Proteome-Wide Characterization of Seed Aging in Arabidopsis: A Comparison between Artificial and Natural Aging Protocols[W][OA]

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A variety of mechanisms have been proposed to account for the extension of life span in seeds (seed longevity). In this work, we used Arabidopsis (*Arabidopsis thaliana*) seeds as a model and carried out differential proteomics to investigate this trait, which is of both ecological and agricultural importance. In our system based on a controlled deterioration treatment (CDT), we compared seed samples treated for different periods of time up to 7 d. Germination tests showed a progressive decrease of germination vigor depending on the duration of CDT. Proteomic analyses revealed that this loss in seed vigor can be accounted for by protein changes in the dry seeds and by an inability of the low-vigor seeds to display a normal proteome during germination. Furthermore, CDT strongly increased the extent of protein oxidation (carbonylation), which might induce a loss of functional properties of seed proteins and enzymes and/or enhance their susceptibility toward proteolysis. These results revealed essential mechanisms for seed vigor, such as translational capacity, mobilization of seed storage reserves, and detoxification efficiency. Finally, this work shows that similar molecular events accompany artificial and natural seed aging.

Before aging ultimately and irreparably leads to seed death, punctual and progressive accumulation of alterations during storage are likely to affect the potential ability of seeds to germinate. This deterioration process can occur even under the ''best'' storage conditions. The life span of seeds is determined by their genetic and physiological storage potential and by any deteriorating events that occur prior to or during storage as well as by the interaction with environmental factors (Bewley and Black, 1994). Since seed storage is often accompanied by a progressive loss of germination vigor, storage conditions must be optimized for both the preservation of genetic resources and commercial applications. For orthodox or desiccation-tolerant seeds, low seed moisture content, low temperature, or cryopreservation seems to result in an increase in storage life span (Abdalla and Roberts, 1968; Walters, 2004; Walters et al., 2004). However, a recent investigation reported a large heterogeneity and inequality for longevity between seeds descended from different plant species (Walters et al., 2005). Genetic approaches in rice (*Oryza sativa*; Miura et al., 2002) and Arabidopsis (*Arabidopsis thaliana*; Bentsink et al., 2000; Clerkx et al., 2004b) showed that seed longevity is controlled by several genetic factors. For both plants, several quantitative trait loci were identified as affecting viability, and these were located on different chromosomes. This behavior suggests that seed longevity is a multigenic trait including various seed traits, including germination under various stresses or Suc and seed oligosaccharide contents (Clerkx et al., 2004b). Several molecular studies also support this notion of the complex genetic basis of seed longevity, as diverse mechanisms were documented to play a role. For example, Arabidopsis seed mutants affected in flavonoid (Debeaujon et al., 2000) or in tocopherol (Sattler et al., 2004) biosynthetic pathways display reduced longevity, a finding that agrees with data showing that protection against reactive oxygen species (ROS) production and attack are important features of Arabidopsis seed longevity (Clerkx et al., 2004a). Furthermore, Bentsink et al. (2006) recently showed that mutations within the *DOG1* gene, which specifically controls seed dormancy in Arabidopsis, were associated with a seed longevity phenotype, indicating that the absence of

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dormancy might also be a factor that reduces seed longevity. Also, transgenic Arabidopsis seeds overaccumulating a heat stress transcription factor exhibit enhanced accumulation of heat shock proteins (HSPs) and improved resistance to aging (Prieto-Dapena et al., 2006). On the contrary, a high level of a membrane lipid-hydrolyzing phospholipase D (PLDα1) seems detrimental for seed quality (Devaiah et al., 2007). Finally, the fantastic multicentenarian longevity of sacred lotus (*Nelumbo nucifera*) seeds (Shen-Miller et al., 1995; Shen-Miller, 2002) has been correlated with the extractible activity of the protein L-isoaspartyl methyltransferase, an enzyme repairing abnormal L-isoaspartyl residues accumulated in proteins during aging, notably during oxidative stress (Ingrosso et al., 2002; Clarke, 2003; Xu et al., 2004).

We are interested in determining the molecular basis of seed longevity. For that purpose, the model plant Arabidopsis can be viewed as a reference species allowing a molecular dissection of this trait. Indeed, achievement of the Arabidopsis genome sequence (Arabidopsis Genome Initiative, 2000) markedly increased our knowledge and understanding of the large complexity of plant growth regulation and development (Somerville and Koornneef, 2002). Global approaches such as transcriptome profiling (Ogawa et al., 2003; Nakabayashi et al., 2005; for review, see Holdsworth et al., 2008) have proved useful for the characterization of potential biomarkers of seed quality and germinative capacity. However, the functional components of a biological system are proteins and metabolites. Thanks to the availability of genomic sequence information and based on the progress achieved in sensitive and rapid separation of proteins and in their high-throughput identification by electrophoresis and mass spectrometry, proteomic approaches have opened up new perspectives to analyze the complex functions of model plants and crop species (Cánovas et al., 2004; Park, 2004; Agrawal et al., 2005a, 2005b, 2005c; Rossignol et al., 2006; Jorrín et al., 2007). In this way, previous proteomic studies revealed the requirements of RNA and protein synthesis for Arabidopsis seed germination (Gallardo et al., 2001, 2002a, 2002b; Rajjou et al., 2004, 2007a). In particular, these studies revealed that proteins and mRNAs stored in the dry mature seeds are sufficient for germination sensu stricto (Rajjou et al., 2004).

Based on these previous findings, in this work we have used proteomics and a seed deterioration treatment known as controlled deterioration (CDT) that is presumed to mimic natural aging (Delouche and Baskin, 1973; Bentsink et al., 2000; Clerkx et al., 2004b) to unravel the mechanisms of seed vigor loss during storage. Sensitivity of seeds to CDT has been successfully used for the rapid evaluation and prediction of seed vigor and longevity (Powell, 1995; TeKrony, 1995; Lanteri et al., 1996; McDonald, 1999; Bentsink et al., 2000; Halmer, 2000; Clerkx et al., 2004a, 2004b; Sattler et al., 2004; Job et al., 2005; Prieto-Dapena et al., 2006). Accordingly, this treatment is widely used by seed companies as a vigor assay for numerous seed species and has been described for Arabidopsis seeds (Tesnier et al., 2002). Here, we compared Arabidopsis seed samples submitted to CDT for different times up to 7 d. A comparison of the dry seed proteome for each sample was carried out to reveal changes in the accumulation of specific proteins during the treatment. The proteome of 1-d-imbibed seeds was also characterized for all seed samples to analyze the behavior of the treated seeds during early steps of the germination process. Since CDT and prolonged seed storage are known to entail an oxidative stress (Goel et al., 2003; Bailly, 2004; Job et al., 2005; Kibinza et al., 2006), which can lead to the formation of oxidatively modified proteins (Terskikh et al., 2008), we also analyzed the oxidized proteome in the deteriorated seeds. Finally, we discuss our results in comparison with natural aging conditions.

RESULTS AND DISCUSSION

CDT Entails a Seed Vigor Loss

The CDT protocol described by Tesnier et al. (2002) was used to alter seed vigor of wild-type Arabidopsis ecotype Landsberg *erecta* (L*er*) seeds for up to 7 d (see "Materials and Methods"). The germination ability of each seed sample was assessed by germination assays (Fig. 1). CDT led to a rapid decline of seed vigor, affecting the speed, homogeneity, and final extent of Arabidopsis seed germination (Fig. 1).

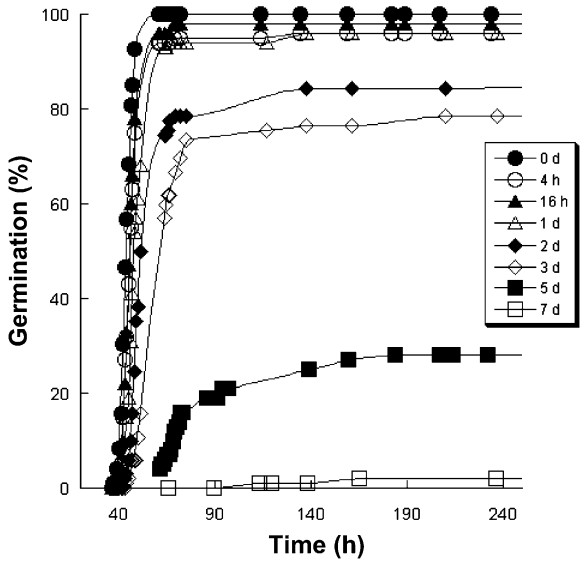

Figure 1. Influence of CDT on Arabidopsis seed germination. Seeds were submitted to CDT for different periods (black circles, 0 d; white circles, 4 h; black triangles, 16 h; white triangles, 1 d; black diamonds, 2 d; white diamonds, 3 d; black squares, 5 d; white squares, 7 d) as described in "Materials and Methods." The graph shows a representative experiment carried out three times in triplicate.

Rationale of the Proteomic Approach

To reveal molecular mechanisms associated with the loss of seed vigor induced by CDT, a differential proteomic approach was used under two different protocols. In the first, we hypothesized that CDT can directly affect the proteome of the seeds, and hence their vigor. In the second, we analyzed whether the loss in seed vigor imposed by CDT resulted from incapacity of the deteriorated seeds to appropriately set up the protein changes normally accompanying early germination (Gallardo et al., 2001; Rajjou et al., 2004).

Total soluble protein extracts from all seed samples (control and deteriorated seeds) were separated by two-dimensional (2D) PAGE. Following silver nitrate staining, protein patterns were determined by image analysis, and protein spots were quantified by Image Master 2D Elite software. Because of the very high reproducibility of 2D protein patterns compared with our previous work (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005), a number of proteins of interest could be readily identified using previously established reference maps for the Arabidopsis seed proteome (http://www.seed-proteome.com). Besides, this study allowed the identification of 87 novel proteins that accumulate in Arabidopsis seeds (Supplemental Table S2).

The Proteome Is Markedly Affected in Seeds Submitted to CDT

A typical 2D gel corresponding to the control nondeteriorated seeds is shown in Figure 2A. Image analysis of 2D protein patterns from control and deteriorated seed samples revealed 12 protein spots that were more abundant in the deteriorated seeds, corresponding to one chloroplastic and 10 nuclear genes. In parallel, six protein spots showed a lower abundance in the deteriorated seeds, corresponding to five genes (Fig. 2; Tables I and II). The time needed to reach 50% of the change in spot accumulation ($T_{1/2}$) during the progress of CDT is a good index of the sensitivity of each of these protein spots toward deterioration. This kinetic analysis disclosed that proteins whose accumulation was altered by CDT displayed a wide range of sensitivity toward this treatment (Tables I and II), with $T_{1/2}$ values ranging from 0.4 to more than 7 d. This finding is illustrated in Figure 3 for protein spots whose relative volume increased (spots 253 and 312) or decreased (spots 146 and 311) during the progress of CDT.

Our results clearly reveal that, despite an expected metabolic quiescent state and a relatively low water content characteristic of the seeds, proteome variations can occur under the low hydration conditions of CDT, as CDT only increases the seed water content from 5.8% to 10.5% (Tesnier et al., 2002). It remains to be determined whether these changes arose from de novo transcription, translation of stored mRNAs, or nonenzymatic modifications of the seed proteins, altering their migration on 2-D gels.

