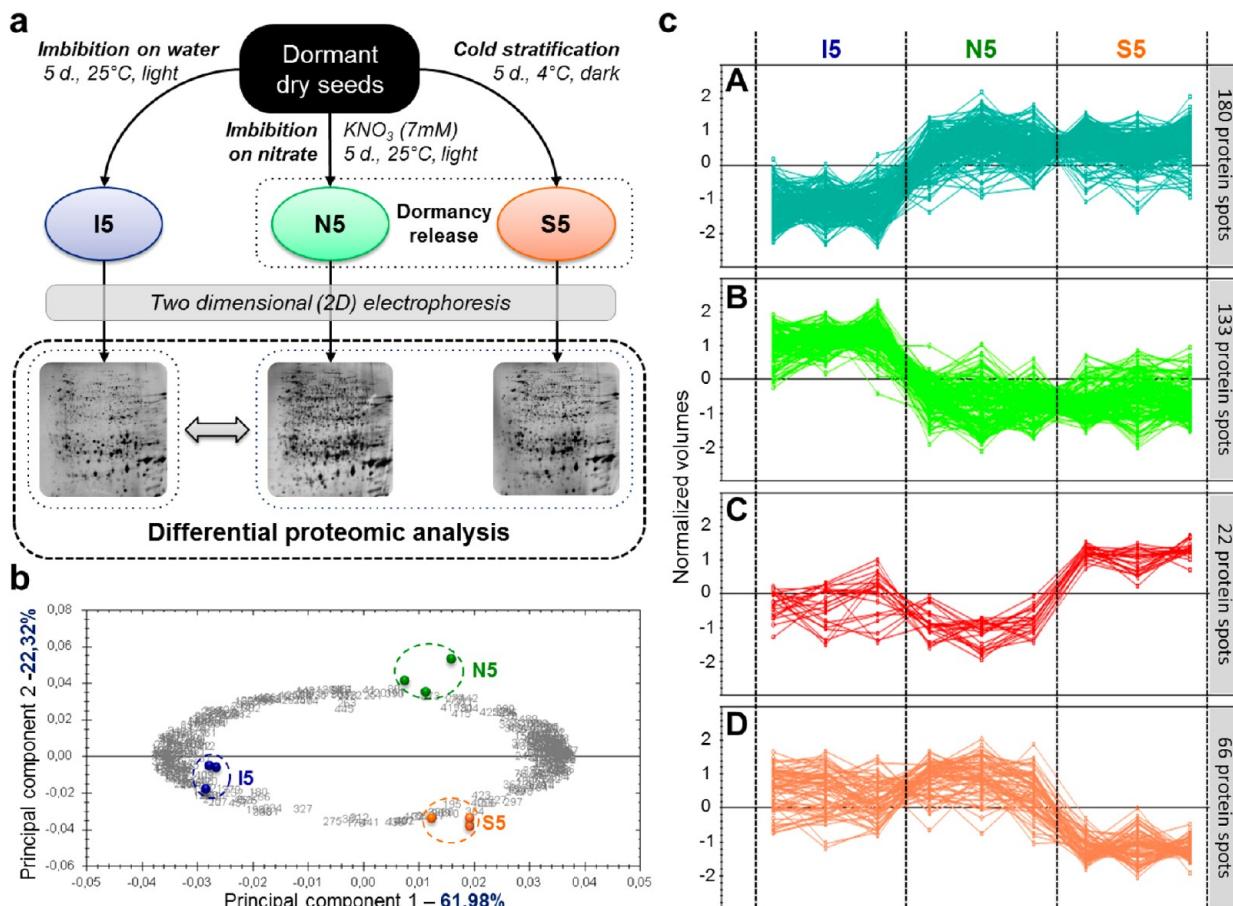


Figure 2. Impact of the dormancy releasing treatments (i.e., cold stratification or nitrate treatments) on the proteome of imbibed *Arabidopsis* seeds (accession Cape Verde Islands; Cvi). (a) Experimental design; (b) Principal Components Analysis (PCA) issued from the comparison between the profile of the 401 differentially abundant protein spots (Anova p -value $<0,05$; fold >1.5 in the Progenesis SameSpot software). Each dot correspond to one of the replicates (blue dots, imbibed D seed, I5; orange dots, stratified seeds, S5; green dots, seeds treated with nitrate, N5) and each number correspond to a single protein spot; (c) clusters of protein accumulation patterns (A, B, C and D) established by applying the correlation analysis module of SameSpot software to the defined spot selection. Each line corresponds to a spot and each point corresponds to the spot normalized volume of one biological replicate in the given condition.

A cluster analysis allowed to dispatch the 401 differentially accumulated protein spots into four groups according to their normalized volumes in each condition (Figure 2c). The clusters A and B represent, respectively, 180 and 133 spots that seem affected in the same way in both N5 and S5 compared to I5 seed samples. The size of these two clusters, corresponding to more than 75% of the selected spots, is in accordance with the position of the different samples on the PCA (Figure 2b). It suggests that both stratification and nitrate treatments have highly similar effects on the proteome of Cvi seeds that correlate with the change in dormancy status. On the contrary, the clusters C and D, which exhibit differing protein accumulation in response to the two dormancy releasing-treatments, only represent respectively 22 and 66 proteins spots. Among the 313 spots found in clusters A and B, 234 were statistically validated as up- or down-accumulated in both pairwise comparisons (N5 vs I5 and S5 vs I5). Therefore, we decided to focus on these spots in order to precise the characteristics of both physiological states (D vs ND) without interferences due to the treatments applied.

Ontological Classification of the Seed Proteins Differentially Expressed during Dormancy Release

Unfortunately, it was not possible to identify all the spots of interest as several of them were in very low abundance. Consequently, we restricted our selection to 150 spots based on their intensity as determined by image analysis. Accordingly, 107 spots were successfully assigned to proteins (Tables 1 and 2; Table S1 and Figure S1), which were then categorized using the ontological classification of Bevan et al.³¹ (Figure 3).

In imbibed D seeds, a higher abundance of stress related proteins and seed storage proteins (SSP) was mainly observed. Such prevalence of stress response proteins in D seeds was previously noted at the transcriptome level.²² The maintenance of these proteins at high level in imbibed D seeds can contribute to their survival until the requirements for dormancy breaking are fulfilled. The same also holds for the SSPs as they could behave as preferential targets for oxidative damages accompanying seed imbibition.³² On the other hand, the dormancy releasing treatments resulted in the up-accumulation of proteins involved in proteolysis and amino-acid, protein and energetic metabolisms. The parallel disappearance of protein spots corresponding to SSPs and up-accumulation of peptidases/proteases suggests that SSP degradation has already started in S5 and N5 seeds. In this context, the induction of

Table 1. Protein Spots Displaying a Decreased Abundance Following Dormancy Release^a