The Glycolytic Pathway Is Affected during Seed Aging

Among the 12 proteins that were more abundant in deteriorated seeds than in control seeds, four of them belonged to the glycolytic pathway (Table I). Thus, the abundance of these protein spots, corresponding to glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12; spots 253 and 302; and EC 1.2.1.13; spot 312) and to phosphoglucomutase (EC 2.7.5.1; spot 305), significantly increased in seeds submitted to CDT. Interestingly, a recent study demonstrated that Arabidopsis cells exposed to oxidative stress react by substantially increasing the levels of hexose phosphates, Glc-6-P and Fru-6-P, as well as 3-phosphoglycerate (Baxter et al., 2007). Our results thereby confirmed that seeds underwent an oxidative stress during CDT (Goel et al., 2003; Bailly, 2004; Sattler et al., 2004; Job et al., 2005; Kibinza et al., 2006) and mounted a protective response through modification of the glycolytic pathway.

The β-Mercaptopyruvate Sulfurtransferase Exhibits Varying Accumulation Levels during Seed Aging

The protein β-mercaptopyruvate sulfurtransferase (MST; EC 2.8.1.2; spot 146; Figs. 2 and 3; Table II) was abundant in control high-vigor seeds (0 d). However, during CDT, the level of this protein showed an important decline. The MST catalyzes the transfer of sulfur from mercaptopyruvate to sulfur acceptors such as thiols and cyanide (Papenbrock and Schmidt, 2000a, 2000b), presumably contributing to cyanide detoxification (Cipollone et al., 2007). Despite the fact that at low concentrations cyanide is beneficial for releasing seed dormancy and improving germination (Taylorson and Hendricks, 1973; Bethke et al., 2006), its production is often associated with deleterious mechanisms and therefore must be controlled. Also, cyanide can inhibit the activity of heme proteins such as peroxidases (Ellis and Dunford, 1968; Job and Ricard, 1975) and catalases (Tejera García et al., 2007). Furthermore, this molecule is a potent inhibitor of mitochondrial ascorbate synthesis in plants (Bartoli et al., 2000), thus potentially impeding plant defense against ROS attack. Most frequently, cyanide production in plants results from the catabolism of cyanogenic glycosides or during cyanolipid hydrolysis (Poulton, 1990). It has been shown that the cyanide potential of sorghum (*Sorghum* spp.), a plant containing high levels of hydrogen cyanide, shows a rapid transient increase during germination and early seedling formation (Busk and Møller, 2002). Similarly, a recent work showed that imbibition of dormant and nondormant sunflower (*Helianthus annuus*) embryos entails a substantial rise in cyanide content (Oracz et al., 2008). However, no data are available on the metabolism of cyanogenic compounds in dry and germinating Arabidopsis seeds. In plants, cyanide can also be released as a by-product of ethylene biosynthesis (Adams and Yang, 1981; Peiser et al., 1984). However, temporal patterns of accumulation of enzymes involved in Met and *S*-adenosyl-Met

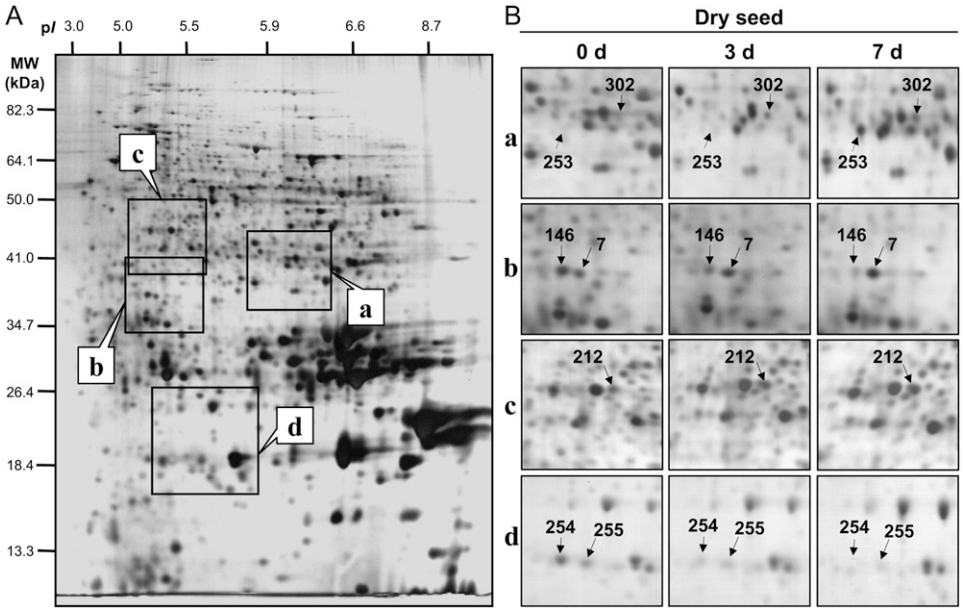

Figure 2. Influence of CDT on the proteome of Arabidopsis seeds. An equal amount (150 μg) of total soluble protein extracts was loaded on each gel. A, Silver-stained 2D gel of total soluble proteins from nondeteriorated seeds (0 d, control seeds). The indicated portions of the gel (a, b, c, and d) are reproduced in B. B, Enlarged windows (a–d) of 2D gels as shown in A for nondeteriorated seeds (left), 3-d deteriorated seeds (middle), and 7-d deteriorated seeds (right). The seven labeled protein spots (spots 7, 146, 212, 253, 254, 255, and 302) were identified by mass spectrometry and by comparison with Arabidopsis seed protein reference maps (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com; Tables I and II; Supplemental Table S2). Protein spot quantitation was carried out as described in ''Materials and Methods'' from at least five gels for each seed sample.

synthesis in seeds are consistent with an essential role of endogenous ethylene in Arabidopsis only after radicle protrusion (Gallardo et al., 2002b), findings that do not favor the hypothesis that cyanide accumulates to high toxic levels through the ethylene pathway during germination sensu stricto. Finally, the decomposition of glucosinolates has also been suggested as a possible source of cyanide production in brassicaceous plants (Cipollini and Gruner, 2007). The glucosinolates of Arabidopsis seeds are distinguished by their high concentration, unique aliphatic constituents, and low level of indole compounds (Brown et al., 2003). However, the cyanide production from stored-seed glucosinolates has never been observed but should be investigated in the context of seed biology. Besides cyanide detoxification, proposed roles for sulfurtransferases are sulfur metabolism (Donadio et al., 1990) and the mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Pagani et al., 1984; Bonomi et al., 1985). Also, MST plays a physiological role in the protection against oxidative stress, and it particularly contributes to the maintenance of cellular redox homeostasis via the metabolic regulation of Cys degradation (Nagahara and Katayama, 2005). In plants, the mobilization of sulfur for transport processes in older leaves was also proposed (Papenbrock and Schmidt, 2000a, 2000b).

Our results reveal, for the first time to our knowledge, that a loss in seed vigor is associated with a decreased level of MST, highlighting further the importance of sulfur metabolism and homeostasis in seeds (Gallardo et al., 2002b). Furthermore, our data suggest as yet unknown important role(s) of this enzyme in seed physiology and quality.

The Dehydrin/RAB Group of LEA Proteins Contributes to Seed Vigor

Our proteomic analysis revealed two protein spots (spots 254 and 255) progressively disappearing in seeds according to the time of CDT. These spots correspond to two isoforms of the RAB18 (Responsive to ABA18) dehydrin, belonging to the LEA group 2 (D11) protein family. This result was unexpected, because a previous work showed an absence of correlation between accumulation of the dehydrin/RAB group of LEA proteins and seed longevity (Wechsberg et al., 1994). These proteins are inducible by dehydration and abscisic acid (Skriver and Mundy, 1990) and have been suggested to play a role in desiccation tolerance, particularly during seed development (Dure, 1993; Close, 1996, 1997). Expression of the RAB18 gene is high in Arabidopsis dry mature seeds at both the mRNA and protein levels (Lång and Palva, 1992; Parcy et al., 1994; Rajjou et al., 2004). Such a high expression could correspond to a remnant accumulation during the maturation program of seed development, in response to the acquisition of desiccation tolerance. However, it is also likely that RAB18 expression is needed to prevent environmental stress that may

Table I. *Arabidopsis proteins whose abundance significantly increased in dry mature seeds according to controlled deterioration time*
AGI, Arabidopsis Genome Initiative.

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]	$T_{1/2}$
	kD			*kD*				*ratio 7 d:0 d*	*d*
7[c]	37.55	5.09	60S acidic ribosomal protein	34.37	5.08	At3g11250	24%	3.4 ± 0.1	2.2 ± 0.3
212[d]	42.57	5.18	Actin 2	41.21	5.43	At3g18780	33%	2.5 ± 0.4	5.2 ± 2.7
253[d]	40.29	5.84	Glyceraldehyde-3-P dehydrogenase, cytosolic	36.99	6.34	At3g04120	18%	>16	>7
293[d]	96.69	5.61	Peptidase M1 family protein	103.40	6.09	At1g63770	4%	>5.1	>7
302[d]	40.34	6.03	Glyceraldehyde-3-P dehydrogenase	36.90	7.21	At1g13440	30%	7.7 ± 1.4	6.1 ± 2.0
305[d]	68.25	5.70	Phosphoglucomutase	63.46	5.57	At1g70730	34%	2.2 ± 0.1	1.3 ± 0.4
308[c]	28.52	5.89	α-Cruciferin 12S (seed storage protein fragment)	50.56	6.53	At1g03880	10%	3.4 ± 0.5	4.8 ± 1.7
312[c]	14.21	3.82	NADP-dependent glyceraldehyde-3-P dehydrogenase (fragment)	53.04	6.61	At2g24270	8%	6.0 ± 0.3	1.8 ± 0.3
320[c]	57.36	5.82	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	24%	>2.7	>7
321[c]	57.36	5.84	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	28%	>2.8	>7
322[c]	35.21	5.20	60S acidic ribosomal protein	33.65	4.93	At2g40010	15%	>4.4	>7
376[d]	57.35	4.92	Tubulin β-8 chain	50.59	4.46	At5g23860	9%	5.0 ± 0.2	1.5 ± 0.2

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Data obtained from densitometric analysis of individual spots from proteins on 2D gels stained with silver nitrate (see Fig. 2A for an example of a 2D gel): normalized spot volume in the deteriorated seeds (7 d of CDT) divided by the normalized spot volume in the nondeteriorated control seeds (0 d of CDT), from five different gels and three independent extractions. [c]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005). [d]Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2.

occur at the start of the germination process (Lopez-Molina et al., 2002; Rajjou et al., 2004, 2006a). The high hydrophilicity and thermostability of dehydrins suggest their involvement in large-scale hydrophobic interactions, such as those of membrane structures or hydrophobic patches of proteins with chaperone-like properties (Ismail et al., 1999; Borovskii et al., 2002). Interestingly, in seeds from cabbage (*Brassica oleracea*), a related cruciferous species, mRNA levels of the RAB18 homolog are correlated with seed stress tolerance (Soeda et al., 2005). Thus, its transcript level increased during seed maturation and declined during

Table II. *Arabidopsis proteins whose abundance significantly decreased in dry mature seeds according to controlled deterioration time*

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]	$T_{1/2}$
	kD			*kD*				*ratio 7 d:0 d*	*d*
4[c]	57.35	4.89	Tubulin β2β3 chain	50.73	4.7	At5g62700	35%	0.26 ± 0.01	0.17 ± 0.05
146[c]	37.67	5.03	MST	41.89	5.95	At1g79230	35%	0.33 ± 0.05	1.12 ± 0.33
254[c]	22.1	4.96	Dehydrin	18.44	7.95	At5g66400	6%	0.13 ± 0.04	0.93 ± 0.15
255[c]	21.65	5.18	Dehydrin	18.44	7.95	At5g66400	6%	<0.22	>7
304[c]	69.3	5.56	Phosphoglucomutase	63.44	5.73	At1g70730	37%	0.30 ± 0.04	0.37 ± 0.12
311[c]	14.1	3.2	β-Cruciferin 12S (seed storage protein fragment)	21.20	6.19	At4g28520	11%	0.19 ± 0.01	0.56 ± 0.04

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Data obtained from densitometric analysis of individual spots from proteins on 2D gels stained with silver nitrate (see Fig. 2A for an example of a 2D gel): normalized spot volume in the deteriorated seeds (7 d of CDT) divided by the normalized spot volume in the nondeteriorated control seeds (0 d of CDT), from five different gels and three independent extractions. [c]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005).