Spot n°	Locus (AGI)	Protein name (Modified from TAIR)	MW (kDa) & pI				ANOVA p-value	Log ₂ Ratios	
			MW _{Th}	MW _{Exp}	pI _{Th}	pI _{Exp}		N5/I5	S5/I5
• Stress response and detoxification-related proteins									
1951	At1g54870	Chloroplast Aldehyde Reductase (ChlADR)	36.76	29.7	8.76	5.4	1.0E-03	-0.61	-0.60
2066	At1g54870	Chloroplast Aldehyde Reductase (ChlADR)	36.76	25.3	8.76	4.9	6.9E-06	-1.01	-1.65
e8	At1g54870	Chloroplast Aldehyde Reductase (ChlADR)	36.76	18.6	8.76	4.6	8.0E-04	-1.06	-1.62
L516 ^c	At1g70840	Major Latex like Protein 31 (MLP31)	19.14	17.1	6.34	5.8	1.4E-04	-2.60	-1.18
L569 ^c	At1g70840	Major Latex like Protein 31 (MLP31)	19.14	17.7	6.34	5.8	3.3E-04	-0.61	-0.89
1240	At2g36640	Embryonic Cell Protein 63 (ECP63)	48.49	58.4	5.19	5.3	9.8E-06	-1.40	-2.21
1077	At2g42560	LEA domain-containing protein - SBP65	67.20	61.6	5.97	5.7	1.0E-03	-0.84	-0.94
1137	At2g42560	LEA domain-containing protein - SBP65	67.20	58.9	5.97	5.7	3.0E-03	-1.18	-0.83
1300	At3g12580	Heat Shock Protein 70.4 (HSP70-4)	71.10	51.9	4.88	5.9	4.0E-03	-1.41	-1.35
1909 ^c	At3g22500	Embryonic Cell Protein 31 (ECP31)	26.78	31.2	5.20	5.3	4.3E-05	-0.49	-0.80
2086 ^c	At3g56350	Iron/manganese superoxide dismutase (Fe/Mn-SOD)	26.89	24.5	6.76	6.3	6.0E-03	-0.65	-0.66
2100 ^c	At3g56350	Iron/manganese superoxide dismutase (Fe/Mn-SOD)	26.89	24.9	6.76	6.4	7.1E-04	-0.82	-1.07
L554 ^c	At5g12030	Heat Shock Protein 17.6A (HSP17.6A)	17.68	18.2	5.40	5.1	6.4E-04	-0.52	-0.85
• Seed storage proteins									
2093	At1g03880	12S Cruciferin (CRB) - α-subunit	27.24	23.2	6.34	7.4	9.3E-04	-2.19	-1.88
2266	At1g03880	12S Cruciferin (CRB) - α-subunit	27.24	17.0	6.34	4.9	5.8E-04	-2.23	-1.75
k145 ^b	At1g03880	12S Cruciferin (CRB) - α-subunit	27.24	26.6	6.34	5.4	1.1E-02	-0.65	-0.48
2336	At1g03880	12S Cruciferin (CRB) - β-subunit	20.80	15.0	7.03	5.6	4.7E-05	-1.72	-1.60
k188 ^b	At1g03880	12S Cruciferin (CRB) - β-subunit	20.80	17.9	7.03	7.9	7.3E-04	-1.69	-1.53
L264 ^c	At1g03880	12S Cruciferin (CRB) - β-subunit	20.80	19.8	7.03	6.4	7.0E-03	-0.48	-0.39
2075	At1g03890	RmlC-like cupins superfamily protein	49.67	25.2	5.45	4.9	2.8E-04	-0.69	-0.72
2154	At1g03890	RmlC-like cupins superfamily protein	49.67	21.9	5.45	4.9	5.0E-03	-1.21	-1.35
2257	At1g03890	RmlC-like cupins superfamily protein	49.67	16.7	5.45	6.3	4.1E-04	-1.76	-1.38
e3	At3g22640	PAP85 - RmlC-like cupins superfamily protein	55.06	17.7	7.15	5.8	8.8E-04	-2.39	-2.16
1892	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	31.2	6.42	7.4	2.0E-04	-0.99	-1.18
2010	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	27.4	6.42	5.8	2.0E-03	-1.31	-1.26
2021	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	27.0	6.42	5.9	8.0E-03	-2.01	-1.42
L159 ^c	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	22.9	6.42	5.2	2.0E-02	-0.60	-0.71
L76 ^c	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	23.3	6.42	5.5	2.0E-03	-0.47	-0.57
L77 ^c	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	22.7	6.42	5.7	1.7E-02	-0.66	-0.60
k161 ^b	At4g28520	12S Cruciferin (CRC) - α-subunit	34.68	31.6	6.42	7.1	4.0E-03	-0.98	-0.71
k163 ^{b,c}	At4g28520	12S Cruciferin (CRC) - β-subunit	21.20	21.1	6.19	7.1	1.8E-02	-0.59	-0.63
L497 ^c	At4g28520	12S Cruciferin (CRC) - β-subunit	21.20	22.4	6.19	7.2	4.4E-02	-0.61	-0.42
1369	At5g44120	12S Cruciferin (CRA1) - Precursor	52.59	46.2	7.88	7.6	1.1E-02	-1.95	-2.11
2301 ^c	At5g44120	12S Cruciferin (CRA1) - α-subunit	31.75	15.5	6.49	6.3	1.0E-03	-1.58	-1.53
k165 ^{b,c}	At5g44120	12S Cruciferin (CRA1) - α-subunit	31.75	25.7	6.49	6.4	6.0E-04	-1.14	-1.38
• Miscellaneous									
1964	At1g13440	Glyceraldehyde-3-phosphate dehydrogenase C1 or C2	33.91	29.5	7.26	6.0	6.0E-03	-1.58	-1.35
	At3g04120	GAPC1 or GAPC2							
2203	At3g05260	NAD(P)-binding Rossmann-fold superfamily protein	31.45	18.9	6.51	5.3	3.1E-05	-1.27	-1.15
e154	At3g21370	β-Glucosidase 19 (BGLU19)	60.01	47.0	6.63	6.2	6.0E-03	-1.58	-1.59
2159	At5g09810	Actin 7 (ACT7) - Fragment	41.74	21.6	5.16	5.0	5.9E-05	-2.35	-2.35
2136	At5g50700	Hydroxysteroid Dehydrogenase 1 (HSD1) - Fragment	39.09	21.8	6.15	6.4	5.0E-03	-1.20	-1.37

^aSpot n°, spot number on the reference maps; MW and pI, theoretical (Th) and experimental (Exp) molecular weight (MW) and isoelectric point (pI). Experimental MW and pI were determined using the 2D calibration module of pg240 software (Non Linear Dynamics); ANOVA p-values (SameSpot), ANOVA p-values obtained with the Progenesis SameSpot software (NonLinear Dynamics) when comparing the indicated groups of biological replicates; Log₂ Ratios, logarithm of the indicated ratios in base 2. Conditional color formatting was used to highlight the highest differences. ^bPreviously identified in Chibani et al.²⁶ ^cPreviously identified in Ler seeds: <http://www.seed-proteome.com/>.

amino-acid and protein metabolism could indicate a preparation for reserve remobilization for *de novo* protein synthesis. In agreement, Finch-Savage et al.²⁰ observed an accumulation of transcripts related to protein synthesis and reserve mobilization

during the relief of dormancy by cold, nitrate or light. In addition, Chibani et al.²⁶ reported that ABA treatment to Cvi after-ripened seeds prevented the accumulation of proteins involved in energetic and protein metabolism. Thus, we can

Table 2. Protein Spots Displaying an Increased Abundance Following Dormancy Release^a

Spot n°	Locus (AGI)	Protein name (Modified from TAIR)	MW (kDa) & pI				ANOVA p-value	Log ₂ Ratios	
			MW _{Th}	MW _{Exp}	pI _{Th}	pI _{Exp}		N5/15	S5/15
• Proteolysis									
359 ^{b,c}	At1g63770	Peptidase M1 family protein	113.95	96.9	6.77	5.3	1.0E-03	1.05	1.63
L293 ^c	At1g63770	Peptidase M1 family protein	113.95	96.1	6.77	5.4	4.0E-03	1.28	2.15
L294 ^c	At1g63770	Peptidase M1 family protein	113.95	97.4	6.77	5.4	4.5E-04	1.46	1.71
L378 ^c	At3g14067	Peptidase S8/S53 - Subtilase family protein	81.82	82.3	6.76	5.7	8.0E-03	0.77	0.79
e78	At2g14260	Peptidase S33 - Proline Iminopeptidase (PIP)	37.43	39.8	5.74	5.6	5.0E-03	0.51	0.65
1075	At3g02090	Peptidase M16 - Metalloendopeptidase (MPPBETA)	59.16	61.8	6.76	5.7	5.0E-03	1.24	1.34
567	At1g50380	Peptidase S9 - Prolyl oligopeptidase family protein	80.94	88.0	5.57	5.5	1.6E-02	0.62	0.63
915	At4g20850	Peptidase S8 - Tripeptidyl peptidase II (TPP2)	152.37	71.4	5.93	5.5	6.0E-03	0.67	0.64
• mRNA metabolism and protein synthesis									
1349 ^c	At3g13920	Eukaryotic Translation Initiation Factor 4A1 (EIF4A1)	45.70	52.8	6.19	5.5	8.2E-04	0.56	0.60
1365	At1g54270	EIF4A1 or EIF4A2	46.19	49.2	5.35	5.6	1.3E-02	1.29	1.05
	At3g13920	Eukaryotic Translation Initiation Factor 4A							
e672	At2g23350	Poly(A) Binding Protein 4 (PAB4)	71.65	72.0	6.85	6.7	1.3E-02	1.01	0.74
k97 ^{b,c}	At5g11200	DEAD/DEAH box RNA helicase family protein	52.86	55.5	5.77	5.5	3.0E-03	0.36	0.58
e137	At1g11650	RNA binding protein 45b (RBP45B)	44.11	46.2	5.66	5.4	2.8E-05	0.80	0.95
1425 ^{b,c}	At4g20360	Rab GTPase Homolog E1B (RABE1b)	51.63	46.7	6.09	5.4	1.4E-05	0.97	0.65
k85 ^b	At3g09200	Ribosomal protein L10 family protein	30.62	36.8	4.48	5.0	1.0E-03	0.35	0.41
k46 ^{b,c}	At1g56070	Translation elongation factor 2-like protein - LOS1	93.89	94.0	6.16	6.1	4.0E-03	1.31	1.33
k47 ^b	At1g56070	Translation elongation factor 2-like protein - LOS1	93.89	94.0	6.16	6.2	3.8E-02	0.95	1.36
L841 ^c	At1g29880	glycyl-tRNA synthetase / glycine--tRNA ligase	81.94	83.3	7.01	5.9	5.0E-03	0.54	0.36
• Protein destination and folding									
k148 ^{b,c}	At2g33210	Heat shock protein 60-2 (HSP60-2)	61.98	72.6	6.59	5.3	2.8E-02	0.54	0.54
956	At3g20050	T-Complex Protein 1 (TCP-1) α-subunit	59.23	67.7	6.22	5.9	1.0E-03	0.84	0.96
969	At3g11830	TCP-1/cpn60 chaperonin family protein	59.78	66.9	6.30	5.9	2.2E-04	1.19	1.47
992 ^{b,c}	At5g20890	TCP-1/cpn60 chaperonin family protein	57.29	66.1	5.67	5.7	3.8E-04	0.76	0.92
1034	At1g24510	TCP-1/cpn60 chaperonin family protein	59.38	66.7	5.34	5.5	9.0E-03	0.70	0.76
k225 ^b	At4g24280	Chloroplast heat shock protein 70-1 (cpHsc70-1)	76.51	83.2	4.81	4.6	1.0E-03	0.70	1.14
L211 ^c	At4g24280	Chloroplast heat shock protein 70-1 (cpHsc70-1)	76.51	82.5	4.81	4.5	3.4E-02	0.58	0.53
L810 ^c	At5g50920	Heat shock protein 93-V / CLPC Homologue 1 (CLPC1)	103.45	95.2	6.73	5.4	6.0E-03	0.54	0.74
2246	At3g62030	Rotamase CYP 4 (ROC4)	28.08	17.5	8.99	5.2	1.0E-03	0.64	0.90
e257 ^c	At2g04030	Heat shock protein 90-5 (HSP90-5 / CR88)	88.26	89.4	4.64	4.5	5.0E-03	1.70	1.09
• Amino-acid metabolism									
1141	At4g29840	Methionine Over-Accumulator 2 (MTO2) Threonine synthase	57.78	58.2	7.45	6.0	3.0E-03	0.63	0.55
1496	At4g33680	Aberrant Growth and Death 2 (AGD2) L,L-diaminopimelate aminotransferase	50.40	45.4	7.39	5.8	1.0E-03	1.54	1.64
1151	At1g17290	Alanine Aminotransferase (AlaAT1)	59.82	62.5	6.38	5.1	3.0E-02	0.94	0.97
1187	At1g17290	Alanine Aminotransferase (AlaAT1)	59.82	60.1	6.38	5.1	1.7E-02	0.62	0.80
k49 ^{b,c}	At5g17920	Methionine Synthase 1 (MS1)	84.36	84.3	6.47	5.9	7.2E-05	1.09	1.17
k50 ^{b,c}	At5g17920	Methionine Synthase 1 (MS1)	84.36	84.3	6.47	6.1	2.0E-03	1.08	1.21
955	At3g23940	Dehydratase family	64.91	71.0	6.15	5.5	8.7E-04	0.67	0.87
L592 ^c	At3g58610	Ketol-acid reductoisomerase	63.81	64.7	6.79	5.7	1.9E-02	0.34	0.41
988	At3g05190	D-aminoacid aminotransferase-like PLP-dependent enzyme	62.21	63.9	6.88	6.3	2.0E-03	0.96	0.71
L648 ^c	At2g26080	Glycine Decarboxylase P-Protein 2 (GLDP2)	113.77	100.6	6.63	5.7	1.0E-02	1.07	1.29
• Metabolism / Energy									
e191 ^c	At2g05710	Aconitase 3 (ACO3)	108.20	95.9	7.17	5.7	6.0E-03	1.14	1.00
k76 ^b	At3g21720	Isocitrate Lyase (ICL)	64.24	66.4	7.20	6.9	4.1E-02	0.55	0.55
L167 ^c	At5g03860	Malate Synthase (MLS)	63.89	65.8	8.03	7.9	4.1E-02	0.91	0.87
k102 ^b	At3g47520	Malate Dehydrogenase (MDH)	42.41	37.4	8.81	5.9	2.6E-02	0.77	1.48
672 ^c	At4g37870	Phosphoenolpyruvate Carboxykinase 1 (PCK1)	73.40	74.0	7.08	6.7	2.0E-02	0.59	0.73
675	At4g37870	Phosphoenolpyruvate Carboxykinase 1 (PCK1)	73.40	73.0	7.08	6.4	2.9E-04	1.02	1.14