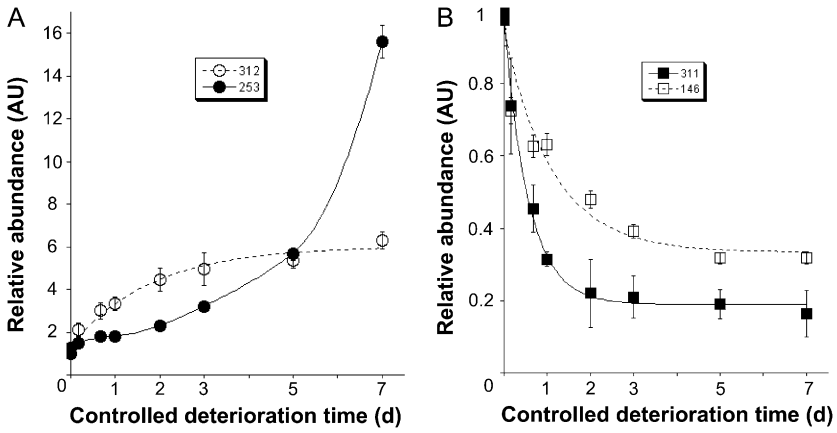

Figure 3. Dynamic evolution of protein spot abundance in Arabidopsis seeds submitted to CDT. The relative abundance of protein spots was calculated by dividing the normalized spot volumes in the deteriorated seeds (4 and 16 h, and 1, 2, 3, 5, and 7 d) by the corresponding normalized spot volumes in the nondeteriorated seeds (0 d). A, Time courses of relative abundance increase in deteriorated seeds during CDT for the two protein spots (spots 253 and 312). B, Time courses of relative abundance decrease in deteriorated seeds during CDT for the two protein spots (spots 146 and 311). Protein spot quantitation was carried out as described in ''Materials and Methods'' from at least five gels for each seed sample. Solid lines were obtained by nonlinear regression analysis using the following equations: relative abundance of spot = $a - b.\exp(-t/T_{1/2})$ and relative abundance of spot = $a + b.\exp(-t/T_{1/2})$, for increase and decrease in relative abundance, respectively, where a and b are constant parameters, $T_{1/2}$ is the time (days) needed to reach half variation in spot volume during the CDT, and t is the time of CDT (days). $T_{1/2}$ values are listed in Tables I, II, IV, and V.

priming (an invigoration treatment of seeds based upon their controlled hydration; Heydecker et al., 1973) and germination. Furthermore, the mRNA can be reinduced during a slow and warm drying treatment of primed seeds to increase their storability (Soeda et al., 2005). As in cabbage, our results suggest that RAB18 protein abundance in dry mature Arabidopsis seeds is strongly correlated with seed aging. One possible explanation is that the decreased level of this protein in deteriorated seeds is associated with membrane destabilization and/or alterations in protein structure. It is noted that RAB18 protein displays both cytosolic and nuclear localization, suggesting multiple functionalities as yet unclear. In this proteomic study, we have not identified dehydrins other than the RAB18 dehydrin. This could mean that only this member of the large LEA protein family (Bies-Ethève et al., 2008) plays a role in seed vigor. Alternatively, we cannot exclude the possibility that other LEA proteins are also involved in seed vigor but are present at too low levels, so their detection escaped our analysis.

Seed Deterioration Entails a Massive Increase in Carbonylated Proteins

In all organisms, oxidative stress has been postulated to be a causal factor in aging processes (Harman, 1956). The extent of oxidative damage to nucleic acids, lipids, and proteins has been found to increase with age, providing support for this basic tenet (Levine and Stadtman, 2001). Indeed, a progressive accumulation of oxidative damage of these macromolecules in aged tissues is thought to contribute to the decline in biological functions characteristic of the aged phenotype (Stadtman, 2001, 2004). There is strong evidence that proteins are the most important targets for oxidants (Davies, 2005). Protein carbonylation has been widely used as an indicator of oxidative damage in several organisms and has been shown to increase in aged tissues (Nyström, 2005; Møller et al., 2007). It results from oxidative attack on Arg, Lys, Pro, or Thr residues of proteins (Levine et al., 1990), which can affect enzyme activities or alter susceptibility of the modified proteins to proteolysis (Berlett and Stadtman, 1997; Davies, 2005). We characterized the influence of CDT on the oxidized proteome of Arabidopsis seeds (Fig. 4). Carbonylated proteins were then identified by matching the 2,4-dinitrophenylhydrazone (DNP)-derivatized protein spots to master gel maps of Arabidopsis seed proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com). The results revealed that protein carbonylation strongly increased in deteriorated seeds, indicating the occurrence of ROS during CDT. In agreement with previous data (Job et al., 2005; Rajjou et al., 2006a, 2007a, 2007b), several polypeptides corresponding to the α- and β-subunits of the 12S cruciferins (legumin-type seed storage proteins) were strongly carbonylated in deteriorated seeds compared with control seeds. However, the present work also revealed that several other proteins ought to be oxidized in Arabidopsis seeds submitted to CDT (Fig. 4; Table III). Among them, several isoforms of the Rubisco large subunit (spots 10, 319, 320, and 321) proved to be oxidized. Rubisco catalyzes the first step in net photosynthetic CO_2 assimilation and photorespiratory carbon oxidation. This protein has already been shown

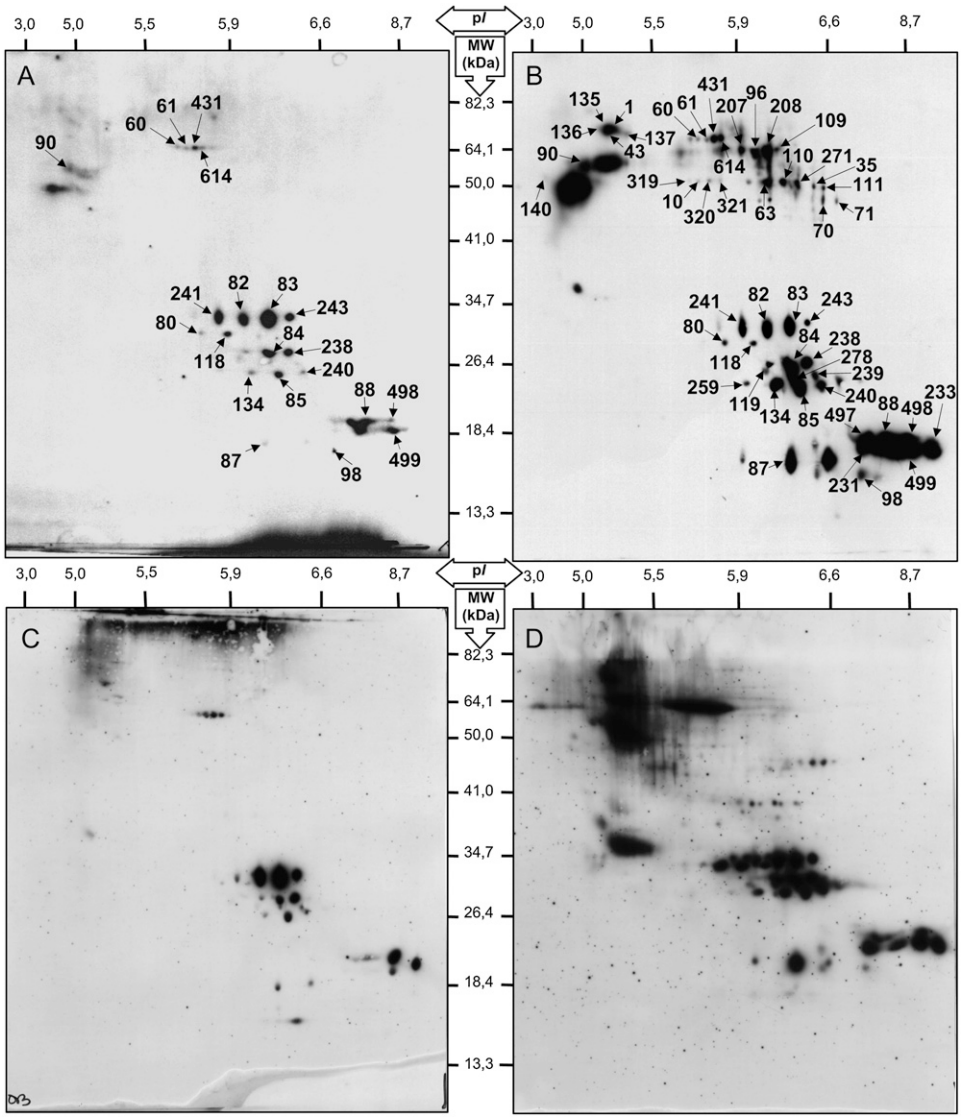

Figure 4. Protein carbonyl patterns in Arabidopsis seeds following CDT or natural aging. Protein extracts were prepared as described in "Materials and Methods" from the dry mature seeds and analyzed by 2D PAGE. Carbonylated proteins were characterized in nondeteriorated seeds (0 d, control seeds; A), 7-d deteriorated seeds (B), freshly harvested seeds (C), and 11-year-old dry mature seeds (D). Proteins were separated by 2D gel electrophoresis as shown in Figure 2A. Following transfer to nitrocellulose, the appearance of carbonyl groups in proteins was analyzed by immunodetection of protein-bound DNP after derivatization with the corresponding hydrazine, as described (Job et al., 2005). Proteins undergoing carbonylation are labeled with black arrows; they are listed in Table III. These findings show that both CDT and natural aging induce an oxidative stress on specific proteins, presumably through the generation of ROS.

to be a preferential target of ROS in Arabidopsis (Johansson et al., 2004; Job et al., 2005). Also, previous work demonstrated that one of the first apparent symptoms of leaf senescence is the aggregation and the deterioration of Rubisco (Feller and Fischer, 1994). Finally, deterioration of Rubisco has also been observed during oxidative stress or ozone treatment (Pell et al., 1997). All of these observations point out the hypersensitivity to oxidative stress of this protein, which was also found in our study.