Table 2. continued

Spot n°	Locus (AGI)	Protein name (Modified from TAIR)	MW (kDa) & pI				ANOVA p-value	Log ₂ Ratios	
			MW _{Th}	MW _{Exp}	pI _{Th}	pI _{Exp}		N5/I5	S5/I5
L296 ^c	At4g15530	Pyruvate Orthophosphate Dikinase (PPDK)	105.14	96.8	6.32	5.3	2.0E-03	0.50	1.27
e161	At2g29560	Cytosolic Enolase (ENO _c)	51.60	60.4	5.18	5.2	3.0E-03	0.53	0.82
919	At3g08590	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	60.76	73.1	5.63	5.5	4.2E-04	0.59	0.96
949	At3g22960	Chloroplast pyruvate kinase α -subunit (PKP α)	65.13	71.4	5.72	4.9	2.5E-02	0.80	0.80
L3 ^c	At1g74030	Enolase 1 (ENO1)	51.47	55.1	5.98	5.1	1.4E-05	0.37	0.65
L427 ^c	At1g50480	10-Formyltetrahydrofolate Synthetase (THFS)	67.80	76.1	6.70	6.2	2.1E-02	0.53	0.72
e131	At3g15730	Phospholipase D α 1 (PLD α 1)	91.85	91.6	5.71	5.5	6.0E-03	1.15	1.53
L106 ^c	At1g77120	Alcohol Dehydrogenase 1 (ADH1)	41.18	45.7	6.19	5.8	4.0E-03	0.68	1.26
1160	At1g16350	Aldolase-type TIM barrel family protein	54.05	56.9	6.44	6.0	6.0E-03	0.49	0.48
582	At5g37510	Mitochondrial NADH dehydrogenase complex subunit EMB1467	81.52	84.3	6.59	5.6	3.0E-03	0.83	0.77
L344 ^c	At5g37510	Mitochondrial NADH dehydrogenase complex subunit EMB1467	81.52	79.5	6.59	5.6	4.0E-03	0.95	1.01
k28 ^b	AtCg00480	ATP Synthase β -subunit	53.93	60.9	5.15	5.4	1.3E-02	0.93	0.95
L171 ^c	At3g60750	Transketolase	79.84	84.4	6.40	5.3	1.5E-02	1.05	1.13
683	At2g45290	Transketolase	79.92	79.7	6.55	5.7	3.2E-04	0.75	1.13
L320 ^c	AtCg00490	Large subunit of RUBISCO (RBCL)	52.95	56.4	6.24	5.8	1.0E-02	1.02	0.91
L321 ^c	AtCg00490	Large subunit of RUBISCO (RBCL)	52.95	55.7	6.24	5.9	6.0E-03	0.89	0.87
• Miscellaneous									
682	At5g19090	Heavy metal transport/detoxification family protein	59.62	79.7	6.79	5.5	8.2E-04	1.05	1.34
L297 ^c	At3g09840	Cell Division Cycle 48 (CDC48)	89.39	99.5	4.88	5.0	2.0E-03	1.40	1.47
L42 ^c	At3g09840 At5g03340	Cell Division Cycle 48 (CDC48)	89.39	97.3	4.88	5.0	7.6E-04	0.59	1.33
k180 ^{b,c}	At1g03880	12S Cruciferin (CRB) - β -subunit	20.80	16.3	7.03	5.6	4.4E-06	1.29	1.00
1408	At1g03890	RmlC-like cupins superfamily protein	49.67	51.5	5.45	5.4	2.0E-03	0.48	0.55
481	At4g03200	Hypothetical protein	91.83	91.6	6.29	5.5	8.0E-03	0.89	0.99
508	At5g66420	Unknown protein	70.03	90.1	5.95	5.4	2.0E-03	1.12	1.24

^aSpot n°, spot number on the reference maps; MW and pI, theoretical (Th) and experimental (Exp) molecular weight (MW) and isoelectric point (pI). Experimental MW and pI were determined using the 2D calibration module of pg240 software (NonLinear Dynamics); ANOVA p-values (SameSpot), ANOVA p-values obtained with the Progenesis SameSpot software (NonLinear Dynamics) when comparing the indicated groups of biological replicates; Log₂ Ratios, logarithm of the indicated ratios in base 2. Conditional color formatting was used to highlight the highest differences. ^bPreviously identified in Chibani et al.²⁶ ^cPreviously identified in Ler seeds: <http://www.seed-proteome.com/>.

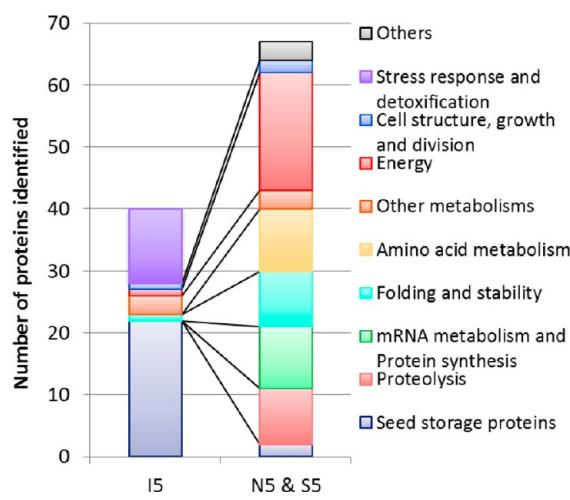


Figure 3. Ontological classification of proteins up-accumulated in dormant (D) imbibed seeds (I5) or in nondormant (ND) imbibed seeds (S5 and N5). Proteins were classified according to the classification established by Bevan et al.³¹ (refer to Table S1 for the individual classification of each protein).

hypothesize that the ABA levels are responsible for some of the differences observed.

The functional categories more represented upon dormancy release are important either during germination or for successful seedling establishment. Therefore, the present results are consistent with the occurrence of an important developmental switch once dormancy is broken. These functional categories are discussed in more details below.

Dormant Seed Proteome Exhibits Enhanced Footprints of the Late Maturation Program

As stated above, the imbibed D seeds (IS) proteome is marked by the abundance of SSPs, late-embryogenesis abundant (LEA) and stress response related proteins (Table 1). The majority of these proteins correspond to highly abundant transcripts detected in both D and ND dry seeds from the Col-0 and Cvi accessions (Figure S2; Table S1) and are usually associated to the late maturation program.^{20,22,33–35} During ND seed germination, most of these stored mRNA progressively disappear (Figure S3A).³⁴ Moreover, GAs repress the expression of a significant part of the corresponding genes (Figure S4c; Table S1)³⁶ while most are either up-regulated in the ABA overaccumulating triple mutants *cyp707a1a2a3* and/