Many proteins with chaperone activities were also favored targets for oxidation (spots 1, 43, 90, 135, 136, 137, and 140; Table III). Among them, three HSP70 proteins (spots 43, 136, and 137) are described as being abundant in dry and imbibed seeds (Gallardo et al., 2001; Rajjou et al., 2006a; http://www.seed-proteome.com). Molecular chaperones are known to be targets of carbonylation in yeast and bacteria challenged by oxidative stress (Tamarit et al., 1998; Cabiscol et al., 2000), presumably because they act as shields protecting other proteins against ROS damage (Cabiscol et al., 2000). Other chaperone proteins associated with the endoplasmic reticulum, such as the luminal binding protein BiP (spots 1 and 135), calreticulin (spot 140), and protein disulfide isomerase (spot 90; Laboissière et al., 1997; Freedman et al., 1998; Wilkinson and Gilbert, 2004), are also oxidized in deteriorated dry seeds (Table III). The fundamental role of the ER and associated proteins in the stress response and aging was recently reviewed in human (Yoshida, 2007). Our results support this hypothesis in plants.

It is worth noting that three LEA protein isoforms (spots 60, 61, and 431), encoded by the At2g42560 gene, appeared to be more oxidized in deteriorated seeds than in control seeds (Table III). The protein sequence of this LEA protein displays strong homology with that of previously described seed biotinylated proteins from pea (*Pisum sativum*; Duval

Table III. *Identification and relative carbonyl content of oxidized proteins during Arabidopsis seed aging*

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]
	kD			*kD*				*ratio 7 d:0 d*
Protein metabolism								
140[c]	60.26	4.15	Calreticulin 1 (precursor)	48.51	4.2	At1g56340	15%	2.3
1[c]	79.36	5.08	dnaK-type molecular chaperone BiP	71.17	5.08	At5g42020	20%	>100
135[c]	80.5	5.04	dnaK-type molecular chaperone BiP	71.17	5.08	At5g42020	20%	>100
136[c]	77.87	4.87	Heat shock cognate 70-kD protein 1	71.37	5.03	At5g02500	19%	>100
43[c]	78.9	5.06	Heat shock cognate 70-kD protein 3	71.13	4.69	At3g09440	15%	>100
137[c]	76.06	5.07	Heat shock protein 70	71.37	5.03	At3g12580	38%	>100
614[d]	68.68	5.83	Aspartyl-tRNA synthetase	62.90	6.12	At4g31180	26%	3.8
90[c]	64.11	4.81	Protein disulfide isomerase	55.60	4.81	At1g21750	39%	2.1
Energy metabolism and photosynthesis								
109[c]	64.79	6.35	Malate oxidoreductase or malic enzyme	64.28	6.32	At2g19900	14%	>100
63[c]	57.09	6.11	Isocitrate lyase	50.42	6.29	At3g21720	30%	>100
10[c]	57.36	5.77	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	30%	>100
319[c]	57.36	5.57	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	26%	>100
320[c]	57.36	5.82	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	21%	>100
321[c]	57.36	5.84	Ribulose bisphosphate carboxylase large chain	53.47	6.25	AtCg00490	30%	>100
Stress response								
60[c]	66.68	5.74	Late embryogenesis abundant (LEA)	67.19	5.78	At2g42560	25%	1.2
61[c]	66.68	5.78	Late embryogenesis abundant (LEA)	67.19	5.78	At2g42560	43%	1.5
431[c]	66.68	5.79	Late embryogenesis abundant (LEA)	67.19	5.78	At2g42560	32%	2.2
Hydrolase								
96[c]	64.62	6.08	Glycosyl hydrolase family 1 protein	57.83	6.02	At3g21370	11%	>100
207[c]	64.96	5.92	Glycosyl hydrolase family 1 protein	57.83	6.02	At3g21370	21%	>100
208[c]	64.96	6.23	Glycosyl hydrolase family 1 protein	57.83	6.02	At3g21370	14%	>100
Seed storage proteins								
35[c]	56.47	6.84	12S seed storage protein (precursor)	55.86	6.36	At4g28520	16%	>100
70[c]	49.43	7.19	12S seed storage protein (precursor)	52.59	7.68	At5g44120	22%	>100
71[c]	50.44	7.67	12S seed storage protein (precursor)	52.59	7.68	At5g44120	34%	>100
110[c]	57	6.4	12S seed storage protein (precursor)	55.86	6.36	At4g28520	19%	>100
111[c]	56.89	7.19	12S seed storage protein (precursor)	55.86	6.36	At4g28520	16%	>100
271[d]	59.96	6.52	12S seed storage protein (precursor)	55.86	6.36	At4g28520	30%	>100
239[c]	28.95	6.64	α-Cruciferin 12S (seed storage protein fragment)	48.03	6.56	At1g03880	18%	>100
80[c]	32.64	5.85	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	33%	3.29
82[c]	33.89	6.24	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	42%	1.6
83[c]	34.35	6.42	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	33%	1
84[c]	30.46	6.61	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	42%	2.1
85[c]	27.2	6.5	α-Cruciferin 12S (seed storage protein fragment)	27.24	6.34	At1g03880	32%	2.9
118[c]	29.21	6.04	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	26%	1
119[c]	30.66	6.16	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	28%	2.9
134[c]	26.35	6.34	α-Cruciferin 12S (seed storage protein fragment)	27.24	6.34	At1g03880	21%	3.9
238[c]	29.42	6.5	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	28%	2.1
240[c]	27.28	6.74	α-Cruciferin 12S (seed storage protein fragment)	27.24	6.34	At1g03880	16%	3.57

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]
241[c]	34.11	5.87	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	32%	1.2
243[c]	34.92	6.62	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	19%	1
278[c]	27.24	6.44	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	24%	>100
98[c]	18.08	6.12	β-Cruciferin 12S (seed storage protein fragment)	20.8	7.03	At1g03880	45%	4.9
498[c]	22.82	8.85	β-Cruciferin 12S (seed storage protein fragment)	21.2	6.19	At4g28520	79%	12.4
87[c]	18.43	6.36	β-Cruciferin 12S (seed storage protein fragment)	20.8	7.03	At1g03880	33%	>100
88[c]	22.82	8.68	β-Cruciferin 12S (seed storage protein fragment)	21.2	6.19	At4g28520	44%	1.8
231[c]	19.71	8.82	β-Cruciferin 12S (seed storage protein fragment)	21.2	6.19	At4g28520	39%	>100
233[c]	18.45	9.1	β-Cruciferin 12S (seed storage protein fragment)	20.84	9.06	At5g44120	52%	>100
497[c]	18.45	6.12	β-Cruciferin 12S (seed storage protein fragment)	21.2	6.19	At4g28520	64%	>100
499[c]	20.67	8.85	β-Cruciferin 12S (seed storage protein fragment)	20.84	9.06	At5g44120	53%	5.3
Other processes								
259[c]	27.01	6.1	Expressed protein	27.28	6.7	At1g05510	21%	5.7

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Data obtained from densitometric analysis of individual spots from carbonylated proteins on 2D gels revealed by anti-DNP immunoassay (examples are shown in Fig. 4): normalized spot volume in the deteriorated seeds (7 d of CDT) divided by the normalized spot volume in the nondeteriorated control seeds (0 d of CDT; ratio of carbonylation in deteriorated dry seeds [7 d of CDT] over carbonylation in nondeteriorated dry seeds [0 d of CDT]) from three different and independent protein extractions. >100 means that the accumulation level of the corresponding carbonylated protein in the nondeteriorated dry seeds (0 d of CDT) was close to background. [c]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005). [d]Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2.

et al., 1994b; Job et al., 2001), soybean (*Glycine max*; Franca Neto et al., 1997; Shatters et al., 1997; Hsing et al., 1998), and barley (*Hordeum vulgare*; March et al., 2007). This type of LEA protein exhibits a characteristic biochemical feature in that it binds in vivo the vitamin biotin (Duval et al., 1994a; Alban et al., 2000; Job et al., 2001). Biotin (vitamin B7), also known as vitamin H, is a fundamental molecule for all living organisms, being the cofactor of housekeeping enzymes involved in carboxylation, decarboxylation, and transcarboxylation reactions (Patton et al., 1998; Alban et al., 2000; Nikolau et al., 2003). It has been proposed that the seed biotinylated LEA proteins may play a role in sequestering this vitamin late in embryogenesis for subsequent use during germination and/or to maintain a metabolic quiescent state in dry seeds by biotin deprivation (Alban et al., 2000; Job et al., 2001). It is also important to note the existence of an ortholog of this biotinylated LEA protein in *Medicago truncatula* seeds, whose accumulation level is strongly associated with the reinduction of desiccation tolerance in radicles (Boudet et al., 2006). Hence, a specific carbonylation of this biotinylated LEA protein caused by CDT could entail an alteration of Arabidopsis seed survival in the dry state.

Overall, our results document that CDT generates an oxidative stress, which in turn induces chemical modifications of proteins by carbonylation, thus providing an explanation for the decrease in seed vigor associated with this treatment.

CDT Exerts an Influence on Proteome Expression during Germination

The evolution of the seed proteome during germination was also analyzed 1 d after imbibition for the control and deteriorated seeds. This stage corresponds to the germination sensu stricto of the Arabidopsis (L*er*) high-vigor control seeds, as none of these seeds showed radicle protrusion at that time (Fig. 1). Of 45 protein spots presenting reproducible variations in their accumulation level, 29 protein spots were less abundant, while 16 protein spots were more abundant, in germinating deteriorated seeds than in control seeds (Fig. 5; Tables IV and V). One of the specific features observed is the maintenance of a high level of

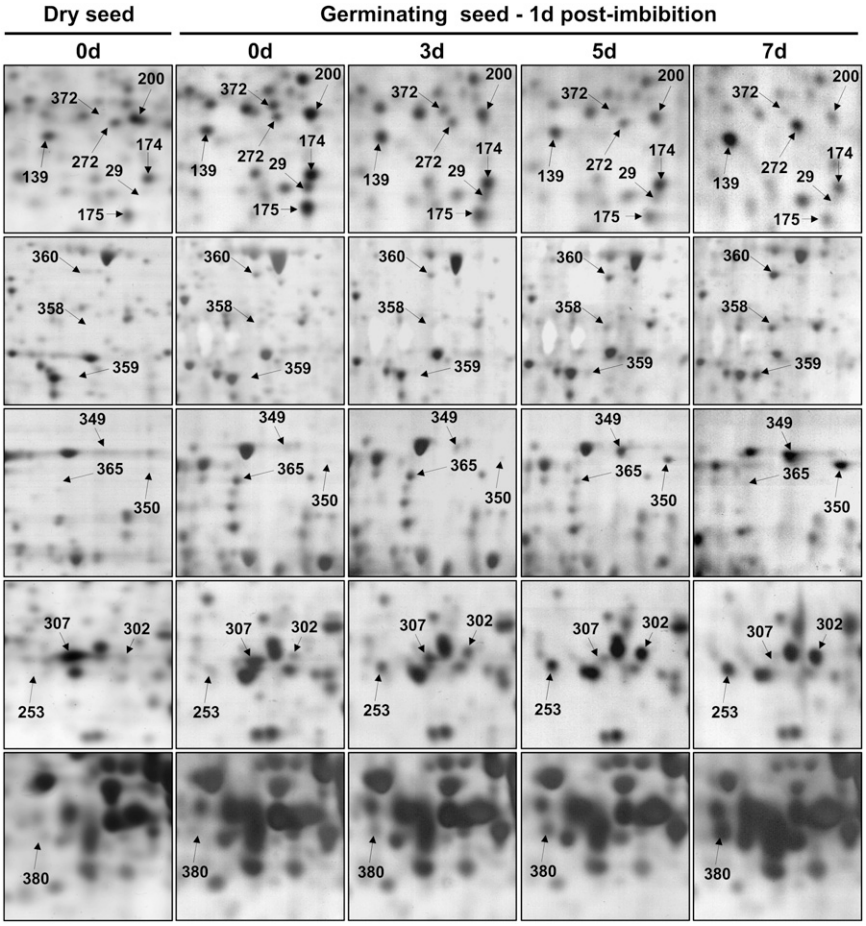

Figure 5. Changes in protein accumulation patterns in deteriorated seeds during germination sensu stricto (1 d after imbibition). An equal amount (150 μg) of total soluble protein extracts was loaded on each gel. Proteins were separated by 2D gel electrophoresis. A representative silver-stained 2D gel of total soluble proteins from nondeteriorated dry mature seeds is presented in Figure 2A. The analysis was carried out on nondeteriorated seed samples (0 d, control seeds) and deteriorated seeds (3, 5, and 7 d of CDT) imbibed in water for 1 d. The 17 labeled protein spots (spots 29, 139, 174, 175, 176, 200, 253, 272, 302, 349, 350, 358, 359, 360, 365, 372, and 380) were identified by mass spectrometry and by comparison with Arabidopsis seed protein reference maps (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com; Tables IV and V; Supplemental Table S2). Protein spot quantitation was carried out as described in ''Materials and Methods'' from at least five gels for each seed sample.