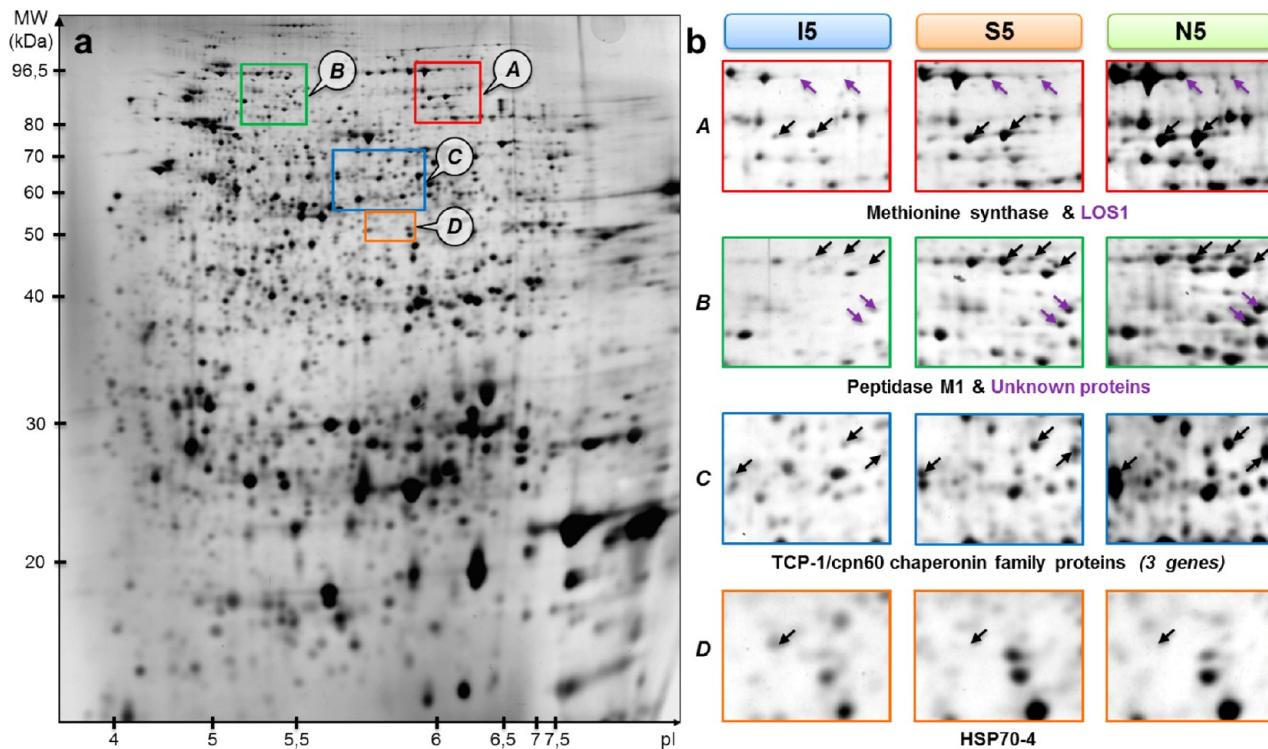


Figure 4. Illustration of proteins whose abundance is affected by the dormancy releasing treatments. (a) A representative silver nitrate-stained 2D gel of total soluble proteins from *Arabidopsis* imbibed seeds (accession Cape Verde Islands; Cvi). The indicated portions of the gel (A, B, C, D) are reproduced in the enlarged windows displayed in panel b. (b) Comparison of selected areas of 2D gels as shown in panel a in the three conditions: Seeds imbibed during 5 days on basal germination medium (I5) or with 7 mM potassium nitrate (N5) or seeds cold stratified during 5 days (S5). A: Protein spots corresponding to Met synthase (MS1; n°k49 and k50; At5g17920) and to a translation elongation factor 2-like protein (LOS1; n°k46 and k47; At1g56070). B: Protein spots corresponding to a peptidase M1 (359, L293 and L294; At1g63770) and to two unknown proteins (481 and 508; At4g03200, At5g66420). C: Protein spots corresponding to TCP-1/cpn60 chaperonin proteins (956, 969 and 992; At3g20050, At5g20890 and At3g11830). D: Protein spot corresponding to the heat shock protein 70-4 (HSP70-4; 1300; At3g12580).

or down-regulated in the ABA deficient mutant *aba2* after 24-h of seed imbibition (Figure S4a; Table S1).³⁷ In imbibed ND seeds, late embryogenesis genes can be reactivated by ABA during a short development window.³⁸ Thus, we suggest that our observations reflect an ABA-dependent recapitulation of the late maturation program.

The functional classification of the differentially accumulated proteins (Figure 3) also indicates that the maintenance of imbibed seeds in the dormant state could be associated with an increased potential to cope with penalizing environmental conditions. For instance, three spots corresponding to a plastidial aldehyde dehydrogenase (*ChlADR*; n°e8, 2066 and 1951; At1g54870) are more abundant in I5 seeds. *ChlADR* can be induced by ABA through the *ABI3* transcription factor.³⁹ In accordance with our results, dormancy release by after-ripening also resulted in the disappearance of this protein from Cvi imbibed seed proteome.²⁶ *ChlADR* is involved in the detoxification of reactive carbonyls that can result from lipid peroxidation in response to oxidative stress.⁴⁰ In this regard, we can notice the elevated abundance of two spots corresponding to Mn-superoxide dismutase (MnSOD; n°2086 and 2100; At3g56350), a pivotal enzyme of oxidative stress responses. MnSOD transcripts were previously assigned to the “dormant” cluster established by Cadman et al. (2006). The expression products of the gene encoding the small class I heat shock protein 17.6A (Hsp17.6A, n°L554, At5g12030) have also already been associated to the D state.^{22,26} This gene is under *ABI3* control and would be associated with environmental

adaptation and desiccation tolerance maintenance in D seeds.⁴¹ Another member of the Hsp family, the Hsp cognate 70-4 (HSC70-4, n°1300, At3g12580) was also only detected in I5 seeds (Table 1, Figure 4b). HSC70-4 is involved in the degradation of unimported plastid-destined proteins thereby preventing cytosolic precursor accumulation.⁴² Transgenic lines overexpressing one member of this gene family, *HSC70-1*, show increased expression of *HSC70-4*⁴³ and are hypersensitive to ABA during seed germination.⁴⁴ Therefore, HSC70-4 might also impact ABA signaling during dormancy release.

Finally, a spot corresponding to the hydroxysteroid dehydrogenase 1 (HSD1, n°2136, At5g50600 or At5g50700) was found at a lower molecular weight than expected (Table 1). The analysis of the position of the identified peptides on HSD1 amino-acid sequence suggests that the spot corresponds to an N-terminal fragment of the protein that could result from its cleavage. The overexpression of *HSD1* was shown to result in a reduced dormancy and a decreased sensitivity to ABA probably due to an increase in the catabolism of this hormone.^{45,46} Therefore, a degradation of the mature protein would be coherent with the maintenance of dormancy.

The finding that several of the proteins that were more abundant in I5 seeds correspond to genes whose expression can be induced by ABA and/or repressed by GA supports the existence of a connection between our results and previous studies.¹⁵ It reinforces the assumption that the proteome of imbibed seeds with contrasted depth of dormancy reflects the state of the dynamic balance between ABA and GA.

Additionally, the presence of bifunctional proteins participating in dormancy maintenance and stress responses could be expected as the result of an adaptation to environmental fluctuations.

Dormancy Release Is Associated with an Increase in Protein Synthesis Potential and Post-Translational Processing Capacity

Chibani et al.²⁶ showed that *Arabidopsis* D seeds are equally competent for protein synthesis as after-ripened seeds after 24-h of imbibition. However, up to now, none of the proteomic analyses undertaken assessed the translation potential during prolonged imbibition of D seeds. Dry seeds already contain most of the proteins required for polypeptide synthesis so that mRNA translation can proceed shortly upon imbibition.³⁰ But the maintenance of this capacity over several days implies a progressive renewal of the implicated proteins. Transcriptome analyses highlighted a correlation between dormancy release and the accumulation of mRNAs associated with the translation machinery leading to the assumption that protein translation plays a key role in the transition toward germination.^{19,20,22,47} In accordance, we observed the accumulation of several proteins involved in this pathway in response to cold stratification or exogenous nitrate (Table 2). This implies that either the turnover of these specific proteins is affected when seeds stay dormant or that the synthesis/stability of the corresponding mRNAs is enhanced upon dormancy release. The differentially abundant protein spots related to the translation machinery included one structural component of the ribosome (n^o k85, At3g09200) and several spots corresponding to translation initiation (n^o e672, 1349 and 1365; At3g13920, At2g23350) and elongation (LOS1, RABE1b; n^o k46, k47 and 1425; At1g56070, At4g20360) factors (Figure 4b). LOS1 (low expression of osmotically responsive genes 1) encodes a translation elongation factor 2-like protein that is required for protein synthesis at low temperature thereby playing a critical role in cold response.⁴⁸ Thus, the presence of this protein may be a prerequisite for cold stratification to release seed dormancy. Apart from their implication in polypeptide synthesis, some of the translation factors up-accumulated following dormancy-releasing treatments may participate in mRNA stability or favor a selective mRNA processing. Indeed, RNA helicases play pivotal roles in RNA metabolism.⁴⁹ eIF4A, an ATP-dependent RNA helicase, and the poly (A) binding protein 4 (PAB4) are components of the cap-dependent translation initiation complex. The assembly of specific initiation factor isoforms in this complex can differentially promote the translation of specific mRNAs.^{50,51} Additionally, the activity of several translation factors, including eIF4A and LOS1 can be modulated by phosphorylation.⁵² Therefore, the presence of several spots for both proteins in the 2D gels (Figure 4b; Table 2) may indicate a regulation by PTMs. This kind of regulation modulates the translation activity during stress responses as a means to control this costly process.⁵³ The DEAD box RNA helicase encoded by the gene At5g11200 is the *Arabidopsis* ortholog of human DDX39 that is involved in alternative splicing and RNA export from the nucleus.⁵⁴ Owing to the high conservation of the amino acid sequence of this protein, we can assume that it has a similar impact on RNA metabolism in *Arabidopsis*. Therefore, the increased abundance of these spots upon dormancy release (Table 2) strongly suggests an important modulation of RNA metabolism and of the potential for selective translation. In

addition, several of these proteins were found down accumulated in 24-h imbibed after-ripened Cvi seeds when ABA was included in the germination medium, which led to the hypothesis that exogenous ABA could promote targeted proteolysis of these polypeptides.²⁶ Thus, ABA may play a pivotal role in the turnover of these proteins.