storage protein precursors (spots 151, 177, and 354) in germinating deteriorated seeds, implying that deteriorated seeds are less active than control seeds in mobilizing their storage protein reserves. In the same way, lipid storage mobilization was also strongly affected. Thus, isocitrate lyase (threo-D-isocitrate-glyoxylate lyase; EC 4.1.3.1), which is the key enzyme in seed lipid mobilization via the glyoxylate cycle (Graham, 2008), increased about 5-fold during germination sensu stricto of the control seeds (0 d), whereas its relative accumulation decreased steadily in deteriorated seeds according to the time of CDT (spot 365; Fig. 5; Table V). Isocitrate lyase plays a crucial role in the synthesis of carbohydrates from storage lipids during seed germination and seedling establishment (Eastmond and Graham, 2001). Also, it has been proposed that glyoxylate cycle activity is a good indicator of seedling emergence potential and seed vigor in sugar beet (*Beta vulgaris*) and Arabidopsis, notably under stress conditions (de los Reyes et al., 2003; Rajjou et al., 2006a). This suggests that a rapid onset of the glyoxylate pathway during germination can facilitate the mobilization of the lipid storage reserves, enabling the fast establishment of a vigorous seedling. Our results are in perfect agreement with these previous studies (de los Reyes et al., 2003; Rajjou et al., 2006a) and further document the fundamental role of the glyoxylate cycle for germination and seed vigor. Moreover, isocitrate lyase is regarded suitable to determine the transition from late embryogenesis to germination (Goldberg et al., 1989). In summary, this enzyme is a very good candidate as a diagnostic marker of seed vigor.

Among proteins accumulating to lower amounts in germinating deteriorated seeds than in corresponding control seeds, two isoforms of the cytosolic *O*-acetyl-Ser(thiol)lyase (EC 2.5.1.47), encoded by the At4g14880 gene, were identified (spots 174 and 175; Fig. 5; Table V). This enzyme forms a complex with Ser acetyltransferase to catalyze the last step of Cys synthesis (Droux et al., 1998). It is noted that in Arabidopsis, the cytosolic form encoded by At4g14880 is the major contributor to the total *O*-acetyl-Ser(thiol)lyase activity of the plant (Lopez-Martin et al., 2008). Our observations, therefore, suggest that Cys synthesis is an important feature of germination potential. Cys is the essential precursor of all organic molecules containing reduced sulfur, ranging from the amino acid Met to peptides such as glutathione or phytochelatins, proteins, vitamins, cofactors such as *S*-adenosyl-Met, and hormones (Höfgen et al., 2001). All of these sulfur compounds play fundamental roles in plant metabo-

Table IV. *Arabidopsis proteins whose abundance was significantly greater in deteriorated seeds than in control seeds during germination sensu stricto (1 d after imbibition)*

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]	$T_{1/2}$
	kD			*kD*				*ratio 7 d:0 d*	*d*
Energy metabolism									
253[c]	40.29	5.84	Glyceraldehyde-3-P dehydrogenase, cytosolic	36.99	6.34	At3g04120	18%	>6.2	>7
302[c]	40.34	6.03	Glyceraldehyde-3-P dehydrogenase	36.90	7.21	At1g13440	30%	>7.6	>7
368[d]	40.36	6.24	Glyceraldehyde-3-P dehydrogenase	36.90	7.21	At1g13440	17%	>4.5	>7
369[d]	40.33	6.20	Glyceraldehyde-3-P dehydrogenase	36.90	7.21	At1g13440	22%	>5.0	>7
349[d]	64.85	6.77	Malate oxidoreductase	64.24	6.68	At2g19900	12%	>32.0	>7
350[d]	64.70	7.21	Malate oxidoreductase	64.24	6.68	At2g19900	2%	>39.0	>7
Amino acid metabolism									
359[c]	56.90	5.57	*S*-Adenosyl-L-homo-Cys hydrolase	53.36	5.83	At4g13940 or At3g23810	13%	>20.0	>7
370[c]	41.66	6.50	Glutamate dehydrogenase	44.51	6.02	At5g18170 or At3g03910	13%	>2.5	>7
Seed storage proteins									
354[d]	42.61	6.60	12S seed storage protein (precursor)	52.56	8.08	At5g44120	25%	>9.2	>7
177[d]	45.47	6.07	12S seed storage protein (precursor)	48.03	6.56	At1g03880	17%	>3.0	>7
151[d]	46.11	5.38	Cupin family protein	49.66	5.45	At1g03890		>6.0	>7
Hydrolase and protease									
378[c]	80.28	5.77	Subtilisin-like Ser proteinase	81.80	6.76	At3g14067	19%	>3	>7
360[c]	64.96	5.75	Glycosyl hydrolase family	84.29	7.71	At5g64570	14%	>3.0	>7
Other processes									
139[d]	40.07	5.56	Reversibly glycosylated polypeptide	40.7	5.61	At3g02230 or At5g15650	7%	>2.5	>7
377[d]	38.42	6.58	Potassium channel β-subunit	36.52	7.49	At1g04690	15%	>3.8	>7
380[d]	26.31	5.89	Nucleoside diphosphate kinase II, chloroplast	25.53	9.30	At5g63310	12%	>3.8	>7

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Data obtained from densitometric analysis of individual spots from proteins on 2D gels stained with silver nitrate (see Fig. 2A for an example of a 2D gel): normalized spot volume in the deteriorated seeds (7 d of CDT) incubated for 1 d in water divided by the normalized spot volume in the nondeteriorated control seeds (0 d of CDT) incubated for 1 d in water, from five different gels and three independent extractions. [c]Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2. [d]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005).

lism (Ravanel et al., 1998). Furthermore, Met and/or Met derivatives have been shown to play an important promotive role in Arabidopsis seed germination and early seedling growth (Gallardo et al., 2002b). Finally, knocking out of the At4g14880 gene showed that Cys is an important determinant of the antioxidative capacity of the cytosol in Arabidopsis, as the resulting mutant appeared to be oxidatively stressed, accumulated ROS, and exhibited lesions characteristic of spontaneous cell death in the leaves (Lopez-Martin et al., 2008). We conclude that the reduced efficiency of deteriorated seeds in synthesizing Cys can induce an irreparable loss of seed vigor, owing to its general implication in metabolism and antioxidative potential in plants.

2-Alkenal reductase (EC 1.3.1.74), encoded by the At5g16970 gene, also showed a strongly depressed accumulation in germinating deteriorated seeds (spot 29; Table V). Interestingly, conditional overexpression of the 2-alkenal reductase gene in Arabidopsis results in increased salt tolerance during germination (Papdi et al., 2008). The 2-alkenal reductase possesses NADPH-dependent oxidoreductase activity, which has been shown to play a key role in the detoxification of reactive carbonyls occurring during the degradation of lipid peroxides and, hence, in the protection of cells

Table V. *Arabidopsis proteins whose abundance was significantly smaller in deteriorated seeds than in control seeds during germination sensu stricto (1 d after imbibition)*

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage	Relative Abundance[b]	$T_{1/2}$
	kD			*kD*				*ratio 7 d:0 d*	*d*
Translation and protein metabolism									
128[c]	47.45	5.45	Elongation factor 1B-γ	46.66	5.36	At1g09640	26%	<0.28	>7
129[c]	47.20	5.50	Eukaryotic initiation factor 4A-1	46.70	5.47	At3g13920	28%	<0.49	>7
105[c]	48.63	5.61	Elongation factor 1-γ2	46.4	5.55	At1g57720	41%	<0.5	>7
314[d]	37.54	5.22	60S acidic ribosomal protein P0-C	34.37	4.78	At2g40010	7%	<0.25	>7
200[c]	40.57	5.72	Protein disulfide isomerase-like (PDIL)	39.50	5.80	At2g47470	20%	<0.44	>7
364[d]	65.22	5.56	T-complex protein 1, θ-subunit (TCP-1-θ)	58.92	5.01	At3g03960	2%	<0.30	>7
Energy metabolism									
39[c]	38.52	6.23	Glyceraldehyde-3-P dehydrogenase	36.91	6.62	At3g04120	26%	<0.48	>7
40[c]	38.55	6.26	Glyceraldehyde-3-P dehydrogenase	36.91	6.62	At3g04120	27%	<0.44	>7
307[c]	40.30	5.87	Glyceraldehyde-3-P dehydrogenase	36.91	6.62	At3g04120	19%	<0.21	>7
365[c]	62.77	6.30	Isocitrate lyase	50.42	6.29	At3g21720	16%	<0.21	>7
126[c]	27.16	5.51	Triose phosphate isomerase, cytosolic	27.15	5.24	At3g55440	10%	<0.36	>7
353[c]	55.70	6.60	Phosphoglucomutase	63.44	5.73	At1g70730	6%	<0.10	>7
372[c]	41.08	5.71	Phosphoglycerate kinase	42.11	5.49	At1g79550	21%	<0.35	>7
194[c]	45.5	6.15	3-Oxoacyl-[acyl-carrier-protein] synthase I	50.41	8.29	At5g46290	44%	<0.49	>7
Cell detoxification and stress response									
23[c]	56.48	6.64	Catalase	56.93	6.63	At4g35090	16%	<0.25	>7
146[c]	37.67	5.03	MST	41.89	5.95	At1g79230	35%	<0.30	>7
29[c]	38.35	5.72	2-Alkenal reductase	38.13	5.81	At5g16970	14%	<0.26	>7
284[d]	25.34	5.43	Reduced glutathione-dependent dehydroascorbate reductase	23.62	5.79	At1g19570	57%	<0.21	>7
94[c]	34.92	5.21	Late embryogenesis abundant (LEA)	31.31	5.21	At3g17520	21%	<0.27	>7
Amino acid metabolism									
196[c]	41.46	6.57	Asp aminotransferase	44.3	6.8	At5g19550	28%	<0.31	>7
174[c]	38.7	5.73	*O*-Acetyl-Ser-thiol-lyase	33.8	5.9	At4g14880	9%	<0.26	>7
175[c]	37.85	5.71	*O*-Acetyl-Ser-thiol-lyase	33.8	5.9	At4g14880	29%	<0.22	>7
Cell division									
24[c]	42.94	5.06	Actin 7	41.73	5.31	At5g09810	23%	<0.43	>7
4[c]	57.35	4.89	Tubulin β2β3 chain	50.73	4.70	At5g62700	35%	<0.32	>7
Seed storage proteins									
504[c]	60.87	6.20	12S seed storage protein (precursor)	55.86	6.36	At4g28520	7%	<0.18	>7
311[c]	14.1	3.2	β-Cruciferin 12S (seed storage protein fragment)	58.23	6.53	At4g28520	11%	<0.21	>7
Other processes									
182[c]	50.76	5.53	DEAD box RNA helicase	48.38	5.49	At5g11200	32%	<0.42	>7
361[c]	54.65	5.22	4-Methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein	41.84	5.08	At3g14990	6%	<0.16	>7
116[c]	30.24	5.77	Expressed protein	28.78	5.92	At5g45690	40%	<0.35	>7

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Data obtained from densitometric analysis of individual spots from proteins on 2D gels stained with silver nitrate (see Fig. 2A for an example of a 2D gel): normalized spot volume in the deteriorated seeds (7 d of CDT) incubated for 1 d in water divided by the normalized spot volume in the nondeteriorated control seeds (0 d of CDT) incubated for 1 d in water, from five different gels and three independent extractions. [c]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005). [d]Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2.

against oxidative stress (Mano et al., 2005). As lipid peroxides accumulate during seed aging (Devaiah et al., 2007), our data highlight the role of this enzyme in seed vigor.

Protein Metabolism and Translation Are Major Components of Seed Vigor

It has been shown that seed germination has an absolute requirement for protein synthesis. Thus, cycloheximide, an inhibitor of protein translation, induces a complete inhibition of Arabidopsis seed germination (Rajjou et al., 2004). In this study, an interesting feature supports these previous observations and concerns the apparent correlation between protein metabolism and translation and the reduction of seed vigor induced by CDT (Table V). Indeed, several proteins associated with translation, such as initiation factor 4A-1, elongation factor 1-γ2, elongation factor 1B-γ, and ribosomal protein 60S (spots 129, 128, 105, and 314), are less abundant in deteriorated seeds during germination sensu stricto (Table V). Moreover, we already observed in this study that many other proteins involved in protein metabolism are altered by carbonylation in deteriorated seeds (spots 1, 43, 90, 135, 136, 137, and 140). Protein metabolism regroups several biological functions, such as protein folding, protein translocation, thermotolerance, oligomeric assembly, and switching between active and inactive protein conformations. Our results demonstrate that simultaneous impairment of these functions is closely linked with the loss of seed vigor. Our conclusion is supported by a recent study showing that transgenic seeds overaccumulating a heat stress transcription factor, which enhances the accumulation of HSPs, exhibit improved seed resistance to CDT (Prieto-Dapena et al., 2006). Moreover, the preservation of a robust stress response and protein disposal by the action of HSPs is indispensable for health and longevity in all organisms (for review, see Söti and Csermely, 2007).

To get direct insight on the importance of protein synthesis activity in seed vigor, proteins that were neosynthesized in vivo following seed imbibition were labeled in the presence of radioactive [^{35}S]Met. The control seeds (0 d), which had a maximum germination of 100%, exhibited a very high extent of [^{35}S]Met incorporation, testifying to a high translational activity during germination sensu stricto. As shown in Figure 6, the extent of [^{35}S]Met incorporation declined dramatically in the deteriorated seed samples. For example, seeds deteriorated for 3 d of CDT presented an 8-fold decrease in [^{35}S]Met incorporation compared with control seeds, although under these conditions the aged seeds still kept good vigor, with a maximum germination of about 80% (Fig. 1). This result disclosed that translational capacity can be an excellent feature for the estimation of seed vigor, a finding that is in good agreement with previous work demonstrating a loss in translational capacity during seed aging in soybean (Pillay, 1977). The seed samples deteriorated for 5 and 7 d had germination maxima of about 28% and 2%, respectively, and showed almost no translational activity. The consequences of the observed reduction of protein synthesis can be diverse, for example, affecting the systems necessary for the maintenance, repair, and normal resumption of metabolism and cell cycle activity, the efficiency of detoxification, the efficiency of the signaling pathways, and/or the production and secretion of several metabolites and plant hormones like gibberellins.

To investigate more closely this question and to reveal seed proteins whose neosynthesis during germination sensu stricto was altered by CDT, we characterized the neosynthesized proteome of three seed samples submitted to this treatment for 0, 2, and 7 d. Radiolabeled proteins were separated by 2D electrophoresis and reveled by autoradiography (Fig. 7). Translational activity of the nondeteriorated seeds (0 d) was high, as shown previously (Fig. 6). The analysis of this neosynthesized protein pattern revealed 1,272 protein spots (Fig. 7A), of which 217 proteins could be identified using our Arabidopsis seed reference maps (Supplemental Table S1). These proteins are involved in a large number of plant metabolic processes, in cell division, and in translation and protein metabolism, and interestingly, 28 protein spots match with 12S and 7S storage proteins. These seed storage proteins neosynthesized during germination sensu stricto are likely translated from stored mRNA. Indeed, it has been shown that in dry seeds, a large amount of stored mRNAs are translated during germination sensu stricto and play a fundamental role for the metabolic restart in the initialization of the germination program (Aspart et al., 1984; Rajjou et al., 2004). Deteriorated seeds submitted to 2 d of CDT displayed reduced translational activity during germination sensu stricto (Fig. 6A). Autoradiography analysis revealed 836 neosynthesized proteins (Fig. 7B), of which a large number were also neosynthesized in the nondeteriorated seeds (0 d). Yet, some proteins were specifically translated in these seeds deteriorated for 2 d, although unfortunately, they could not be identified from our Arabidopsis seed protein reference maps, because of their very low abundance. In contrast, de novo synthesis of many proteins as seen in the nondeteriorated control seeds (0 d) was abolished in 2-d deteriorated seeds. Some of them could be identified by comparison with Arabidopsis reference maps (Table VI). Interestingly, a large number of these proteins are generally associated with the end of the seed maturation program and not with the germination program, such as 12S seed storage proteins, LEA proteins, or dehydrins (Finkelstein, 1993; Bewley and Black, 1994; Cuming, 1999). It has been shown that the seed maturation program can be reinduced during early steps of seed germination (Lane, 1991; Lopez-Molina et al., 2002; Rajjou et al., 2006a, 2006b). This recruitment of the late maturation program either by de novo transcrip-

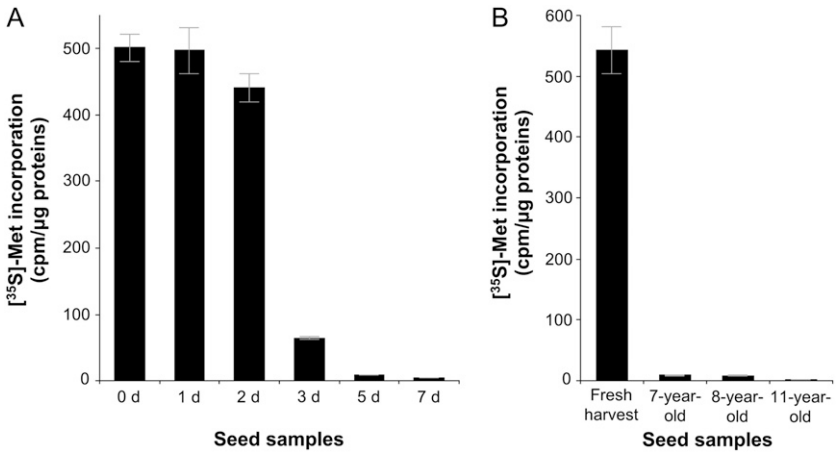

Figure 6. Influence of CDT and natural aging on de novo protein synthesis during germination sensu stricto (1 d after imbibition). Arabidopsis seeds were incubated in water containing [^{35}S]Met for 1 d. Protein synthesis was measured by TCA precipitation of aliquots of reaction mixtures spotted on Whatmann GF/C filters; after 10 washing steps in cold 5% TCA and 0.04 M sodium pyrophosphate and two washing steps in absolute ethanol, filters were dried and counted for radioactivity in a liquid scintillation counter. A, Incorporation of [^{35}S]Met in proteins synthesized de novo during germination sensu stricto of deteriorated seeds (0, 1, 2, 3, 5, and 7 d of CDT). B, Incorporation of [^{35}S]Met in proteins synthesized de novo during germination sensu stricto of naturally aged seeds (freshly harvested seeds as well as 7-, 8-, and 11-year-old seeds).

tion (Lopez-Molina et al., 2002) or by translation of stored mRNAs (Rajjou et al., 2004, 2006a) is a strategy to mount appropriate defense mechanisms in response to the vagaries of nature's water supply and to protect the embryo during the transition from a metabolic quiescent state to active metabolism. For a longer time of CDT (7 d), seeds became almost unable to support de novo protein synthesis (Fig. 7C). In summary, our results clearly indicate that seed vigor is closely associated with the ability of the seeds to reinduce the late maturation program during early stages of germination.

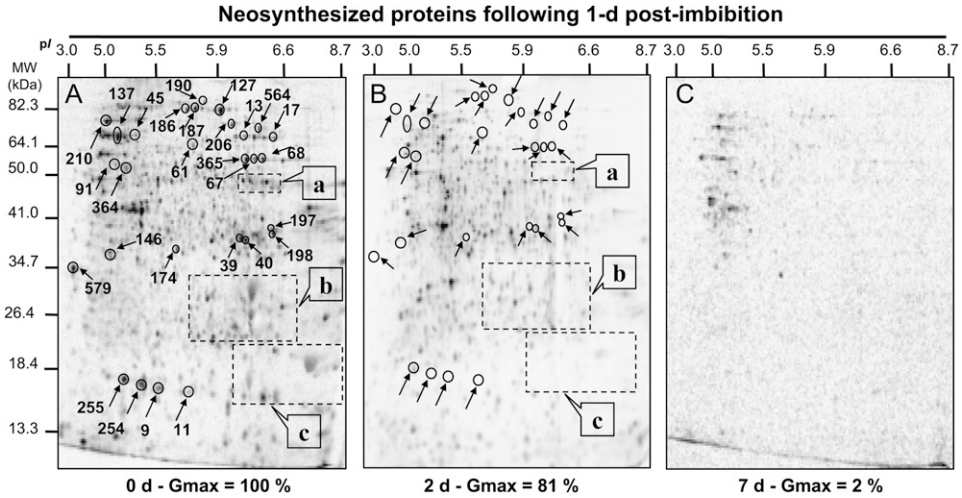

Figure 7. Comparison of de novo protein synthesis patterns during germination sensu stricto (1 d after imbibition) of deteriorated seeds. A, Protein profiles of de novo synthesized proteins in nondeteriorated seeds (0 d, control seeds). B, Protein profiles of de novo synthesized proteins in 3-d deteriorated seeds (3 d of CDT). C, Protein profiles of de novo synthesized proteins in 7-d deteriorated seeds (7 d of CDT). Radiolabeling of proteins was carried out by introducing [^{35}S]Met in the germination assays, as described in ''Materials and Methods.'' Soluble proteins were extracted after 1 d of imbibition and submitted to 2D gel electrophoresis, and the radiolabeled proteins were revealed as described in ''Materials and Methods.'' The 33 labeled protein spots were identified by mass spectrometry and by comparison with Arabidopsis seed protein reference maps (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com; Table VI; Supplemental Table S2). a, 12S seed storage protein precursor; b, α-subunit of 12S seed storage protein; c, β-subunit of 12S seed storage protein. Several radiolabeled spots exhibiting contrasting specific accumulation in deteriorated seeds could not be identified because they had no match with proteins detected in Arabidopsis seed protein reference maps (http://www.seed-proteome.com).