Translational capacity is a key element in proteome dynamics. However, it does not account for all the physiological changes presently observed as the contribution of post-translational processing and (targeted) proteolysis needs to be taken into account. Additionally, even if an mRNA is efficiently translated, the activation of the resulting polypeptides may require further processing. One of the consequences of both dormancy-releasing treatments is the accumulation of a wide range of proteins involved in post-translational processing (Table 2; Figures 3 and 4d). Enhanced protein folding may be required for the activation of stored and/or newly synthesized polypeptides thereby contributing to a metabolic switch. Among the eight protein chaperones identified as being more abundant in NS and SS seeds compared to IS seeds, four belong to the TCP-1/cpn60 family (n^o 956, 992, 969, 1034; At3g20050, At5g20890, At3g11830, At1g24510). They are part of an hetero-oligomeric complex that assists the folding of target proteins in the cytosol.⁵⁵ Each complex can comprise eight different subunits.⁵⁶ The concomitant up-accumulation of several subunits of this complex upon dormancy release strongly suggests that it could play a central role in the commitment toward seed germination. However, the physiological role of the members of this family has not yet been precisely described in plants.

Another important element in polypeptide fate during or after translation is their translocation into the various cell compartments. The chloroplastic Hsp 70-1 (cpHsc70-1, n^o k225 and L211, At4g24280) and the Hsp93-V/CLPC1 (n^o L810, At5g50920) participate in the import of proteins into plastids through their interaction with the translocon complex in the stroma.⁵⁷ Our results suggest that the translocation of unfolded polypeptides into plastids is compromised in D imbibed seeds. Moreover, the induction of components of the translocation system appears to be concomitant with the accumulation of a substantial number of plastid proteins following dormancy release (Table 2). The cleavage of the transit peptide, upon translocation into plastids, suggested by the position of several spots on the 2D gels corresponding to plastidial proteins supports this hypothesis (Table S1). In addition to their participation in precursor protein import, both cpHsc70-1 and Hsp93-V/CLPC1 participate in protein folding in plastids.⁵⁷ Another stromal protein, ROC4/CYP20-3, which is a member of the peptidyl-prolyl cis/trans isomerase family,⁵⁸ was also up-accumulated in NS and SS seeds (Table 2). It was previously shown that ROC4 could play a role in oxidative stress tolerance due to its interaction with the chloroplast serine acetyltransferase 1, the rate-limiting enzyme in cysteine biosynthesis.⁵⁹ By this mean, ROC4 can affect sulfur metabolism and in particular glutathione synthesis and Met metabolism. Overall, the data accumulated on this protein suggest that it plays an essential role in the chloroplast functions.^{58,60} Therefore, its accumulation upon dormancy release together with the other chaperones discussed above may reflect the activation of plastidic metabolism and a preparation of the transition toward autotrophic growth through photosynthesis during subsequent seedling establishment.⁶¹

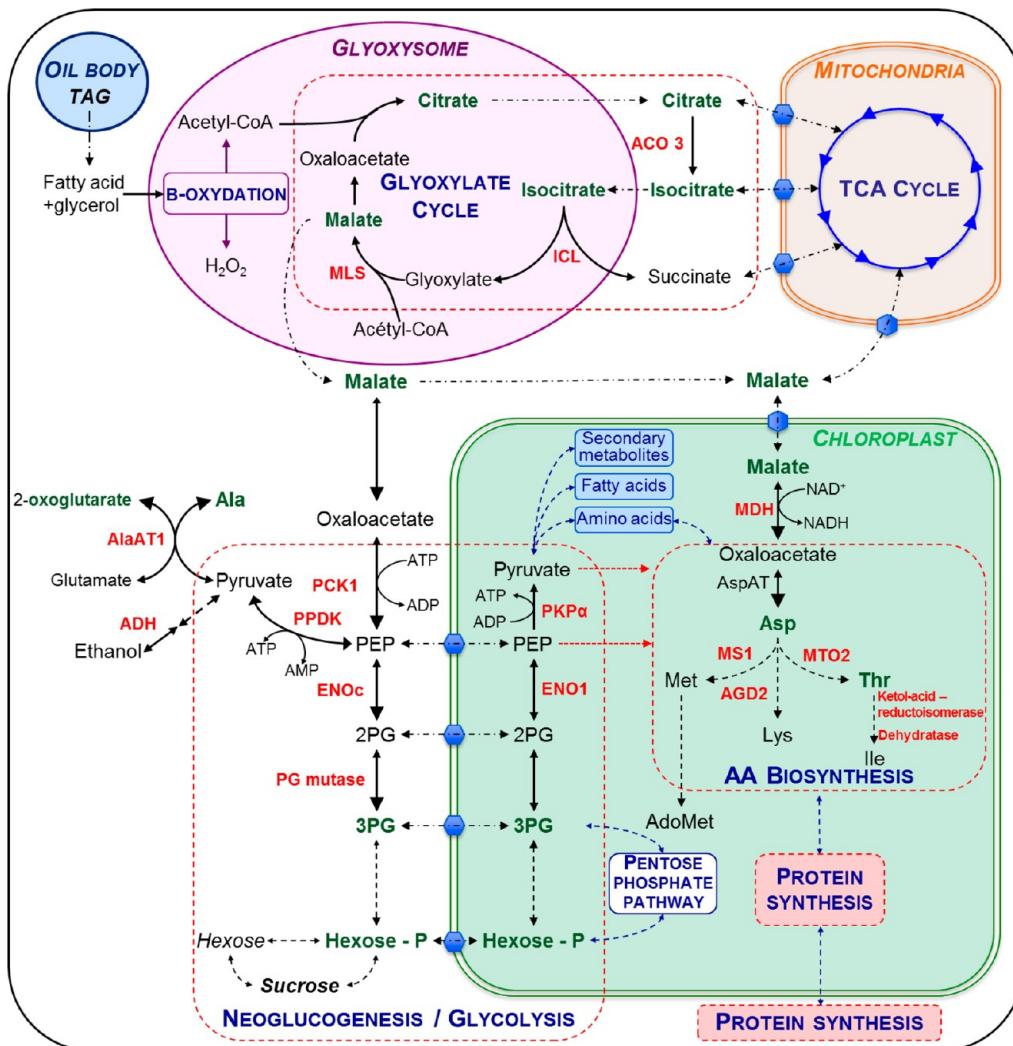


Figure 5. Representation of the metabolic pathways triggered upon dormancy release in *Arabidopsis* seeds. The enzymes identified as being more abundant upon dormancy release by exogenous nitrate or cold stratification are displayed in red. The metabolites previously found to be up-accumulated during the germination of ND seeds are displayed in green (Fait et al.⁶⁴). TAG, Triacylglycerol; 2PG, 2-Phosphoglycerate; 3PG, 3-Phosphoglycerate; PEP, Phosphoenolpyruvate; P, Phosphate; ACO3, Aconitase 3, n°e191; ICL, Isocitrate lyase, n°k76; MLS, Malate synthase, n°L167; MDH, Malate dehydrogenase, n°k102; ADH, Alcohol dehydrogenase, n°L106; PCK1, PEP carboxykinase 1, n°672 and 675; PPDK, Pyruvate orthophosphate dikinase, n°L296; ENOc, Cytosolic enolase, n°e161; ENO1, Enolase 1, n°L3; PKP α , Plastidial pyruvate kinase α -subunit, n°949; PG mutase, Phosphoglycerate mutase 2,3 biphosphoglycerate-independent, n°919; AlaAT1, Ala aminotransferase 1, n°1151 and 1187; MS1, Met synthase, n°k49 and k50; AGD2, L,L-diaminopimelate aminotransferase, n°1496; MTO2, Thr synthase, n°1141 (refer to Table 2 and Table S1 for further details).

Only Specific Energetic Pathways Are Activated upon Dormancy Release

The reserves accumulated during *Arabidopsis* seed filling are progressively mobilized during seed germination and subsequent seedling establishment.^{62–64} Previous reports indicated that oil and protein reserves are mainly mobilized following radicle protrusion.^{63,64} However, the relative contribution of each of the various stored compounds in the metabolic activity of imbibed D seeds or upon dormancy release remains uncertain as well as the involved energetic pathways. Our results indicate that many of the enzymes up-accumulated upon dormancy release are involved in these pathways (Figure 5; Table 2). For example, the key limiting enzymes of the glyoxylate cycle, isocitrate lyase (ICL, n°k76, At3g21720) and malate synthase (MLS, n°L167, At5g03860) are both more abundant in NS and S5 seed samples along with other enzymes involved in this pathway such as the cytosolic aconitase 3

(ACO3, n°e191, At2g05710). Although studies carried on *icl* and *mls* mutants (in a Col-0 background) showed that this cycle is not necessary either for dormancy release or radicle protrusion,⁶⁵ these enzymes contribute to seed vigor under stress conditions.^{66,67} Metabolites generated through the glyoxylate cycle (shared with the TCA cycle) such as citrate, malate and succinate accumulate during stratification and subsequent germination of Col-0 ND seeds.⁶⁴ Malate could well be the main four-carbon product released in the cytosol by both the glyoxylate and the TCA cycles. It can further be metabolized by malate dehydrogenases (MDH) to yield oxaloacetate. At least eight genes encode MDH in *Arabidopsis*, four of which having previously been identified in the germinating seed proteome.^{26,68} In our experiments, a protein spot corresponding to the plastidial MDH (MDH, n°k102, At3g47520) was up-accumulated in S5 and NS seed proteomes suggesting that the malate generated could be used in plastids.

The oxaloacetate resulting from MDH activity can subsequently fuel the gluconeogenesis pathway in the cytosol to produce soluble carbohydrates. In this context, it is noted that several enzymes of this pathway are also more abundant after dormancy breaking by cold stratification or exogenous nitrates (Figure 5; Table 2). These include a pyruvate orthophosphate dikinase (PPDK, n° L296, At4g15530), the cytosolic enolase (ENOc, n° e161, At2g29560), a phosphoglycerate mutase (n° 919, At3g08590) and the phosphoenolpyruvate (PEP) carboxykinase 1 (PCK1, n° 672 and 675, At4g37870). PPDK, which catalyzes the reversible reaction converting pyruvate and ATP to PEP and AMP is surmised to participate in the conversion of protein reserves into carbon skeletons in the cotyledons.^{69,70} PCK1 links the glyoxylate cycle to gluconeogenesis by catalyzing the formation of PEP from oxaloacetate. In germinating seeds or during early postgerminative growth, PCK1 is therefore associated with the ability to mobilize storage lipids and proteins.⁷¹ According to previous studies, PCK1 plays a key regulatory role in the production of hexose phosphates and is required for seedling establishment.^{71,72} Moreover, ABA inhibits PCK1, MLS and ICL expression in the embryo but not in the endosperm, while in both tissues, GAs are still required for the induction of these genes at radicle emergence.⁷³ Accordingly, exogenous ABA application during ND Cvi seed imbibition (for 24 h) resulted in the repression of ICL, MDH, PCK1 and PPDK.²⁶ Therefore, the concomitant up-accumulation of all these enzymes upon dormancy release most presumably reflects the adjustment of the dynamic balance between ABA and GA. Furthermore the specific accumulation of pivotal elements of the glyoxylate cycle and of the gluconeogenesis pathway strongly supports the assumption that reserve mobilization is activated. However, whether these pathways are induced mainly in the endosperm, in the embryo or in both tissues remain unclear in our context.

A possible fate for the carbon derived from seed storage compound mobilization is for chloroplast development.⁷³ In our experiments, the specific up-accumulation of the plastidial MDH seems concomitant with that of several other enzymes targeted to this compartment (Figure 5; Table 2). Among these proteins several are implicated in plastid energetic metabolism such as the plastid-encoded ATP synthase subunit (n° k28AtCg00480), the enolase 1 (ENO1, n° L3, At1g74030) and the pyruvate kinase subunit α (PK α , n° 949, At3g22960). PK α catalyzes the irreversible formation of pyruvate and ATP from PEP and ADP in the stroma.^{74,75} In plastids, PK α and β subunits could assemble into a heterohexamer composed of stoichiometric amounts of both polypeptides.⁷⁶ The *pkp* *Arabidopsis* mutants display delayed seed germination and are compromised in seedling establishment,^{74,75} due to the failure of these mutants to efficiently metabolize seed reserves.⁷⁴ Therefore, the accumulation of PK α along with ENO1 upon dormancy release suggests an induction of plastidial glycolysis that would be fuelled by the stored and neosynthesized carbohydrates. This would lead to the synthesis of PEP and pyruvate, two essential precursors for anabolic reactions such as amino-acid, fatty acid or secondary metabolites biosynthesis.⁷⁷ PK α activity is also important for chlorophyll synthesis.⁷⁴ In addition, dormancy release triggers the accumulation of several enzymes of the Calvin-Benson cycle such as phosphoribulokinase (PRK, n° k81, At1g32060), RUBISCO large subunit (RBCL, n° L320 and L321, AtCg00490) and two transketolases (n° 683 and L171, At2g45290 and At3g60750). Thus, our results strongly indicate a progressive built-up of plastidial

metabolism in preparation of the transition toward autotrophic growth.

The phospholipase D α 1 (PLD α 1, n° e131), was up-accumulated upon dormancy breaking (Table 2). This cytosolic enzyme is involved in ABA signaling in guard cells where it mediates ABA-induced stomatal closure.⁷⁸ During this process, PLD α 1 could act through two distinct mechanisms via phosphatidic acid (PA), its lipid product. First, PA could bind to the ABI1 protein phosphatase 2C thereby inhibiting its activity.⁷⁹ Second, PA could stimulate the NADPH oxidases RbohD and RbohF thus leading to the production of ROS and nitric oxide (NO),⁸⁰ species that can promote seed dormancy release in *Arabidopsis*.^{81,82} Moreover, freshly harvested seeds of the *rbohD* mutant are deeply dormant,⁸² while a PLD antagonist, 1-butanol, proved to block *Arabidopsis* seed germination.⁸³ From these results, one could have expected the *pld α 1* KO mutant seeds to display a higher depth of dormancy. Yet, contrarily to expectation, these mutant seeds are less sensitive to ABA than the WT *Arabidopsis* seeds.^{78,84} In light of our results, we can hypothesize that this protein could play a dual role during seed germination. Depending on the presence of ABI1 and RbohD/RbohF, PLD α 1 may either enhance ABA signaling via ABI1 inhibition or promote dormancy release via NADPH oxidase-mediated ROS production.

Dormancy Releasing Treatments May Activate Amino Acid Biosynthesis and/or Recycling Pathways

One possible fate for the carbon skeletons derived from seed storage compound mobilization is the biosynthesis of amino-acids. As in plants many of them are synthesized in plastids, the specific accumulation of the plastidial MDH discussed above may drive the malate produced to oxaloacetate, which can further be transformed into Asp by the plastidial Asp aminotransferase (Figure 5), from which Lys, Met, Thr and Ile can subsequently be synthesized.⁸⁵ Several enzymes of these pathways are more abundant in S5 and N5 proteomes than in I5 proteome (Figure 5; Table 2), as is the case for a Thr synthase (MTO2, n° 1141, At4g29840) and two isoforms of the Met synthase (AtMS1, n° k49 and k50, At5g17920; Figure 4b). In agreement with this, metabolomic profiling of germinating ND Col-0 seeds revealed a marked accumulation of Thr and Asp during cold stratification.⁶⁴ In addition, Met synthesis is a key element of seed vigor.²³ Exogenous ABA application during imbibition for 24 h of ND Cvi seeds resulted in the repression of the two same isoforms of AtMS1.²⁶ Altogether, this reinforces the finding that the elevated ABA level associated with dormancy maintenance⁷ could be responsible for the repression of Met biosynthesis in plastids. In addition to the plastidial pathways, two isoforms of the cytosolic alanine aminotransferase (AlaAT1, n° 1151 and 1187, At1g17290) are up-accumulated upon dormancy release (Table 2). This enzyme catalyzes the reversible conversion of glutamate and pyruvate into 2-oxoglutarate and Ala. AlaAT1 abundance increases during ND Col-0 seed germination⁸⁶ in parallel with the Ala content.⁶⁴ Overall, it appears that interconversions between amino-acids and their *de novo* synthesis pathways are triggered upon dormancy release. This induction would result in a higher pool of free amino-acids to support protein synthesis.