Table VI. *Arabidopsis proteins whose de novo synthesis was inhibited during germination sensu stricto (1 d after imbibition) of deteriorated seeds submitted to CDT for 2 d compared with nondeteriorated control seeds*

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage
	kD			*kD*			
Translation and protein metabolism							
127[b]	93.92	5.89	Elongation factor EF-2	94.25	5.89	At1g56070	24%
9[b]	17.94	5.50	HSP17.6	17.83	5.22	At5g12030	14%
45[b]	76.38	5.24	HSP70	70.91	5.30	At1g16030	24%
137[b]	76.06	5.07	HSP70	71.37	5.03	At3g12580	19%
190[b]	101.62	5.82	HSP101	101.27	5.99	At1g74310	16%
364[c]	65.22	5.56	T-complex protein 1, θ-subunit (TCP-1-θ)	58.92	5.01	At3g03960	2%
579[b]	36.05	3.98	Gly-rich protein similar to P23 cochaperone	25.47	4.17	At4g02450	24%
Energy metabolism							
17[b]	73.40	6.58	Phospho*enol*pyruvate carboxykinase	73.40	6.61	At4g37870	17%
197[b]	40.91	6.77	Fru-bisphosphate aldolase	38.37	7.46	At2g36460	23%
198[b]	40.72	6.89	Fru-bisphosphate aldolase	38.37	7.46	At2g36460	41%
39[b]	38.52	6.23	Glyceraldehyde-3-P dehydrogenase, cytosolic	36.99	6.34	At3g04120	26%
40[b]	38.55	6.26	Glyceraldehyde-3-P dehydrogenase, cytosolic	36.99	6.34	At3g04120	27%
365[b]	62.77	6.30	Isocitrate lyase	50.42	6.29	At3g21720	16%
188[b]	96.69	5.83	Aconitate hydratase, cytoplasmic	98.13	6.36	At4g35830	10%
Cell detoxification and stress response							
11[b]	19.61	5.75	Em-like protein GEA1	16.61	5.75	At3g51810	26%
61[b]	66.68	5.78	Late embryogenesis abundant (LEA)	67.19	5.78	At2g42560	43%
91[b]	61.88	5.06	Late embryogenesis abundant (LEA)	52.08	5.29	At3g53040	17%
102[b]	60.51	5.22	Late embryogenesis abundant (LEA)	48.49	5.43	At2g36640	16%
210[b]	88.93	4.93	Low-temperature-induced 65-kD protein	65.95	4.81	At5g52300	20%
146[b]	37.67	5.03	MST	41.89	5.95	At1g79230	35%
13[b]	72.97	6.30	MLO-like protein	67.21	10.06	At1g61560	15%
254[b]	17.96	5.20	Dehydrin	18.44	7.95	At5g66400	6%
255[b]	17.96	5.37	Dehydrin	18.44	7.95	At5g66401	6%
DNA metabolism							
186[b]	96.47	5.78	DNA topoisomerase I	102.78	10.05	At5g55300	8%
187[b]	96.47	5.81	DNA topoisomerase I	102.78	10.05	At5g55300	8%
Amino acid metabolism							
174[b]	38.71	5.73	*O*-Acetyl-Ser-thiol-lyase	33.79	5.95	At4g14880	9%
206[b]	82.05	6.09	5-Methyl-tetra-hydropteroyl-tri-Glu-homo-Cys methyl transferase	84.34	6.47	At5g17920	17%
Hydrolase and protease							
67[b]	63.25	6.47	Glycosyl hydrolase family 1 protein	60.26	6.91	At3g09260	46%
68[b]	63.47	6.56	Glycosyl hydrolase family 1 protein	60.26	6.91	At3g09260	27%
564[c]	87.84	6.40	Cucumisin-like Ser protease	78.28	5.91	At5g67360	14%
Seed storage proteins							
69[b]	43.67	6.29	12S seed storage protein (precursor)	48.03	6.56	At1g03880	18%
70[b]	49.43	7.19	12S seed storage protein (precursor)	52.59	7.68	At5g44120	22%
71[b]	50.44	7.67	12S seed storage protein (precursor)	52.59	7.68	At5g44120	34%

(*Table continues on following page.*)

Table VI. (*Continued from previous page.*)

No.[a]	Experimental Molecular Mass	Experimental pI	Arabidopsis Protein Name	Theoretical Molecular Mass	Theoretical pI	AGI No.	Coverage
78[b]	27.26	6.61	12S seed storage protein (precursor)	52.59	7.68	At5g44120	13%
110[b]	57.97	6.40	12S seed storage protein (precursor)	55.86	6.36	At4g28520	19%
245[b]	27.22	6.64	12S seed storage protein (precursor)	50.61	6.77	At1g03880	25%
271[c]	59.96	6.52	12S seed storage protein (precursor)	55.86	6.36	At4g28520	9%
80[b]	32.64	5.85	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	33%
83[b]	34.35	6.42	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	33%
84[b]	30.46	6.61	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	42%
85[b]	27.20	6.50	α-Cruciferin 12S (seed storage protein fragment)	27.24	6.34	At1g03880	32%
97[b]	28.89	7.90	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	40%
159[b]	23.54	5.33	α-Cruciferin 12S (seed storage protein fragment)	27.24	6.34	At4g28520	15%
241[b]	34.11	5.87	α-Cruciferin 12S (seed storage protein fragment)	34.68	6.42	At4g28520	32%
392[c]	13.31	5.89	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	14%
739[c]	31.04	5.81	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	47%
1,154[c]	11.78	9.05	α-Cruciferin 12S (seed storage protein fragment)	31.75	6.49	At5g44120	5%
1,155[c]	10.43	9.02	α-Cruciferin 12S (seed storage protein fragment)	31.64	9.80	At5g44120	17%
25[b]	23.32	6.89	β-Cruciferin 12S (seed storage protein fragment)	21.20	6.19	At4g28520	27%
44[b]	16.14	7.42	β-Cruciferin 12S (seed storage protein fragment)	20.80	7.03	At1g03880	7%
87[b]	18.43	6.36	β-Cruciferin 12S (seed storage protein fragment)	20.80	7.03	At1g03880	33%
88[b]	22.82	8.68	β-Cruciferin 12S (seed storage protein fragment)	21.20	6.19	At4g28520	44%
287[b]	23.44	5.58	β-Cruciferin 12S (seed storage protein fragment)	20.84	9.06	At5g44120	22%
705[b]	11.75	8.90	β-Cruciferin 12S (seed storage protein fragment)	21.20	6.19	At1g03880	44%
1,153[c]	11.51	9.02	β-Cruciferin 12S (seed storage protein fragment)	21.20	6.19	At4g28520	39%
1,156[c]	10.68	9.05	β-Cruciferin 12S (seed storage protein fragment)	20.84	9.06	At5g44120	41%
246[b]	29.45	6.70	Storage proteins 7S	55.05	7.15	At3g22640	28%
247[b]	34.02	6.77	Storage proteins 7S	55.05	7.15	At3g22640	18%

[a]Protein numbering following Arabidopsis seed protein reference maps available at http://www.seed-proteome.com. [b]Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005). [c]Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2.

Similar Events Occur during Accelerated and Natural Aging

There is still uncertainty regarding whether CDT mimics natural aging. This is a major concern of seed companies, because, for practical reasons, they rely on CDT and germination assays to predict seed storability (Delouche and Baskin, 1973). Therefore, it was important to compare the biochemical behavior of seeds submitted to CDT and of seeds that have been naturally aged, namely, seeds that have been stored for several years in tubes in a refrigerator regulated at 5°C. For that purpose, three naturally aged Arabidopsis seed samples were examined. Two of them were 7 and 8 years old and presented maximum germination of about 45% and 23%, respectively. A third sample was

Table VII. *The effect of natural aging on the germination of Arabidopsis seeds*

Seed Samples	Maximum Germination	SD
	%	
Freshly harvested seeds	99.00	1
Seven-year-old seeds	45.33	2.96
Eight-year-old seeds	23.00	2.52
Eleven-year-old seeds	0.00	0

11 years old and did not germinate, even at 30 d after imbibition (Table VII).

Our present proteome analysis revealed common features between the artificially and naturally aged seeds. Indeed, the evolution of the dry seed proteome during natural aging and during CDT displayed common changes, as shown in Figure 8 for two protein spots (spots 146 and 7). Spot 146 (Figs. 2, 3, and 8; Tables II and V), corresponding to MST, was abundant in nondeteriorated seeds (0 d) and in freshly harvested seeds. However, during both CDT and natural aging, the abundance of this protein was strongly reduced in the dry seeds. An opposite behavior was observed for protein spot 7, corresponding to the 60S ribosomal protein, whose level strongly increased during both artificial and natural aging (Figs. 2, 3, and 8; Tables II and V).

Another spectacular similarity observed between natural and artificial aging concerned the oxidation patterns of the seed proteome. As depicted in Figure 4, almost identical protein carbonylation events occurred during natural and artificial aging. In both cases, the extent of protein carbonylation was strongly increased and the protein targets of carbonylation were nearly the same. In particular, these results confirm our previous finding that protein oxidation is not a random process but targets very specific proteins (Job et al., 2005).

Finally, it is remarkable that translational capacity was strongly repressed in naturally aged seeds (Fig. 6), a specific feature also observed with CDT (Figs. 6 and 7).

Overall, our data provide the first molecular indication supporting the usefulness of CDT for the prediction of seed storability.

A Reduction in Amino Acid Pools during Aging Is Not the Cause of Seed Vigor Loss

Aging of the seeds, either by CDT or natural aging, caused large reductions in protein synthesis during the first day of imbibition. One of the hypotheses is that this can be caused by preferential use of amino acids as alternative energy sources, thereby limiting the substrate for protein synthesis. This hypothesis was tested by incubating CDT and control seeds in solutions of different amino acids, pyruvate, or Glc.

We found that Asp, Glu, or Met at 1 M could not stimulate the germination of seeds submitted to CDT. Also, neither Glc nor pyruvate could stimulate the germination of CDT seeds (data not shown).