The Differences between Transcriptome and Proteome Data Sets Reinforce the Importance of the Post-Transcriptional Controls of Gene Expression during Seed Dormancy Release and Germination

The large number of published microarray data on seed dormancy and germination provide unprecedented knowledge on the transcriptional and post-transcriptional regulations of gene expression occurring during these processes. To fill the gap up to physiological phenotypes, it appears important to investigate the translational and post-translational regulations and to establish the links between all these levels contributing to gene expression control. To this end, the proteomic data generated during this work were crossed with different sets of published expression data.

In marked contrast with the previously discussed findings for IS seeds, most of the proteins that accumulated upon dormancy release (i.e., in NS and S5 seed samples) do not match the transcripts prominently abundant in dry seeds (Figure S2; Table S1).²⁰ Indeed, half of these proteins correspond to mRNAs that accumulate at least temporarily upon imbibition during the germination of ND seeds (Figure S3b; Table S1).³⁴ However, the others transcripts were either classified as remaining constant or decreasing in abundance and, only a few genes were reported as being repressed by ABA or induced by GAs (Figure S4b,c; Table S1).^{36,37} Therefore, mRNA and protein abundances do not appear directly correlated to hormone levels upon dormancy release (S5 and NS) whereas the link with ABA levels seems obvious when dormancy is maintained (IS). On the other hand, most of the protein spots previously detected in another proteomic analysis carried on Ler seeds increased in abundance either during germination *sensu stricto* or during radicle protrusion (Tables 1 and 2; Table S1).⁶⁸

Finch-Savage et al.²⁰ carried out a comparison of the transcriptome of different Cvi seed samples exhibiting contrasted depth of dormancy. Among these, three were quite similar to those analyzed in the present work such as the D seeds imbibed for 24-h in the light on water (PDL), with 10 mM KNO₃ (PDLN), or cold-stratified during 3 days in darkness (PDC). Therefore, we used the data set generated by these authors to investigate possible correlations between transcript and protein abundances. For each of the polypeptides identified in this comparison, we first looked at the normalized abundance value of the corresponding transcripts in PDL, PDC and PDLN (Table S1). It appears that the proteins more abundant in IS seeds matched transcripts that were more abundant in PDL than in PDC or PDLN seeds (Figure S5a). On the other hand, no significant differences were observed when comparing the profiles corresponding to the proteins more abundant in the S5 and NS samples (Figure S5c). To further document these findings, the log-ratios PDLN/PDL and PDC/PDL were calculated for all the transcripts, allowing classifying them in three groups (namely corresponding to up-, stable or down-regulation) based on a ± 0.5 threshold (Figure S5b,d). Most of the mRNAs matching the IS proteins were down-accumulated by both dormancy-releasing treatments (Figure S5b). However, most of the transcripts corresponding to the protein spots accumulating in S5 and NS seed samples were not affected by the dormancy-releasing treatments (Figure S5d). Thus, the transcriptome of PDL seeds appears somehow correlated to the proteome of imbibed D seeds (IS) despite the difference of imbibition time used (1 day vs 5 days). On the other hand, no significant correlation is noted between the

abundance of transcripts in PDC/PDLN and the protein spots accumulated upon dormancy release (S5 and NS) even though PDC and S5 corresponded to the most similar physiological conditions in the two studies. These discrepancies between proteome and transcriptome variations could reflect the importance of translational and/or post-translational levels of regulation along with the control of mRNA and protein turnover.⁸⁷ Whether transcription is required for dormancy release remains a matter of debate.¹⁹ Preliminary experiments using α -amanitin, a specific inhibitor of the DNA-dependent RNA polymerase II,⁸⁸ suggests that *de novo* mRNA synthesis is not required for dormancy release by cold stratification (Figure S6).

Chibani et al.²⁶ reported significant differences between the proteome of 24-h imbibed Cvi D and ND seeds in presence of exogenous ABA. However, in our experiments, we found that several of the proteins accumulated (more abundant in IS seeds, Table 1) or repressed (i.e., being more abundant in S5 and NS seeds, Table 2) during prolonged imbibition associated with dormancy maintenance were among those induced by exogenous ABA and/or matched ABA regulated transcripts. Therefore, the exogenous application of a nonphysiological concentration of ABA to ND Cvi seeds may have promoted the induction of molecular mechanisms that normally occur later during the imbibition of D seeds, presumably concomitantly with the reinduction of ABA synthesis.

CONCLUSION

Depending on the time of dry storage and of the environmental conditions, seeds with very similar initial pool of stored mRNA and proteins can either proceed toward radicle protrusion or stay in a dormant state and recapitulate the late maturation program. Thus, the first phases of the germination process constitute a key developmental checkpoint⁸⁹ during which the regulation of ABA metabolism will clearly represent a pivotal element of seed dormancy control. As the maintenance of this blockage can result from inappropriate environmental conditions, the observed induction stress response proteins can be determinant for seed survival. In addition, the metabolic transitions associated with germination seem to be repressed at least by a reduced accumulation of key metabolic enzymes. After prolonged imbibition of D seeds, the proteome is marked by a lower abundance of important components of the energetic, amino acid, and protein metabolisms suggesting that these processes could be reduced to a low level as a way to avoid energy waste and reserve consumption in the absence of germination. Furthermore, preparation of postgerminative growth is not observed at the proteome level when dormancy is maintained. On the contrary, upon dormancy release, the seed potential to mobilize stored reserves to provide energy and carbon for the nascent seedling increases substantially. Finally, the comparison of our results with previously published -omic data sets and the results obtained following the application of a transcription inhibitor during dormancy release reinforce and extend the assumption that post-transcriptional, translational, and post-translational regulation are determinant for germination. In light of our findings, complementary experiments aiming to assess the respective contribution of transcription, mRNA metabolism, and translation (with particular emphasis on its selectivity) in seed dormancy control seem highly required. In addition, our approach revealed new protein candidates whose roles during the germination process remain to be elucidated. However, it did not allow us to identify all the

protein spots detected as affected by dormancy release. Thus, the proteome adjustments underlying this physiological transition go beyond this first glimpse. Further experiment relying on subcellular fractionation and selective enrichment would probably allow to complete our current overview on dormancy release.

■ ASSOCIATED CONTENT

§ Supporting Information

Supporting Information Material S1, protein extraction and separation by 2D electrophoresis; Supporting Information Material S2, detailed protocol for protein identification by mass spectrometry; Supporting Information Table S1, complete protein list including information on identification and correlations with different -omics data set; Supporting Information Figure S1, reference two-dimensional gel maps; Supporting Information Figure S2, correlation between protein and stored mRNA abundances in dry *Arabidopsis* seeds (data from Finch-Savage et al.²⁰); Supporting Information Figure S3, comparison of proteins differentially accumulated upon dormancy release with the corresponding transcript abundance variations during nondormant seed germination (data from Narsai et al.³⁴); Supporting Information Figure S4, comparison of proteins differentially accumulated upon dormancy release with the ABA- and GA-regulated transcripts (data from Okamoto et al.³⁷ and Cao et al.³⁶); Supporting Information Figure S5, comparison of our proteome data set with the transcriptome results obtained on similar samples by Finch-Savage et al.²⁰; Supporting Information Figure S6, *Arabidopsis* seed (accession Cape Verde Islands, Cvi) dormancy release by cold stratification in presence of α -amanitin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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