A Reduction in Template Activity of Stored mRNAs Is Not the Cause of Seed Vigor Loss

As documented above, the potential of de novo protein synthesis was severely reduced during both artificial and natural aging. A possible explanation could be that the translational machinery was damaged, which is supported by our present data (Fig. 6). However, a different explanation to account for this behavior could be that the stored mRNA pool is damaged in seeds challenged by CDT or following natural aging. To further explore this question, stored mRNAs were extracted from nondeteriorated and aged seeds and the translation potential of these mRNAs was evaluated by in vitro translation assays using a commercially available wheat germ translation system, as described in ''Materials and Methods.'' For all seed samples, we found that stored mRNAs can be used as templates in this system (data not shown). It should be noted, however, that this conclusion is based on the use of an in vitro heterologous translation system that might not reproduce all facets of the in vivo situation. Furthermore, this assay allowed only global estimation of the template activity of the extracted pool of stored mRNAs, and at present we cannot exclude the possibility that particular stored mRNAs playing a role in seed quality could be dam-

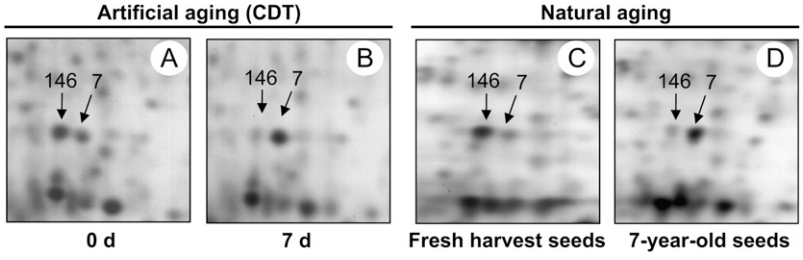

Figure 8. Similar protein pattern evolution during CDT or natural aging. An equal amount (150 μg) of total protein extracts was loaded on each gel. A representative silver-stained 2D gel of total proteins from nondeteriorated seeds (0 d, control seeds) is presented Figure 2A. A, Enlarged window of 2D gels as shown in Figure 2Ab for nondeteriorated seeds. B, Enlarged window of 2D gels as shown in Figure 2Ab for deteriorated seeds (7 d of CDT). C, Enlarged window of 2D gels as shown in Figure 2Ab for freshly harvested seeds. D, Enlarged window of 2D gels as shown in Figure 2Ab for 7-year-old seeds (natural aging).

aged by aging. Nevertheless, our data strongly indicate that reduced activity of the translational machinery is one of the main factors involved in seed longevity integrity, either due to reduced integrity or to an inhibition of this machinery.

CONCLUSION

In conclusion, proteomics provided an innovative and powerful tool for investigating the molecular mechanisms of seed vigor and seed viability during aging. From a methodological point of view, it is worth noting that the proteins analyzed here could be readily identified from our previous studies establishing reference protein maps of Arabidopsis seeds (http://www.seed-proteome.com). On the one hand, this illustrates the robustness of the proteomic approach, notably concerning the reproducibility of protein patterns on 2D gels. On the other hand, this shows the usefulness of establishing such protein maps, especially considering the cost and effort needed for protein identification. From this work, it appears that changes in the regulation of protein synthesis, post-translational modifications, and protein turnover are crucial determinants of the age-related decline in the maintenance, repair, and survival of the seed. The CDT used to mimic natural seed aging was efficient to alter germinative ability, as indicated by germination behavior. A decrease of seed vigor was observed in relation to the duration of treatment. This experimental protocol allowed comparing a differentially deteriorated seed proteome in order to gain a better understanding of complex mechanisms controlling seed aging. In particular, our proteomic analyses revealed that the loss in seed vigor induced by aging can be accounted for both by protein changes in the dry seeds and by an inability of the low-vigor seeds to display a normal proteome during germination. We characterized several proteins whose accumulation levels varied as a consequence of CDT and the loss of the ability to germinate. Therefore, these proteins should play an important role in the expression of seed vigor. In this context, our results revealed essential mechanisms for seed vigor, such as translational capacity, mobilization of seed storage reserves, and detoxification efficiency. Furthermore, the observed increase in protein oxidation, both in artificially and naturally aged seeds, lends support to the finding that oxidative stress accompanies seed aging. The accumulation of oxidative damage in seeds was correlated with the loss of germination vigor. Increased protein oxidation (carbonylation) might induce a loss of functional properties of target seed proteins and enzymes and/or enhance their susceptibility toward proteolysis. Since protein oxidation mainly results from attack by ROS, this suggests an important role of antioxidant systems through detoxification and protection of upstream mechanisms to maintain seed vigor.

Another fundamental feature shown by our study was the dramatic reduction of the protein neosynthesis capacity of the aged seeds. Their translational activity was strongly reduced during the first day of imbibition, corresponding to germination sensu stricto. It may be strongly impaired by aging, through protein oxidation and/or degradation. The 3-d CDT seeds, although having a considerable reduction in translation potential during the first day of imbibition, still kept a high vigor, as 80% of these seeds could germinate under the experimental conditions. Since these seeds would require a functional translational machinery, we assume that their translational machinery is both damaged and repaired, or that its activity is temporary halted, or a combination of both. It can be hypothesized that during germination, the seeds have feedback mechanisms inhibiting mRNA translation until DNA damage is repaired. Induction of DNA damage during seed aging has been demonstrated for a long time (Osborne et al., 1980/1981).

Our results are in agreement with previous experiments showing that Arabidopsis seeds are unable to germinate in the presence of the translational inhibitor cycloheximide, thereby implying that seed germination requires de novo protein synthesis (Rajjou et al., 2004). This study on the model plant Arabidopsis enables a better understanding of the molecular mechanisms underlying loss of germination vigor during seed aging. Finally, for the first time, to our knowledge, we demonstrate that the CDT protocol, extensively used by the seed industry to control seed quality, mimics truly molecular and biochemical events occurring during natural seed aging. Further work is in progress in our laboratories to validate the role of the characterized proteins in seed vigor by reverse genetics.

MATERIALS AND METHODS

Plant Material and Germination Experiments

Nondormant seeds of Arabidopsis (*Arabidopsis thaliana*), accession L*er*, were used in all experiments. Germination assays were carried out at 25°C, with 16 h of light and 8 h of dark daily, as described (Rajjou et al., 2004, 2006a). Naturally aged seeds had been stored for several years in tubes in a refrigerator regulated at 5°C.

CDT

CDT was performed according to Tesnier et al. (2002). Briefly, seeds were equilibrated at 85% relative humidity (20°C) in an electronically controlled environment cabinet (van den Berg Klimaattechniek), and day-0 controls were immediately dried back at 32% relative humidity. Treatment was done by storing the seeds (after equilibration at 85% relative humidity) for various times (0, 4, and 16 h, and 1, 2, 3, 5, and 7 d) at 40°C. Then, seeds were dried back at 32% relative humidity (20°C) and stored at 4°C.

Preparation of Total Protein Extracts

Total protein extracts were prepared from dry mature seeds and from seeds at 1 d after imbibition as described previously (Rajjou et al., 2006a). Following grinding of 100 mg of seeds using mortar and pestle in liquid nitrogen, total soluble proteins were extracted at 4°C in 1.2 mL of thiourea/urea lysis buffer (Harder et al., 1999) containing 7 M urea (GE Healthcare), 2 M thiourea (Merck), 4% (w/v) CHAPS (Amersham Biosciences), and 1% (v/v) Pharmalyte pH 3 to 10 carrier ampholytes (GE Healthcare). This extraction buffer also contained 18 mM Tris-HCl (Trizma HCl; Sigma), 14 mM Trizma base (Sigma), the protease inhibitor cocktail Complete Mini from Roche Diagnostics, 53 units/mL DNase I (Roche Diagnostics), 4.9 Kunitz units/mL RNase A

(Sigma), and 0.2% (v/v) Triton X-100. After 10 min at 4°C, 14 mM dithiothreitol (DTT; GE Healthcare) was added and the protein extracts were stirred for 20 min at 4°C, then centrifuged (35,000*g*, 10 min) at 4°C. The supernatant was submitted to a second clarifying centrifugation as above. The final supernatant corresponded to the total soluble protein extract. Protein concentrations in the various extracts were measured according to Bradford (1976). Bovine serum albumin was used as a standard.

Two-Dimensional Electrophoresis

Proteins were first separated by electrophoresis according to charge. Isoelectric focusing was realized with protein samples with an equivalent to an extract of 100 seeds, corresponding to about 150 mg of protein for all samples. Proteins from the various extracts were separated using gel strips forming an immobilized nonlinear pH gradient from 3 to 10 (Immobiline DryStrip pH 3–10 NL, 18 cm; GE Healthcare). Strips were rehydrated for 14 h at 20°C with the thiourea/urea lysis buffer containing 2% (v/v) Triton X-100, 20 mM DTT, and the protein extracts. Isoelectric focusing was performed at 20°C in the Multiphor II system (Amersham Biosciences) for 1 h at 300 V and for 7 h at 3,500 V. Proteins were then separated according to size. Prior to the second dimension, the gel strips were equilibrated twice for 30 min each in 2 × 100 mL of equilibration solution containing 6 M urea, 30% (v/v) glycerol, 2.5% (w/v) SDS, 0.15 M BisTris, and 0.1 M HCl (Görg et al., 1987; Harder et al., 1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% [w/v]) was added to the second (Harder et al., 1999). Equilibrated gel strips were placed on top of vertical polyacrylamide gels (10% [v/v] acrylamide, 0.33% [w/v] piperazine diacrylamide, 0.18 M Trizma base, 0.166 M HCl, 0.07% [w/v] ammonium persulfate, and 0.035% [v/v] *N,N,N′,N′*-tetramethylethylenediamine [TEMED]). A denaturing solution (1% [w/v] low-melting agarose [Gibco BRL], 0.4% [w/v] SDS, 0.15 M BisTris, and 0.1 M HCl) was loaded onto gel strips. After agarose solidification, electrophoresis was performed at 10°C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine, and 0.1% (w/v) SDS for 1 h at 35 V and for 14 h at 100 V. Ten gels (200 × 250 × 1.0 mm) were run in parallel (Isodalt system from Amersham Biosciences). For each condition analyzed, 2D gels were made at least in triplicate and from three independent protein extractions; kinetic data shown in Figure 2 and Tables I, II, IV, and V were obtained from at least five gels for each seed sample (nondeteriorated and deteriorated seeds). 2D gels were stained with silver nitrate according to Blum et al. (1987) for densitometric analyses. Image analysis was carried out with ImageMaster 2D Elite version 4.01 software (Amersham Biosciences). The kinetics of protein accumulation were analyzed by nonlinear regression analysis using exponential relationships and KaleidaGraph software (Synergy Software).

Detection of Oxidized Proteins and Western Blotting

Detection of oxidized proteins by carbonylation was performed by derivatization of protein extracts with 2,4-dinitrophenylhydrazine and immunological detection of the DNP adducts with monoclonal anti-DNP antibody (OxyBlot Oxidized Protein Detection Kit; Chemicon) as described previously (Korolainen et al., 2002; Johansson et al., 2004; Job et al., 2005).

De Novo Protein Synthesis

Labeled proteins were synthesized in vivo by imbibing seeds on water for 1 d in the presence of [^{35}S]Met (1.85 MBq; ICN Biomedicals). Protein synthesis was measured by TCA precipitation of aliquots of reaction mixtures spotted on Whatmann GF/C filters; after 10 washing steps in cold 5% TCA and 0.04 M sodium pyrophosphate and two washing steps in absolute ethanol, filters were dried and counted for radioactivity in a liquid scintillation counter (Rajjou et al., 2004).

Protein Identification

Proteins of interest correspond to previously identified seed proteins from 2D electrophoresis experiments and localized on 2D electrophoresis reference maps (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com). In this work, the protein identification of each spot of interest was verified by mass spectrometry as described by Rajjou et al. (2006a).

RNA Integrity

Stored RNAs were extracted using hot borate (Wan and Wilkins, 1994). The integrity of the rRNA and mRNA pools was analyzed, respectively, by gel electrophoresis and using a wheat germ extract in vitro translation assay (Promega) with ^{35}S-labeled Met (1.85 MBq; ICN Biochemicals).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Arabidopsis proteins whose de novo synthesis occurs during germination sensu stricto (1 d after imbibition) of nondeteriorated control seeds (0 d).

Supplemental Table S2. Peptide sequences identified by tandem mass spectrometry sequencing and corresponding to novel proteins in Arabidopsis seeds identified in this work.

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